Effect of 25-Hydroxyvitamin D_3 and 1 α,25 Dihydroxyvitamin D_3 on Differentiation and Apoptosis of Human Osteosarcoma Cell Lines

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Received 3 May 2011; accepted 6 October 2011
Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jor.21585

ABSTRACT: Osteosarcoma (OS) is a malignant bone tumor predominantly affecting children and adolescents. OS has a 60% survival rate with current treatments; hence, there is a need to identify novel adjuncts to chemotherapeutic regimens. In this pilot study, we investigated the dose-response to 1α,25-dihdroxyvitamin D_3 (1,α 25$(OH)_2D_3$) and 25-hydroxyvitamin D_3 (25$(OH)D_3$) by human OS cell lines, SaOS-2, and 143B. We hypothesized that 1,α 25$(OH)_2D_3$ and 25$(OH)D_3$ would stimulate differentiation and induce apoptosis in OS cells in a dose-dependent manner. Human OS cell lines, SaOS-2, and 143B, were treated with 1,α 25$(OH)_2D_3$ or 25$(OH)D_3$ or an ethanol control, respectively, at concentrations ranging from 1 to 1,000 nM. Ki67 (a marker of cellular proliferation) immunocytochemistry revealed no significant changes in the expression of Ki-67 or MIB-1 in 1α,25$(OH)_2D_3$ or 25$(OH)D_3$ treated SaOS-2 or 143B cells. Both control and 1α,25$(OH)_2D_3$ treated SaOS-2 and 143B cells expressed vitamin D receptor (VDR). Markers of osteoblastic differentiation in 143B cells and SaOS-2 cells were induced by both 25$(OH)D_3$ and 1α,25$(OH)_2$D, and evident by increases in alkaline phosphatase (ALP) activity, osteocalcin (OCN) mRNA expression, and mineralization of extra-cellular matrix (ECM) by alizarin red staining. An increasing trend in apoptosis in response to 25$(OH)D_3$, in both SaOS-2 and 143B cells was detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining. With 1α,25$(OH)_2D_3$ treatment, apoptosis was evident at higher concentrations only. These preliminary findings suggest that OS cells express VDR and respond to 25$(OH)D_3$ and 1α,25$(OH)_2D_3$ by undergoing differentiation and apoptosis. J Orthop Res

Keywords: osteosarcoma; vitamin D; proliferation; differentiation; apoptosis

Osteosarcoma (OS) is a malignant bone tumor predominantly affecting children and adolescents.[1] Current chemotherapy regimens and surgical interventions have not led to significant improvement in the present 60–70% survival rate. In addition, therapies in OS have remained relatively unchanged over the past 20 years.[2,3] There is a need to identify novel therapeutic regimens to improve survival rates for individuals over those achieved with the current treatment approaches.

The active form of vitamin D, 1α,25-dihdroxyvitamin D_3 (1α,25$(OH)_2D_3$) is increasingly recognized for its anti-cancer properties.[4,5] The main biologic function of 1α,25$(OH)_2D_3$ is to maintain serum calcium levels within normal range by increasing the efficiency of intestinal absorption of dietary calcium[6,7] and mobilizing calcium stores from the bone into circulation.[8–10] Extra-skeletal cells and tissues produce 1α,25$(OH)_2D_3$.[7] By binding to the nuclear vitamin D receptor (VDR), 1α,25$(OH)_2D_3$ regulates expression of genes responsible for cellular proliferation, differentiation, apoptosis, and angiogenesis in local tissues.[11,12] Previous studies demonstrate that 1α,25$(OH)_2D_3$ inhibits proliferation and enhances differentiation of OS cells.[13–16] The mechanisms by which 1α,25$(OH)_2D_3$ regulates proliferation and differentiation in OS are not completely understood. Evidence that 1α,25$(OH)_2D_3$ induces apoptosis in canine OS cells comes from TUNEL studies detecting DNA fragmentation, a hallmark of apoptotic cells.[17] Studies in rodent and human OS cell lines are contradictory. Those studies show that 1α,25$(OH)_2D_3$ either inhibits[15,18,19] or has no effect on apoptosis.[20] There are significant unknowns and contradictions in the current literature on the effects of 1α,25$(OH)_2D_3$ on OS cells. The dose-response to 1α,25$(OH)_2D_3$ by different OS cells is not well-defined or characterized. The mechanisms involving inhibition of proliferation and promotion of differentiation remain unclear.

The objectives of this pilot study were to determine the dose-response to 25$(OH)D_3$ or 1α,25$(OH)_2D_3$ by OS cell lines, and thus, to identify the effects of vitamin D (25D or 1α,25$(OH)_2D_3$) on cellular processes in human OS cell lines: SaOS-2 and 143B. The rationale for choosing the above mentioned cell lines versus other

Additional supporting information may be found in the online version of this article.

Abbreviations: 1α,25$(OH)_2D_3$, 1α,25-Dihydroxyvitamin D_3; 25$(OH)D_3$, 25-Hydroxyvitamin D_3; ALP, Alkaline Phosphatase; DMEM, Dulbecco's Modified Eagle's Medium; ELISA, Enzyme-linked Immunoassay; FBS, Fetal bovine serum; FITC, Fluorescein isothiocyanate; HBSS, Hank's Balanced Saline Solution; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (tetrazolium product); PBS, Phosphate-buffered saline; pNPP, para-nitrophenyl phosphate; RB, retinoblastoma; RT-PCR, Reverse transcriptase polymerase chain reaction; TEM, Transmission electron microscopy; TUNEL, Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling; VDR. Vitamin D receptor.

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cell lines is as follows: SaOS-2 cells are p53 and retinoblastoma (RB) null, metastatic,[21,22] have a high proliferative capacity and are known to possess high affinity receptors for $1\alpha,25(OH)_2D_3$.[23] Both p53 and RB mutations play an important role in the pathogenesis of OS. SaOS-2 cell line is often used as model for studying osteoblastic differentiation. Our objective in this study was to use SaOS-2 as model to study differentiation inducing effects of $1,25(OH)_2D_3$. The 143B cell line is a Ki-ras oncogene transformation of the HOS OS cell line,[24] and highly metastatic. Studies have shown that 143B cells express p53.[25] The RB status is not very clear. The hypothesis of the current study is that both $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ will stimulate differentiation and induce apoptosis of SaOS-2 and 143B in a dose-dependent manner.

METHODS

Cell Culture

Human OS cell lines, SaOS-2, and 143B (American Type Culture Collection; Manassas, VA) were used in this study. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% Penicillin/Streptomycin, 10% fetal bovine serum (FBS), 5% non-essential amino acids, and 5% glutamine. Cells were maintained in standard conditions at 37°C in a humidified (95% air: 5% CO_2) incubator. Culture medium was changed three times per week.

Differentiation Agents

OS cells were cultured in the presence of $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ at concentrations of 1, 10, 100, or 1,000 nM or appropriate ethanol controls. Both $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$ were purchased from Enzo Life Sciences International, Inc. (Plymouth Meeting, PA). Stock solutions were prepared in ethanol and frozen at −20°C until use.

Cell Counting and Viability

Thirty thousand (3×10^4) cells were seeded in six-well plates. Cells were trypsinized and quantified with a Bright-Line Hemocytometer (Sigma-Aldrich, St Louis, MO) using trypan blue exclusion assay after 96 h of proliferation. Trypan blue dye is taken up by non-viable cells whose cell membrane is disrupted.

Cell Proliferation MTS-based Assay

Cell proliferation and viability were determined using the Cell Titer 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). The MTS cell proliferation assay is a colorimetric method to identify viable cells. Metabolism in viable cells produces "reducing equivalents" such as NADH or NADPH. These reducing compounds pass the electrons to an intermediate electron transfer reagent. This reagent reduces the tetrazolium product, 3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), into an aqueous, soluble formazan product. At death, cells rapidly lose the ability to reduce tetrazolium products. The production of the purple formazan product is proportional to the number of viable cells in culture.

Eight thousand cells (8×10^3) per well were seeded in 96-well plates. Cell proliferation was measured after 96 h according to manufacturer's instructions. Plates were read on a microplate reader at 450 nm instead of 490 nm due to filter availability. The absorbance spectrum of the formazan product shows an absorbance maximum at 490 nm but data can be recorded at wavelengths between 450–540 nm.

