THE DOSE-RESPONSE OF VITAMIN D ON CELL PROLIFERATION, DIFFERENTIATION AND APOPTOSIS IN HUMAN OSTEOSARCOMA CELLS

by

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B.S., University of Missouri-Columbia, 2007

Submitted to the graduate degree program in Dietetics and Nutrition and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Osteosarcoma is a malignant bone tumor predominantly affecting children and adolescents. Osteosarcoma has a 60-70% survival rate with current treatments; hence there is a need to identify novel adjuncts to chemotherapeutic regimens. The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25D), is being increasingly recognized for its anti-cancer properties. The dose-response of 1,25D and 25-hydroxyvitamin D_3 (25D) was examined on human osteosarcoma cell lines, SaOS-2 (tumorigenic, p53 null, metastatic) and 143B (tumorigenic, ras gene transformed and highly metastatic). It was hypothesized that both forms of vitamin D would inhibit proliferation, stimulate differentiation and induce apoptosis of these cells in a dose-dependent manner. Osteosarcoma cell lines, SaOS-2 and 143B, were treated with 1,25D, 25D or an ethanol control respectively at concentrations ranging from 1-1000nM. Cellular proliferation was measured after 1,25D or 25D exposure using a cell viability assay (MTS), Ki67 immunocytochemistry and cell cycle analysis by flow cytometry. Osteoblastic differentiation was measured by alkaline phosphatase (ALP) activity, osteocalcin secretion and *in vitro* osteoblastic mineralization. Apoptosis was determined by Terminal Deoxynucleotide Transferase dUTP Nick End Labeling (TUNEL). Neither 25D nor 1,25D inhibited proliferation or affected cell cycle in SaOS-2 or 143B cells, although in 143B cells, proliferation was increased significantly in cells exposed to 25D at 1000nM versus control. Markers of osteoblastic differentiation were upregulated. In particular, a significant increase in ALP in

143B cells and mineralization in SaOS-2 and 143B cells was observed. The effect of 1,25D on apoptosis in SaOS-2 and 143B cells was not significant; 25D at high concentration (1000nM) increased numbers of apoptotic cells significantly ($p<0.05$). Biological differences between SaOS-2 and 143B control cells were observed. Cell cycle analysis revealed significantly more SaOS-2 control cells in G0/G1 than 143B, and significantly fewer SaOS-2 cells in synthesis phase than 143B ($p<0.05$). SaOS-2 control cells had ALP levels significantly higher than 143B cells ($p<0.05$). Both forms of vitamin D (25D and 1,25D) did not inhibit proliferation but acted as differentiation agents in SaOS-2 and 143B cells through activation or upregulation of markers of osteoblastic differentiation, including ALP and/or osteoblastic mineralization. At high concentration (1000nM), 25D increases apoptosis. There are also inherent differences in the biology of SaOS-2 and 143B osteosarcoma cells which modulate 1,25D response.

ACKNOWLEDGEMENTS

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Funding:
School of Allied Health- Start-Up Funds, Rama Garimella, PhD, KU Medical Center, Kansas City, KS
Spears Grant- Department of Dietetics and Nutrition, KU Medical Center, Kansas City, KS
Research Grant for Pilot Studies- Ossama Tawfik, MD, PhD, Department of Pathology, KU Medical Center, Kansas City, KS

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ABBREVIATIONS

1,25D: 1α,25-Dihydroxyvitamin D_3

25D: 25-Hydroxyvitamin D_3

AICR: American Institute of Cancer Research

ALP: Alkaline Phosphatase

DMEM: Dulbecco's Modified Eagle's Medium

ELISA: Enzyme-linked Immunoassay

FBS: Fetal bovine serum

HBSS: Hank's Balanced Saline Solution

KU Medical Center: University of Kansas Medical Center

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

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

CHAPTERS

CHAPTER 1 INTRODUCTION

Osteosarcoma is a malignant bone tumor predominantly affecting children and adolescents (1). Current treatment regimens do not significantly improve the present 60-70% survival rate. Therapies in osteosarcoma 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 those individuals with ineffective treatments.

The active form of vitamin D, 1α,25-dihdroxyvitamin D_3 (1,25D) is increasingly recognized for its anti-cancer properties (4, 5). The main biologic function of 1,25D 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 1,25D (7). By binding to the nuclear vitamin D receptor (VDR), 1,25D regulates expression of genes responsible for cellular proliferation, differentiation, apoptosis and angiogenesis in local tissues (11, 12). *In vitro* and *in vivo*, 1,25D induces anti-neoplastic activity in tumors of the colon (13, 14), prostate (15-17) and breast (18).