Immunolocalization of Ki 67, VDR, p53, and RB Protein

Thirty thousand cells (3×10^4) per well were seeded in six-well plates and treated with or without $1,25(OH_2D_3$ or $25(OH)D_3$. After 96 h, cells were washed 1× with Hank's Balanced Saline Solution (HBSS) and fixed at room temperature with 4% paraformaldehyde for 30 min. Cells were then washed with Tris-buffered saline-Tween, scraped into centrifuge tubes, and centrifuged for 5 min at 8,000*g*. The cell pellet was embedded into a paraffin block. Paraffin embedded cell blocks were made using the Hologic Cellient Automated Cell Block System (Hologic LP, Marlbough MA) according to the manufacturer's protocol. Paraffin blocks were sectioned at 4 μm, mounted on Superfrost + slides (Fisher Scientific, Pittsburgh, PA) and baked in a 65°C oven for 1 h. For VDR, p53 and RB immunocytochemistry, epitope retrieval was done by incubating the slides in citrate buffer (pH 6.0) for 5 min in the Biocare Decloaking Chamber (Concord, CA). The sections were processed for target antigen immunocytochemistry on a Dakocytomation autostainer according to the manufacturer's protocol (Dako, Carpentaria, CA). Primary antibodies to Ki-67 (clone MIB-1) and RB gene product (clone RB1) were purchased from Dako. Antibodies to VDR, and p53 (clone SP 5) were purchased from Santa Cruz, Inc. (Santa Cruz, CA), and Fisher Scientific, respectively. The Ki-67 and VDR antibodies were used at a concentration of 1:500 and immunoexpression was detected using peroxidase conjugated secondary antibody and a peroxidase substrate (3, 5-diaminobenzidine). Primary antibodies for RB gene product and p53 were used at a concentration of 1:100 and immunoexpression was detected using Envision + anti-mouse and DAB + Chromogen (Dako) for RB gene product and Envision + anti-rabbit and DAB + Chromogen for p53 (Dako) per kit instructions. The stained cells were imaged using a digital camera equipped microscope (DP2-BSW).

Cell Cycle Analysis

Three hundred thousand cells (3×10^5) were plated in T-25 flasks. Cell culture medium was removed at subconfluence (96–120 h); cells were trypsinized, resuspended in HBSS, and stored in ethanol at 4°C until further use. For cell cycle analysis, cells ($\geq 10^6$) were pelleted and resuspended in propidium iodide (PI) (50 μg/ml). Cells were then incubated in the dark at 37°C for 45 min after the addition of 100 μl of 1 mg/ml RNase. The cell cycle distribution is represented as the percentage of cells in sub G0, G0/G1, S, or G2/M phase. Samples were analyzed on BD LSR II flow cytometer in the Flow Cytometry Core Laboratory (The University of Kansas Cancer Center Shared Resource and Department of Microbiology, Molecular Genetics and Immunology, The University of Kansas Medical Center, Kansas City, KS).

ALP Enzyme Activity

Para-Nitrophenyl Phosphate (*p*NPP) was used as a substrate to quantify alkaline phosphatase (ALP) activity in cell cultures using colorimetric assay. Upon dephosphorylation by phosphate, *p*NPP turns yellow and is detected on a microplate reader.

Eight thousand cells (8×10^3) per well were plated in 24-well plates. Cells were maintained in a differentiating

medium containing ascorbic acid (50 μg/ml) for 10–12 days. Cells were cultured in serum-free media for 48 h. Cell lysate was collected after trypsinization and homogenization, and ALP activity in cell cultures was determined using colorimetric assay (AnaSpec, Inc. San Jose, CA). The manufacturer's protocol was followed except in the detection step, where 10 μl standards and samples were used per well instead of 50 μl. The change in protocol was made to avoid saturation of the substrate early after addition.

Analysis of In vitro Mineralization

The mineralization potential of OS cell lines in presence of $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$ was assessed by alizarin red (Sigma) staining. Eight thousand (143B) or 16,000 (SaOS-2) cells were seeded per chamber in four-chambered slides with 700 μl medium per well. Cells adhered overnight and ascorbic acid (50 μg/ml) was added to the medium, the following day. Beta-glycerophosphate (5 mM) was added upon confluence. After 21 days of culture, cells were washed with HBSS and fixed with paraformaldehyde (4%). Cells were washed with phosphate buffered saline (PBS) twice and stained with 2% alizarin red (pH 4.1–4.3) for 10 min. The slides were washed, air-dried, and mounted. The stained cells were imaged using a digital camera equipped microscope (Nikon SPOT microscope).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Studies for Evaluating the Osteocalcin (Mineralization Marker), mRNA Expression

Total RNA was extracted from control (untreated) and $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ treated (21 days) SaOS-2 and 143B OS cells using RNeasy Mini Kits (Qiagen; Santa Clara, CA). Reverse transcription and qPCR reactions were performed according to the instructions specified in the reverse transcription kit (Promega). The cDNAs were kept at $-20^{\circ}C$ until further use. Quantitative RT-PCR was performed using the standard protocol of Applied Biosystems 7500 Sequence Detection System and Software (Applied Biosystems; Foster City, CA). The qPCR reaction mixture consisted of 1:10 diluted cDNA, universal PCR Master Mix kit reagents, SYBR green, 900 nM of each primer. Primer pairs of osteocalcin (OCN) gene were synthesized according to the sequence information described by Atkins et al.[26] Relative expression of target gene (X_N) was calculated according to the comparative cycle threshold (ΔC_t) method,[27] using the formula $X_N = 2^{-\Delta Ct}$, where ΔC_t is the difference between C_t of target gene$-C_t$ of the endogenous control gene (GAPDH).

Apoptosis Detection Using Terminal Deoxynucleotide Transferase dUTP Nick End Labeling (TUNEL) and Annexin V-FITC and PI Flow Cytometry

Fragmentation of genomic DNA during apoptosis generates strand breaks, which can be detected by dUTP Nick End Labeling of free 3′ OH ends with modified nucleotides in an enzymatic reaction.

Cells are treated with proteinase K to induce membrane permeability to the reagents and enzymes necessary to label the DNA fragments. After cellular uptake of the reagents, the enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes the addition of labeled deoxyuridine triphosphate nucleotides (Br-dUTP) to 3′ OH ends of the multimers.

OS cells (143B: 8×10^3 or SaOS-2: 1.6×10^4) were plated per chamber in four-chambered slides. After 15 days of culture, cells were fixed with 4% paraformaldehyde $1\alpha,25(OH)_2D_3$. Detection and quantification of apoptosis was determined by TUNEL at the single cell level. Fragmentation was identified by labeling free 3′-OH terminal with modified nucleotides in an enzymatic reaction. Apoptotic cells were stained using the APO-BRDU kit (Phoenix Flow Systems, Inc. San Diego, CA). Manufacturer instructions were followed apart from the Detection Step, where slides were incubated in 3,3 Diamino benzidine (DAB) solution (DAB, H_2O_2\Urea in tap H_2O) for 30 min instead of 15 min. This step was modified to increase contrast in staining.

Annexin V-FITC staining can detect early stage apoptosis as it relies on the changes (like the externalization of phosphatidylserine (PS)) that occur prior to the loss of plasma membrane integrity. Apoptosis was detected using FITC annexin V apoptosis detection kit (BD Biosciences, MD). OS cells were plated in non-adherent 100 mm tissue culture petridish with or without 100 nM $1,25(OH)_2D_3$ or $25(OH)D_3$ for 4 and 15 days, respectively. All the cells were pelleted and prepared for Annexin V-FITC and PI staining according to the instructions provided in the kit. The samples were examined and analyzed on BD LSR II flow cytometer in the Flow Cytometry Core Laboratory (The University of Kansas Cancer Center Shared Resource and Department of Microbiology, Molecular Genetics and Immunology, The University of Kansas Medical Center, Kansas City, KS).

Analysis of Data

Excel and SAS were used for all statistical analysis. Means, standard deviations, and standard errors were calculated for all data. Proc mixed was used for one-way ANOVA to calculate the differences between treatment and control data sets. Comparison between least square means was conducted using Dunnett's adjustment since this the method of choice for samples containing controls. A p-value <0.05 was considered statistically significant.

RESULTS

Effect of $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ on Proliferation and Cell Cycle Distribution of OS Cell Lines

Immunolocalization of Ki 67, VDR, p53, and RB Protein

Ki67 (a marker of cellular proliferation) immunocytochemistry revealed no significant changes in Ki-67 or MIB-1 expression in SaOS-2 or 143B cells after 96 h treatment with $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ (Fig. 1). Both $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$ bind to VDR to mediate their biological effect. Both control and $1\alpha,25(OH)_2D_3$ (100 nM) treated SaOS-2 and 143B cells expressed VDR (Fig. 2). In response to increasing concentrations of $1\alpha,25(OH)_2D_3$, the expression of VDR increased in SaOS-2 cells. The expression of VDR was highly significant at 1,000 nM $1\alpha,25(OH)_2D_3$ when compared to control cells (vehicle treated). In 143 B cells, VDR was expressed in control and $1\alpha,25(OH)_2D_3$ treated cells but the difference was not significant. Both control and $1\alpha,25(OH)_2D_3$ (100 nM) treated SaOS-2 cells did not express p53 or RB tumor suppressor proteins, thereby confirming that SaOS-2 cells are p53 and RB null (Figs. 3 and 4). Both control and $1\alpha,25(OH)_2D_3$ (100 nM) treated 143B cells strongly expressed p53 and RB proteins (Figs. 3 and 4), but the difference in the staining intensity for p53 expression between control versus treated cells was not

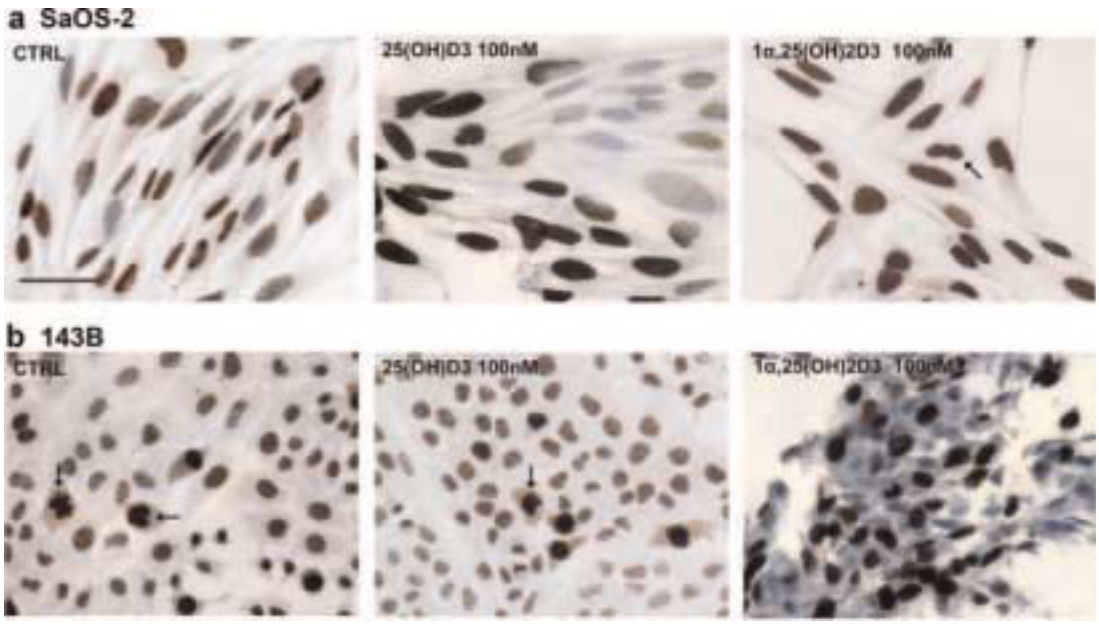

Figure 1. Photomicrographs of Ki67 immunostaining (a proliferation marker) after 96 h of incubation of OS cell lines treated with or without $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$. Top panel represents SaOS-2 control, 100 nM $25(OH)D_3$ and 100 nM $1\alpha,25(OH)_2D_3$ treatments and bottom panel represents 143B control, 100 nM $25(OH)D_3$ and 100nM $1\alpha,25(OH)_2D_3$ treatments. Brown staining indicates Ki67 expression. Arrows indicate an actively dividing cell where Ki67 is maximal (Bar = 50 μm).

significant. There was a slight increase in the expression of RB protein in $1\alpha,25(OH)_2D_3$ treated 143B cells versus control. Whether this difference is more evident at higher concentrations of $1\alpha,25(OH)_2D_3$ remains to be tested.

Cell Proliferation MTS-Based Assay

Neither $1\alpha,25(OH)_2D_3$ nor $25(OH)D_3$ had any significant effects on proliferation in SaOS-2 cells after 96 h, although there was a decrease in proliferation at 100 nM $1\alpha,25(OH)_2D_3$ concentration, compared to control (Fig. 5A). Proliferation was increased significantly (p-value < 0.05) in 143B cells exposed to $\geq$100 nM $25(OH)D_3$ or 10 nM $1\alpha,25(OH)_2D_3$, versus control after 96 h, as detected by MTS assay (Fig. 5B). At other concentrations, neither $1\alpha,25(OH)_2D_3$ nor $25(OH)D_3$ had any significant effect on proliferation.

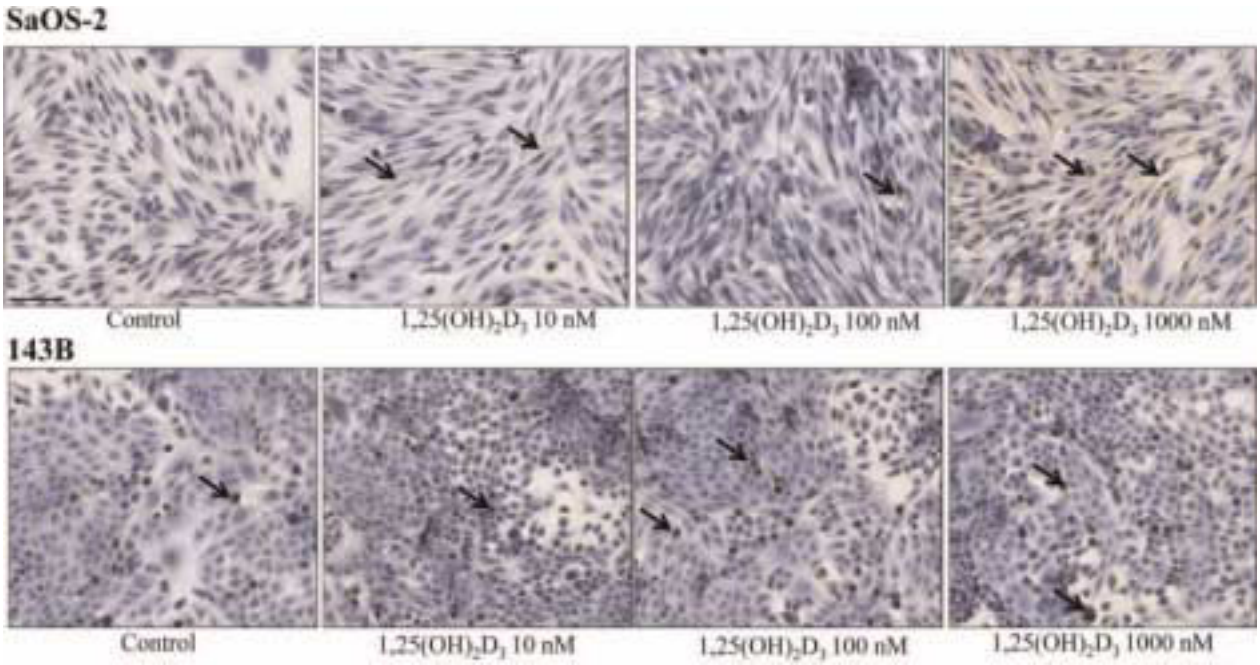

Figure 2. Photomicrographs of VDR immunostaining after 96 h of incubation treated with or without $1\alpha,25(OH)_2D_3$. Top panel represents SaOS-2 control, 10, 100 and 1,000 nM $1\alpha,25(OH)_2D_3$ treatments and bottom panel represents 143B control, 10, 100 and 1,000 nM $1\alpha,25(OH)_2D_3$ treatments. Brown staining indicates VDR expression (also indicated by arrows).