In human osteosarcoma cells, 1,25D inhibits cell proliferation at concentrations 0.1-100nM in a time- and dose-dependent manner (11, 19-28). At concentrations of 10nM-100nM, 1,25D enhances the differentiation of osteosarcoma cell lines into cells that exhibit properties of mature osteoblasts

(20, 21, 23, 24, 26, 28-31). In human osteosarcoma cells, 1,25D stimulates the expression of markers of osteoblastic differentiation, including osteocalcin (21-25, 27, 30, 31), alkaline phosphatase (20, 21, 26, 28, 30, 31), and mineralization (25, 29) through incorporation of calcium into the extracellular matrix. Type I collagen is expressed after exposure to 1,25D (29, 32, 33) early in differentiation of osteosarcoma cells.

The mechanisms by which 1,25D regulates proliferation and differentiation in osteosarcoma are not completely understood. Several studies suggest 1,25D exerts an antiproliferative effect by arresting cells in the G1 phase of the cell cycle (20, 24, 27, 28). It is postulated that G1 arrest might be due to an antiapoptotic effect (34), an antiproliferative effect (24) or a combination of both.

Evidence that 1,25D induces apoptosis in canine osteosarcoma cells comes from TUNEL studies detecting DNA fragmentation, a hallmark of apoptotic cells (33, 35). Studies in rodent and human osteosarcoma cell lines are contradictory. These studies show that 1,25D either inhibits (26, 34) or have no effect on apoptosis (22, 23). Numerous signaling pathways are regulated by 1,25D and 1,25D-induced apoptosis varies among cell types (19, 20, 28). Further study is necessary to explore these pathways and the role of 1,25D in modulating apoptosis of osteosarcoma cells.

Justification for Further Investigation

There are significant unknowns and contradictions in current literature on 1,25D and osteosarcoma. The dose-response of 1,25D on different osteosarcoma cells is not well-identified. The mechanisms involving inhibition of proliferation and promotion of differentiation remain unclear. The effects of 1,25D on apoptosis in osteosarcoma cells are particularly contradictory, warranting further investigation, including examination of pro- and anti-apoptotic protein expression.

The potential of the inactive form of vitamin D, 25-hydroxyvitamin D_3 (25D), to locally convert to active 1,25D and inhibit tumorigenesis and progression is a new finding (7, 25). The effect of exogenous 25D on these cellular processes in human osteosarcoma cells is not clearly defined. The effects of 1,25D or 25D on 143B, a highly metastatic human osteosarcoma cell line, remain largely unknown. Given that osteosarcoma is a highly aggressive and malignant disease, results with regard to this cell line are of significant interest.

A better understanding of the underlying mechanisms of disease is important to identify targets for devising novel therapies given the prevalence of osteosarcoma in children and adolescents. The results from this *in vitro* study will provide the foundation for future studies using *in vivo* animal models and in clinical chemotherapy and treatment.

Statement of Purpose

The objectives of this study were:

a) to determine the dose-response of vitamin D (25D or 1,25D) on osteosarcoma cell lines

b) to identify the effects of vitamin D (25D or 1,25D) on cellular processes in osteosarcoma cells

Research Questions

Primary Question:

What is the dose-dependent response of vitamin D, (25D or 1,25D) on osteosarcoma cell lines, SaOS-2 and 143B?

Secondary Questions:

1. What is the *in vitro* effect of vitamin D (25D or 1,25D) on cellular proliferation in human osteosarcoma?
2. What is the *in vitro* effect of vitamin D (25D or 1,25D) on differentiation in human osteosarcoma, as analyzed by expression of differentiation markers osteocalcin, alkaline phosphatase and mineralization competence?
3. What is the effect of vitamin D (25D or 1,25D) on apoptosis in human osteosarcoma cells?

Hypothesis

It is hypothesized that vitamin D (both 25D and 1,25D) will inhibit proliferation, stimulate differentiation and induce apoptosis of human osteosarcoma cell lines in a dose-dependent manner.