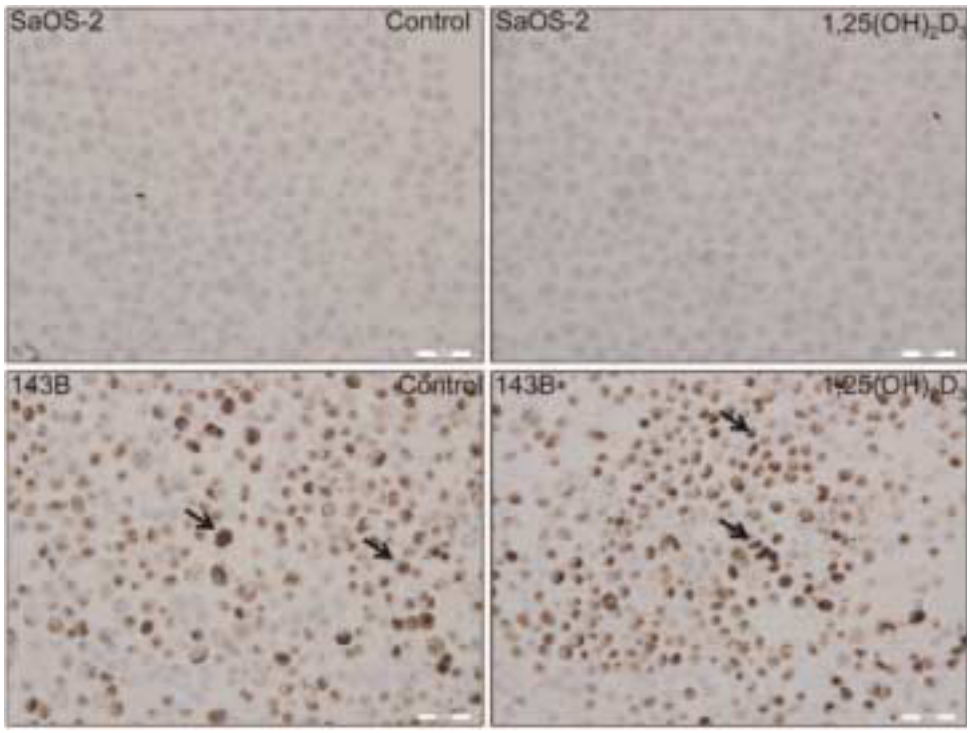

Figure 3. Photomicrographs of p53 immunostaining after 96 h of incubation treated with or without $1\alpha,25(OH)_2D_3$. Top panel represents SaOS-2 control and 100 nM $1\alpha,25(OH)_2D_3$ treatment and bottom panel represents 143B control and 100 nM $1\alpha,25(OH)_2D_3$ treatment. Brown staining indicates p53 expression (also indicated by arrows). (Bar = 50 μm)

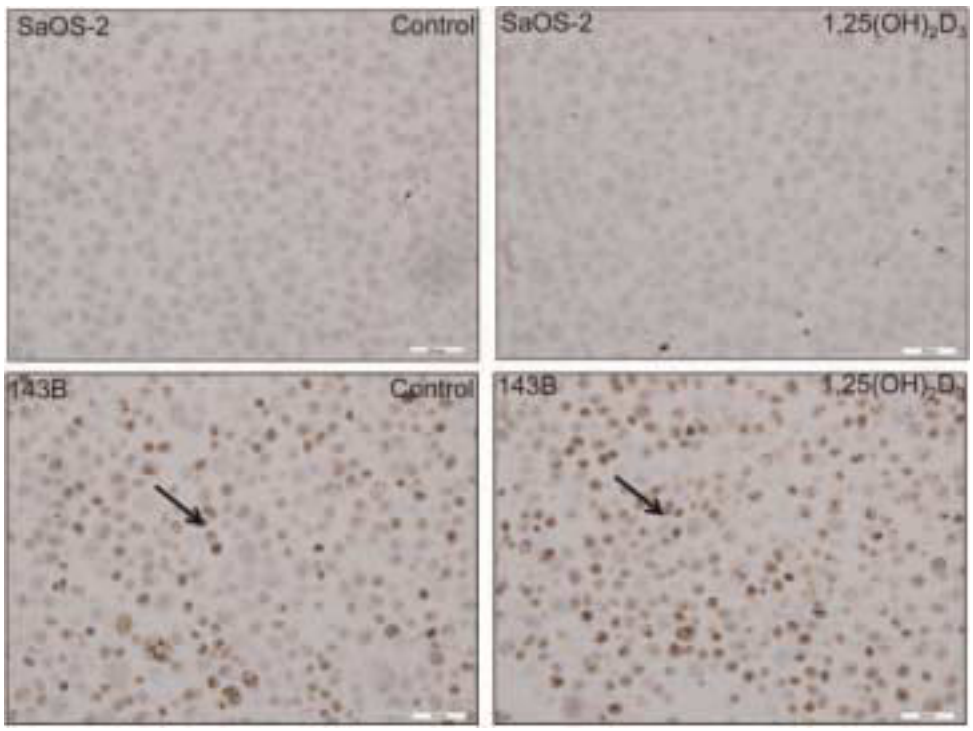

Figure 4. Photomicrographs of RB immunostaining after 96 h of incubation treated with or without $1\alpha,25(OH)_2D_3$. Top panel represents SaOS-2 control and 100 nM $1\alpha,25(OH)_2D_3$ treatment and bottom panel represents 143B control and 100 nM $1\alpha,25(OH)_2D_3$ treatment. Brown staining indicates RB expression (also indicated by arrows). (Bar = 50 μm)

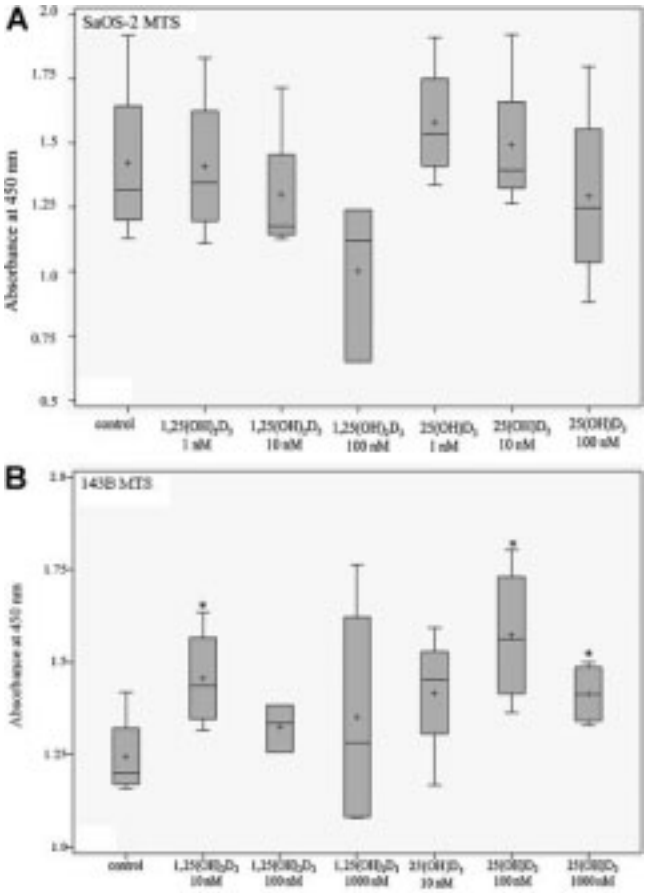

Figure 5. Boxed plots showing the effects of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ on cell proliferation in (A) SaOS-2 and (B)143B. Cells were cultured in 96-well plates and proliferation measured by MTS-based assay after 96 h. Mean and Median are represented by a " + " sign and a horizontal bar, respectively. The vertical bars indicate the range. (*Statistical significance, p value < 0.05)

Cell Cycle Analysis

Cell cycle distribution of SaOS-2 and 143 B cells treated with increasing concentrations of $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ was measured using flow cytometry (Fig. 6). In SaOS-2 cells, treatment with $25(OH)D_3$ at 1,000 nM for 96 h, cell cycle analysis revealed a significant increase (p-value < 0.05) in the percentage of cells in the G2/M phase and a significant decrease in the percentage of cells in G0/G1 phase versus untreated or control cells. Treatment with $1\alpha,25(OH)_2D_3$ at 100 and 1,000 nM resulted in more cells in Sub G0 and G2/M phase versus control untreated SaOS-2 cells. There were no significant effects on cell cycle distribution in 143B cells with $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$ treatment (Figs. 6A,B,C).

Effect of $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ on Differentiation and Apoptosis of OS Cell Lines

ALP Enzyme Activity

ALP, an early marker of osteoblastic differentiation, activity was measured via colorimetric assay. In 143B cells treated with $1\alpha,25(OH)_2D_3$, ALP activity was significantly increased dose-dependently ($p < 0.05$) from 100 nM versus control. In SaOS-2 cells, $1\alpha,25(OH)_2D_3$ was able to significantly increase ALP activity versus control (Fig. 7A,B) only at 100 nM concentration.

OCN mRNA Expression

OCN, a late marker for osteoblastic differentiation and an important marker for matrix mineralization was evaluated by real time qPCR. In SaOS-2 cells, a significant increase in OCN mRNA was observed at 1,000 nM for both $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ versus control (Fig. 8A). At 100 nM concentration, OCN mRNA expression was greater for $1\alpha,25(OH)_2D_3$ than $25(OH)D_3$. In 143B cells, with $25(OH)D_3$, an increase was seen only at 1,000 nM concentration (Fig. 8B). There was an increase in OCN mRNA expression at both 100 and 1,000 nM (significant) concentrations with $1\alpha,25(OH)_2D_3$.

In Vitro Mineralization

Both SaOS-2 and 143B cells formed multiple mineralization nodules in culture after 21 days, and nodule formation peaked at 100 nM concentrations for both $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ as examined by alizarin red staining (Fig. 9). SaOS-2 cells were more mineralization competent than 143B cells as evident by alizarin red staining.

Apoptosis

Evidence of apoptosis was clearly detected by TUNEL staining in both cell lines treated with either $25(OH)D_3$ or $1\alpha,25(OH)2D_3$ at $\geq$100 nM. A significant induction of DNA fragmentation (p-value < 0.05) was observed with 25D at 1,000 nM in both cell lines (Figs. 10 and 11). Annexin V-FITC staining revealed a significant increase in the percentage of late stage apoptotic 143B cells when treated with 100 nM concentrations $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$ for 96 h (See Fig. 1, Supplementary Information in the online version of this article). In SaOS-2 cells we did not see any significant increase in the apoptotic cells in vitamin D treated cells versus control, at 96 h time point.

DISCUSSION

Recent studies have demonstrated that calcitriol ($1\alpha,25(OH)_2D_3$) exerts antineoplastic properties against several cancers including breast,[28,29] prostate,[30,31] and colon[32] cancer. The mode of action underlying the antineoplastic properties of $1\alpha,25(OH)_2D_3$ is mainly by modulating cellular proliferation, differentiation, and apoptosis of cancer cells. In this pilot study, we investigated the dose-response to $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ on proliferation, cell cycle, differentiation, and apoptosis in human OS cell lines.

From our study, it is clearly evident that neither $25(OH)D_3$ nor $1\alpha,25(OH)_2D_3$ inhibit cellular proliferation at the early time points. It was expected from previous investigations[11,13–15,19,20,33–36] that $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ at concentrations as low as 10 nM would inhibit OS cell proliferation in a dose dependent manner. The lack of effect on cellular proliferation by

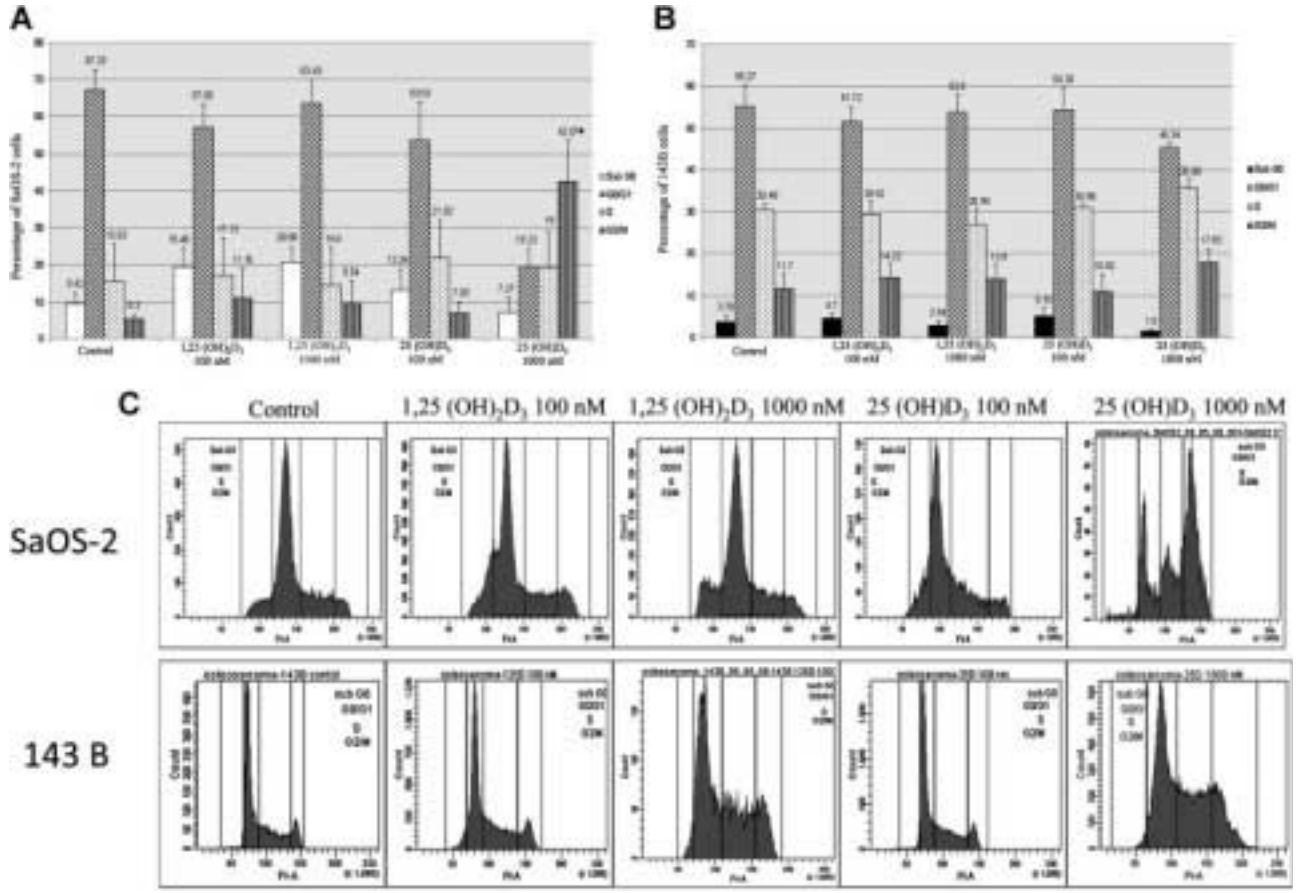

Figure 6. Effects of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ on cell cycle distribution as analyzed by flow cytometry in (A) SaOS-2 cells and (B) 143B cells. Figure 6C shows a representative cell cycle distribution profile. Cells were cultured in T-25 flasks and cell cycle distribution was analyzed at subconfluence (96–120 h). All data points are means ± SEM ($n \geq 3$). (*Statistical significance, p value <0.05)

$1\alpha,25(OH)_2D_3$ has been observed in rat OS cells.[18] There exist several instances in the literature which point toward cell specific mechanisms by which vitamin D modulates proliferation and differentiation of normal and tumor cells. Lutzow-Holm et al.[37] reported that topical application of $1\alpha,25(OH)_2D_3$ and KH1060 (vitamin D analogue) induces hyperproliferation of mouse keratinocytes while Bollag et al.[38] observed a biphasic effect of $1\alpha,25(OH)_2D_3$ (low concentration: Stimulatory and high concentrations: Inhibitory) on proliferation of mouse keratinocytes. Another reason for the lack of antiproliferative effect in our study, possibly might be due to an early endpoint (96-hour post-plating) which was perhaps not long enough for $1\alpha,25(OH)_2D_3$ to mediate its inhibitory effect on OS cells. The transition from proliferation to differentiation stage could be a critical time point for $1\alpha,25(OH)_2D_3$ to mediate its inhibitory effect on SaOS-2 and 143 B OS cell lines. The switch to differentiation mode is not possibly rapid in some cancer cells. Instead, $1\alpha,25(OH)_2D_3$ may cause an initial increase in proliferation followed by an inhibition as seen in in vitro cultures of monocytes, HL60, or U937 cells.[39–41] Other studies have required serum-reduced medium to observe $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$-mediated antiproliferative effects.[20,36] Recent studies by Piek et al.,[42] demonstrate that $1\alpha,25(OH)_2D_3$ does not affect the kinetics of cellular proliferation of human mesenchymal stem cells (hMSCs) (similar to our studies) but, instead induces changes in cell cycle related gene expression during differentiation.