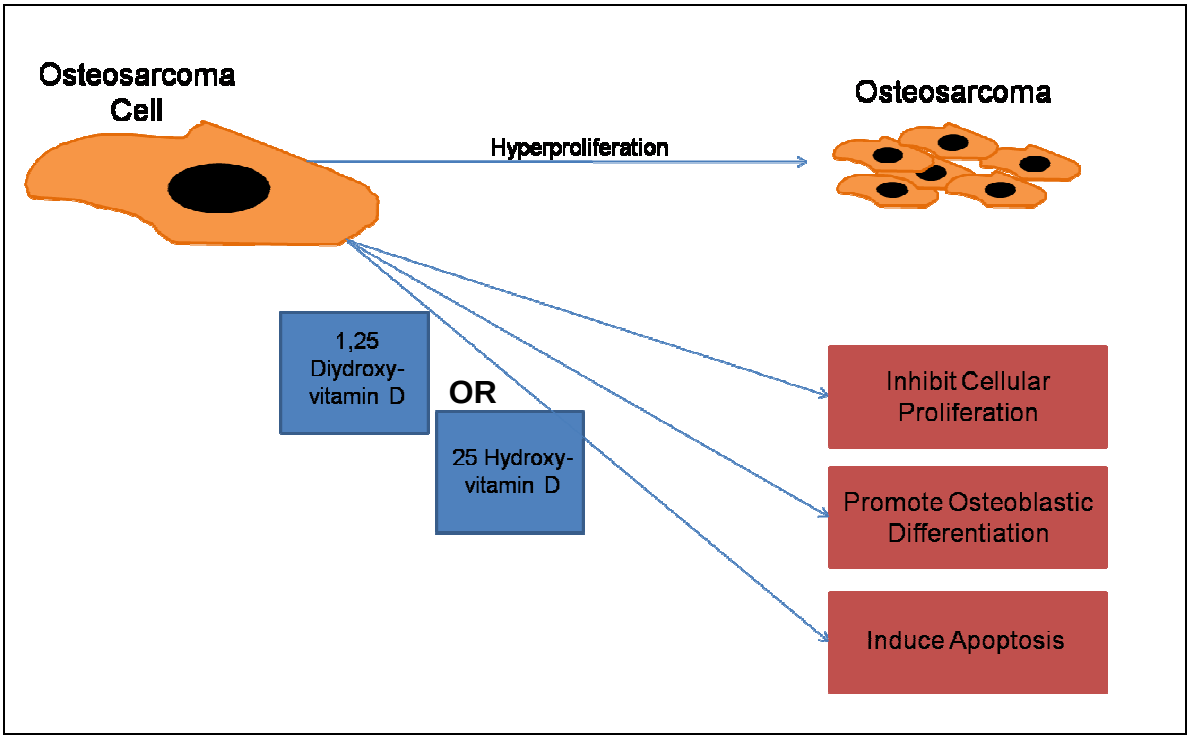


FIGURE 1. Hypothesis schematic

CHAPTER 2 LITERATURE REVIEW

Osteosarcoma

Osteosarcoma is a malignant bone tumor predominantly affecting children and adolescents (1, 36, 37). Osteosarcoma is associated with lung metastases, characterized by uncontrolled proliferation, insensitivity to growth inhibitory signals and evasion of apoptosis (1, 19). As with many other forms of cancer, its etiology is not known and is likely multifactorial.

Prior to the introduction of chemotherapy, osteosarcoma survival rates were only 10% to 20%. Surgical resection, usually amputation, was the only treatment available (3, 36-40). More recently, chemotherapy regimens combined with surgical resection have resulted in improved patient outcomes (41, 42). Long-term survival rates are now 60% to 70% in patients with localized disease (2, 3). The most effective chemotherapeutic agents for OS treatment include high-dose methotrexate, cisplatin, doxorubicin, etoposide and ifosfamide. All are associated with toxicities (43, 44) and may induce debilitating side effects (3). Patients who present with metastatic disease have only a 20% to 30% survival rate five years after diagnosis regardless of treatment (2, 3, 37).

Chemotherapeutic efficacy and patient survival has reached a plateau with currently available treatments. New treatment regimens and novel adjuncts to chemotherapy are necessary for those patients with ineffective treatment regimens. The active form of vitamin D could be a novel adjunct to chemotherapy improving survival rates and decreasing toxicity observed in osteosarcoma.

Vitamin D

Vitamin D is a secosteriod humans acquire from their diet, dietary supplements and exposure to sunlight. Upon ingestion or absorption through the skin, the biologically inert vitamin D_3 is hydroxylated in the liver to form 25D (8, 9, 45). In the kidney, 25D is further hydroxylated by the enzyme 25D-1α-hydroxylase into its active form, 1,25D (8-9, 45). The major circulating metabolite of vitamin D is 25D and blood levels serve as the best indicator for vitamin D status (25). Since the kidneys tightly regulate the production of 1,25D, serum levels do not rise in response to increased exposure to sunlight or increased intake of vitamin D (8).