In general, treatment of cells with $1\alpha,25(OH)_2D_3$ mostly results in the accumulation in the G1 phase.[43,44] In our study we observed that in both cell lines, $25(OH)D_3$ at high concentrations (1,000 nM) resulted in remarkably altered pattern of distribution of cells in various stages (G1, G2/M, and S phase) compared to control. With $1\alpha,25(OH)_2D_3$ an increase in G2/M population in both the cell lines versus control was observed (Fig. 6). Although the exact sequence of events leading to cell-cycle arrest in G1 phase is not clear and may be specific for each cell type, several effectors and their pathways play an important role in orchestrating G1 arrest. These include upregulation of RB,[45] cell cycle dependent kinase inhibitors p21 WAF1/Cip1 and p27 Kip1,[46–48] and inhibition of c-Myc gene expression.[49] G2/M transitions are under the regulatory control of cyclin B-Cdk1 complex. The G2/M arrest induced by $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ is less frequent and possible mechanisms involved in G2/M

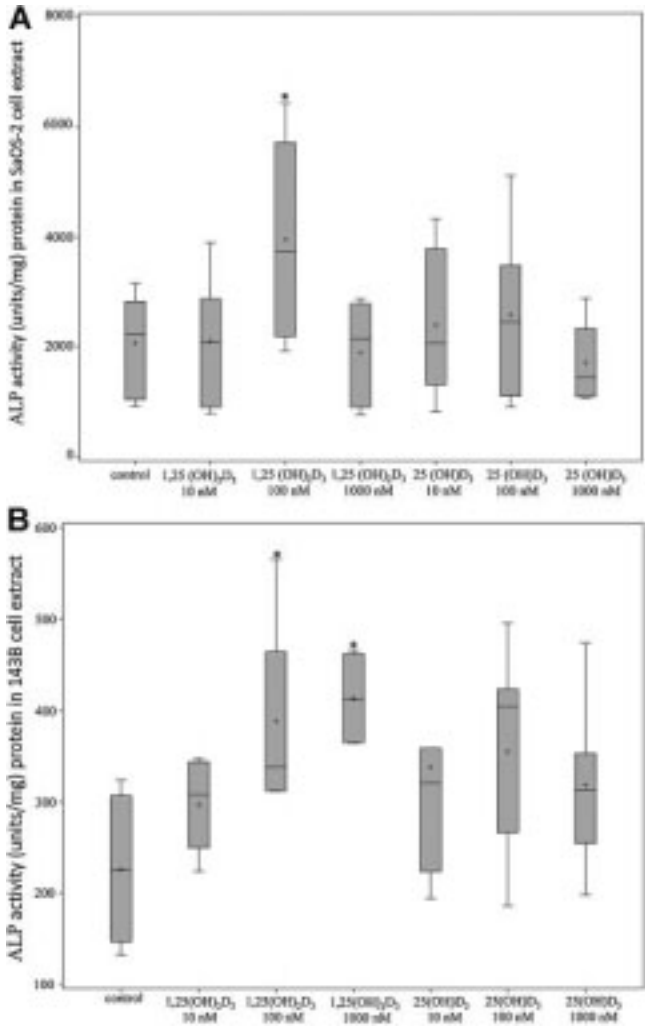

Figure 7. Effects of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ on osteogenic differentiation in (A) SaOS-2 cells and (B) 143B cells. Cells were cultured in 24-well plates in a differentiating medium for 12–14 days. Para-Nitrophenyl Phosphate (pNPP) was used to quantify ALP activity in cell cultures. Mean and Median are represented by a " + " sign and a horizontal bar, respectively. The vertical bars indicate the range. (*Statistical significance, p value < 0.05)

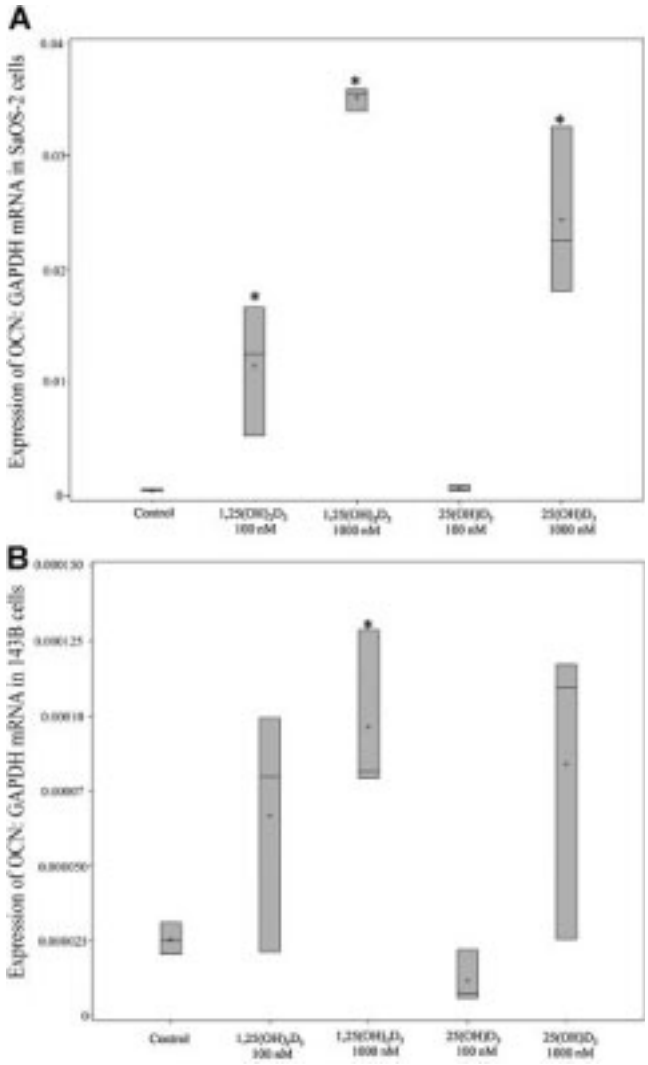

Figure 8. Boxed plots showing the effects of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ on the expression of osteocalcin mRNA in (A) SaOS-2 cells and (B) 143B. Cells were cultured in 6-well plates and total RNA was isolated after 21 days from vitamin D treated and untreated OS cultures. Mean and Median are represented by a " + " sign and a horizontal bar, respectively. The vertical bars indicate the range. (*Statistical significance, p value < 0.05)

arrest might be due to decreased levels of CDK1.[50] Other factors that could possibly contribute toward G2/M arrest include differential expression of cohesion, separase, or Polo-like kinase (all of which play an important role in orderly separation of DNA into daughter cells). In our p53 and RB immunolocalization studies, we confirmed the null status of p53 an RB expression in SaOS-2 cells, and strong expression of the above two tumor suppressor proteins in 143B cells. Both p53 and RB are important regulators of cell cycle progression and play an important role in OS pathogenesis. Studies have demonstrated that loss of p53 or RB increases the incidence of OS[1] and p53 and RB deficient mice models develop bone lesions, and have high incidence of OS.[51,52] Both p53 and RB are also linked to osteogenesis mainly via regulation of runx2 expression and activity.[53–57]

OS results from the malignant transformation of osteoblasts.[58] The use of current chemotherapy regimens primarily results in necrosis of malignant osteoblasts. In the differentiation of osteoblasts, a specific order of gene expression exists. Stimulation of differentiation of SaOS-2 and 143B in the presence of $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ was assessed by measuring ALP enzymatic activity, OCN mRNA expression, and mineralization of extra-cellular matrix (ECM). ALP plays a significant role in facilitating bone mineralization and is an early marker of osteoblast differentiation.[16] In this study, $1\alpha,25(OH)_2D_3$ at higher concentrations significantly increased ALP activity in 143B cells but not in SaOS-2 cells. The stimulation of ALP activity by $1\alpha,25(OH)_2D_3$ at higher concentration in 143B cells supports the study hypothesis that $1\alpha,25(OH)_2D_3$ can promote OS cell differentiation, and

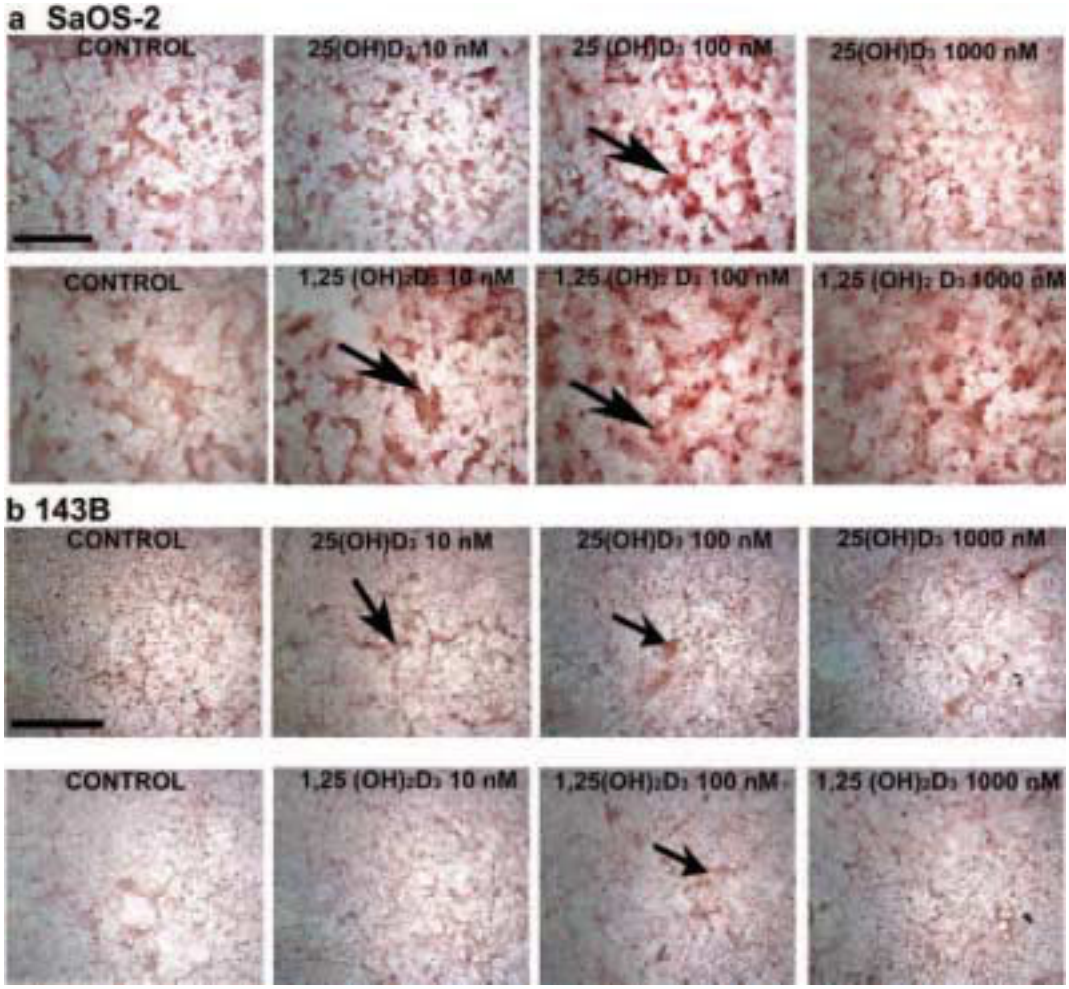

Figure 9. Photomicrograph composite showing alizarin red staining of SaOS-2 (top two panels) and 143B (Lower two panels) (Bar = 500 μm). Mineralization nodules are visualized by reddish colored staining of calcium phosphate deposits (shown by arrow).

conforms with research in other human OS cells.[15,33,36,58–61] The ability of $1\alpha,25(OH)_2D_3$ to promote ALP activity at 100 nM concentration and not at higher concentrations. This finding suggests that SaOS-2 cells show an increased ALP activity in response to an effective threshold concentration of $1\alpha,25(OH)_2D_3$. SaOS-2 cells had 12-fold higher baseline ALP activity levels than 143B cells as well as an elevated expression of other osteoblastic markers found in literature.[19,26,62]

OCN is a marker for osteoblastic differentiation and an important indicator for matrix maturation and mineralization.[63] The present study demonstrated that OCN mRNA expression was upregulated in $1\alpha,25(OH)_2D_3$ treated SaOS-2 and 143B cells. The ability of $1\alpha,25(OH)_2D_3$ to stimulate OCN has been previously reported in bone cells and human dental follicle cells in vitro.[64,65]

Both SaOS-2 and 143B cells formed multiple mineralization nodules in culture by 12 days, and nodules appeared to increase in size by 21 days at higher concentrations for both $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ as examined by alizarin red staining. The presence of greater number of mineralized nodules in SaOS-2 cells suggests that these cells respond better to both forms of vitamin D than 143 B cells, and induce the expression of gene regulating osteoblastic differentiation and mineralization.

Collectively, the results suggest that both 25D and $1\alpha,25(OH)_2D_3$ at higher concentration (100–1,000 nM) have a direct effect on the mineralization functions and induce maturation and osteogenic differentiation of OS cells, in vitro. Both $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ help promote differentiation toward a functionally mature osteoblastic phenotype as suggested by increased ALP activity, OCN expression, and mineralization of ECM. The effects of $1\alpha,25(OH)_2D_3$ on mineralization are species and stage specific. Treatment of mouse osteoblasts with $1\alpha,25(OH)_2D_3$ stimulated ALP activity only in the early phase of differentiation and not during mineralization,[59] whereas in rat osteoblasts, $1\alpha,25(OH)_2D_3$ inhibited the expression of ALP during the proliferative stage, but stimulated ALP activity during mineralization.[66] In human pre-osteoblast cell line SV-HFO, $1\alpha,25(OH)_2D_3$ treatment during pre-mineralization phase only accelerated mineralization

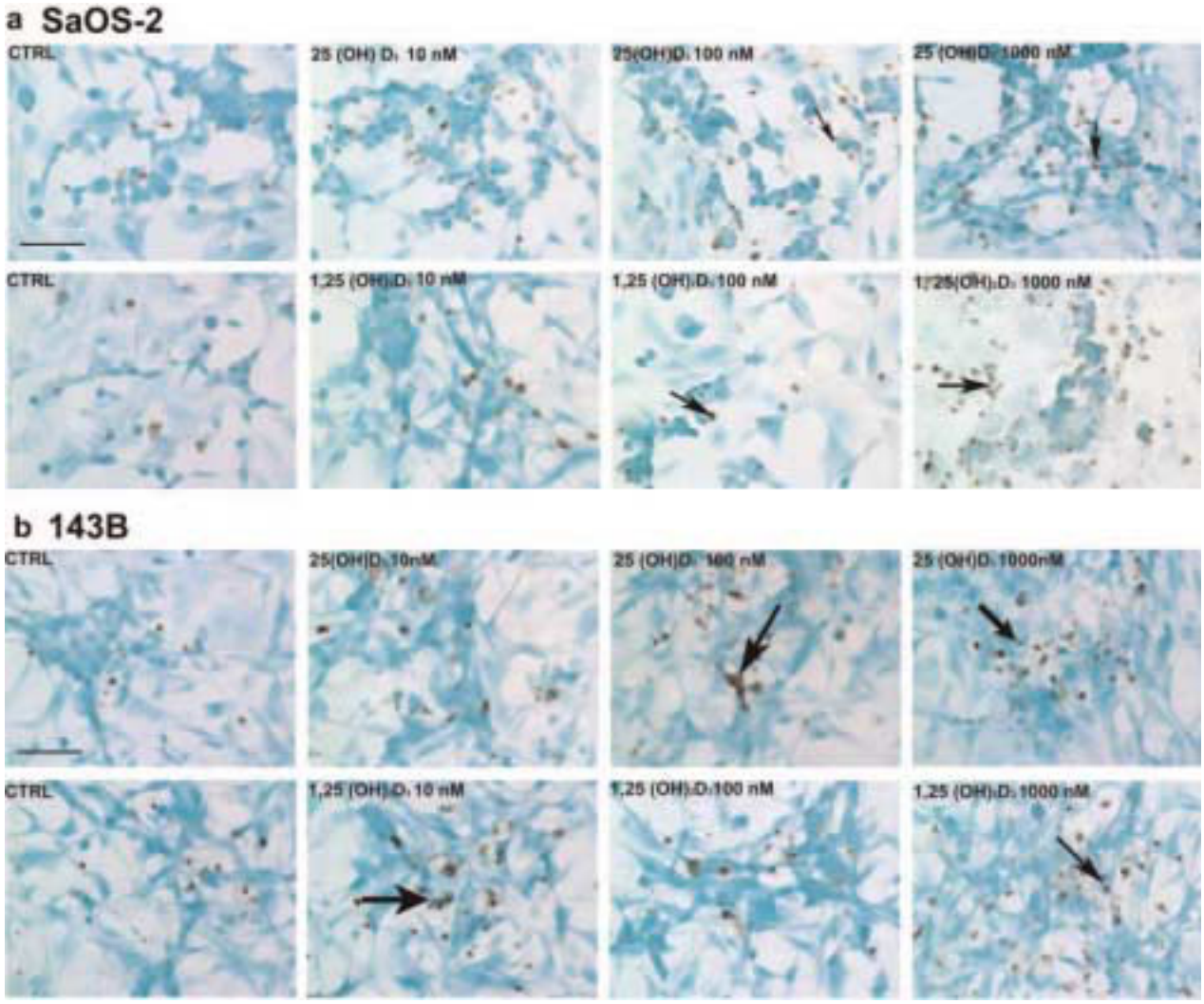

Figure 10. Photomicrograph composite showing TUNEL staining (Fig. 5) of SaOS-2 (top two panels) and 143B (Lower two panels) (Bar = 50 μm). Brown peroxidase staining indicates TUNEL positive apoptotic cells.

by enhancing the production of mineralization competent matrix vesicles.[67]

The mechanism(s) underlying $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ mediated maturation and differentiation of OS cells is not clear and is currently under intense investigation. Atkins et al.[26] reported the presence of 1α hydroxylase, an enzyme that converts $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ in SaOS-2 cells, and demonstrated that silencing of Cyp27B1 mRNA by RNAi, abolishes the synthesis of $1\alpha,25(OH)_2D_3$ and suppresses OCN and CYP24 mRNA expression in HOS cells.[68] Thus, the effects of $25(OH)D_3$ on differentiation of OS cells are likely to be mediated by 1α hydroxylase and VDR activity. Recent studies from Geng et al.[69] reported that the prodifferentiation effects of $25(OH)D_3$ on hMSCs are mainly mediated by CYP 27B1.

Piek et al.[42] suggested a role of c MYC in promoting $1\alpha,25(OH)_2D_3$ induced differentiation of human MSCs. We are currently investigating the expression and activity of RUNX-2, a transcriptional regulator of osteogenesis in $1\alpha,25(OH)_2D_3$ induced differentiation of OS cells. Alternatively, $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ may mediate osteoblastic differentiation of osteoblasts and pre-osteoblasts via upregulation of IGF-1.[70–72]

The current pilot study suggested that cells treated with $25(OH)D_3$ showed evidence of dose-dependent increase in apoptosis in both the cell lines. With $1\alpha,25(OH)_2D_3$ although an increase in apoptosis was observed versus control, the dose-response was not as clear as seen with $25(OH)D_3$ Our TUNEL studies indicate that there is a positive correlation between differentiation and apoptosis, in response to $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$. The increase in calcium ion concentration in mineralizing or differentiating OS cells in response to $1\alpha,25(OH)_2D_3$ could induce apoptosis via activation of calpains.[73] In breast cancer cell line MCF-7, $1\alpha,25(OH)_2D_3$ and analog EB1089 reduce the expression of *bcl*-2 (cell death inhibitor or anti-apoptotic) gene and facilitate the localization of Bax (a cell death promoter or pro-apoptotic) protein to the

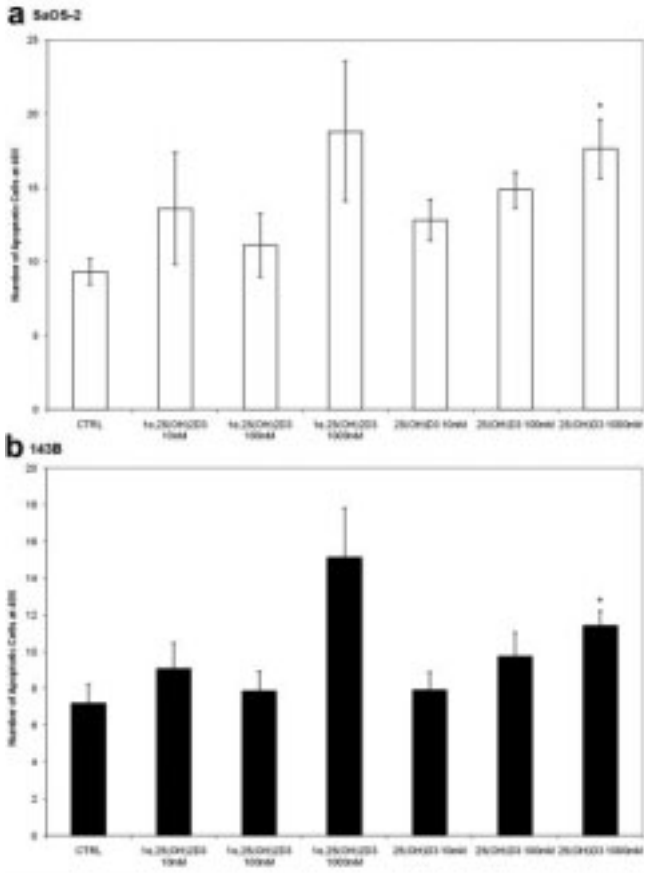

Figure 11. Semiquantitative analysis of apoptotic cells after treatment with $1\alpha,25(OH)_2D_3$ or $25(OH)D_3$ in (A) SaOS-2 and (B) 143B cells. Cells were cultured on chamber slides and apoptotic cells were identified via TUNEL staining after 15 days. Five sites on each slide were selected at random and apoptotic cells were counted at 40× using a scaled standard. All data points are means ± SEM. (N = 5; *Statistical significance, p value <0.05; Student's t-test)

mitochondria and induce apoptosis.[74–76] Another mediator of apoptosis is Calbindin 28K, which is a vitamin D induced protein and induces apoptosis in a number of cells.[77] The mechanism underlying the exact timing and occurrence of major cellular events (i.e., whether a cancer cell undergoes apoptosis or differentiation or apoptosis as a consequence of differentiation in the presence of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$) depends on the cell cycle stage, presence of specific regulators, and relative concentrations of tumor suppressor versus oncogenic proteins. The molecular basis of initiation of apoptosis from a differentiating OS cell is unclear.

There are some limitations in the current study. This study overlooks the complexity of the in vivo tumor environment where there is interplay between different cell types and signaling pathways, and tumor heterogeneity both of which could affect tumor growth and progression and response to therapy. In vitro studies are important for gathering preliminary data on the effect of $1\alpha,25(OH)_2D_3$ on OS cell lines and can provide the foundation for future in vivo and translational studies. A second limitation of the study is the small sample and lack of power. This study serves as a pilot for future study and the results need to be reproduced on a larger scale with adequate statistical power. No adjustment for multiple comparisons was done, as the study was a pilot study with low power.

Knowledge gained from this pilot study provides a better insight into understanding the mechanism of action of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ on OS cells and may provide the framework for future in vivo studies. Given the prevalence of OS in humans and canines, a better understanding of the underlying mechanisms of disease are important for identification of biomarkers that may provide targets for devising novel therapies. The ability of $25(OH)D_3$ or $1\alpha,25(OH)_2D_3$ to promote normal osteogenic differentiation and modulate apoptosis in OS cells may be useful in the future to improve the current treatment regimens and consequent rates of survival.

In conclusion, this pilot study investigated the effects of $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ on differentiation and apoptosis in human OS cell lines, SaOS-2 and 143B. Our studies suggest that $25(OH)D_3$ and $1\alpha,25(OH)_2D_3$ act as differentiation agents in human OS cell lines through activation or upregulation of markers of osteoblastic differentiation, including ALP in 143B cells, mineralization and OCN expression in SaOS-2 and 143B cells. Both SaOS-2 and 143 B cells showed evidence of an increasing trend in the number of TUNEL positive apoptotic cells with $25(OH)D_3$ (dose-dependent) and $1\alpha,25(OH)_2D_3$ treatments.

ACKNOWLEDGMENTS

The authors thank Dr. Slusser and Mr. Hastings for their help with Flow Cytometry, Ms. Roach for help with imaging slides, Ms. Bhamidi for preparing samples for immunocytochemistry and flow cytometry, and Ms. Danley for helping with Ki-67, p53, and RB immunocytochemistry. They also thank Dr. Jinxi Wang for allowing them to use his PCR equipment.

Funding sources for this study include:

(i) School of Health Professions (SHP)—Start-Up Funds, SHP-Research Award to Rama Garimella, PhD, MS, M.Sc., University of Kansas Medical Center, Kansas City, KS.
(ii) Speas Clinical Nutrition Research Student Project for supporting MS Thesis Project of Lindsey Thompson, MS, RD—Department of Dietetics and Nutrition, University of Kansas Medical Center, Kansas City, KS.
(iii) Research Grant for Pilot Studies—Ossama Tawfik, MD, PhD, Department of Pathology, University of Kansas Medical Center, Kansas City, KS.

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