The main biologic function of 1,25D is to maintain serum calcium levels within normal range. It increases the efficiency of intestinal absorption of dietary calcium (6, 7) and mobilizes calcium stores from the bone into the circulation through the action of osteoclasts (8-10). In addition to its calcemic actions, 1,25D and its analogues are now recognized to influence regulation of the cell-cycle (4, 5). While the kidneys are the source of circulating levels of 1,25D (8, 9, 45), bone and other extra-renal tissues synthesize 1,25D (25, 46). This locally produced 1,25D can bind to the VDR and activate or repress gene expression through 1,25D-responsive elements located in the promoter regions of various target genes (11). In an autocrine or paracrine manner, 1,25D influences more than 200 genes, including genes responsible for the regulation of cellular proliferation, differentiation, apoptosis and angiogenesis (9, 12, 25, 46).

Vitamin D and Cancer

The anti-neoplastic activity of 1,25D is seen in the tumors of the prostate (15-17), breast (18) and colon (13, 14) *in vitro* and *in vivo*. The mechanisms underlying the antineoplastic activity of 1,25D could be mediated by both genomic and non-genomic pathways. In osteoblasts, cytoplasmic 1,25D signaling initiates non-genomic pathways via classic VDR mechanisms. This process includes modulation of the electrical state of the plasma membrane, elevation of intracellular calcium and activation of protein kinases (19). The transcriptional and post-transcriptional control of gene expression may be mediated by 1,25D (26). By binding to the nuclear VDR, 1,25D functions as a transcription factor and modulates the expression of genes involved in cell cycle progression (45). Tissues express 25-hydroxyvitamin D-1α- hydroxylase and produce 1,25D locally. It is hypothesized that cancer may be prevented by controlling genes that regulate cellular proliferation and differentiation (12). There is also evidence that cross-talk between non-genomic and genomic pathways occurs (19).

In vitro studies show local 1,25D synthesized in osteoblasts regulates proliferation and induces differentiation in normal osteoblasts of human (29, 47), rodent (48) and canine (32) origin. Human osteoblasts responded to exogenous 1,25D by decreasing their rate of proliferation, and increasing their expression of osteocalcin (7, 47), a marker of osteoblast differentiation. Siggelkow et al. found

similar increases in osteocalcin and alkaline phosphatase in osteoblasts incubated with 1,25D (49).

In the presence of exogenous 25D, osteoblasts were shown to undergo molecular changes at the transcriptional level, following metabolism of 25D to 1,25D (7, 25). Proliferation is inhibited and differentiation induced in normal human osteoblasts after treatment with 25D (25).

Growth Inhibitory Effects of 1,25D in Osteosarcoma Cells

Current literature suggests cancerous cells of bone, including osteosarcoma, synthesize 1,25D (25, 46). Many studies report 1,25D inhibits osteosarcoma cell proliferation in a time- and dose-dependent manner (11, 19-24, 26-28, 50, 51). **Table 1** summarizes the studies demonstrating an antiproliferative effect of 1,25D in osteosarcoma.

The amount of 1,25D required to produce antiproliferative effects in cellular models varies. The majority of studies have found 10nM effective to in inhibiting cellular growth. Employing a range of dosages below 10nM, Ryhanen et al. observed 50% inhibition (IC_{50}) of proliferation in MG-63 osteosarcoma cells was 120pM or 0.12nM (24). Dose-response studies are needed to determine the effective dosages in various cell lines.

TABLE 1. Antiproliferative effects of 1,25D in osteosarcoma cells[1]

Study	Cells	Dose 1,25D	Mechanism
Ryhanen et al. 2003[24]	MG-63	1pM-100 nM	G1 arrest, increased p27, reduced p21, hypophosphorylation of Rb protein
Li et al. 2008[26]	MG-63	0.01-1 nM	Not studied
Finch et al. 2001[51]	MG-63	0.01-1000 nM	Not studied
Barroga et al. 1999[50]	Canine POS	0.1-10 nM	Not studied
Tsuchiya et al. 1993[21]	OST	0.1-1000 nM	Not studied
Witasp et al. 2005[22]	TE-85	1-50 nM	Not studied
Zenmyo et al. 2001[28]	MG-63	1-100 nM	G1 arrest, p-53 independent induction of p21 (Waf1/Cip1)
Chattopadhyay et al. 2003[11]	MG-63	1-1000 nM	Not studied
Matsumoto et al. 1998[20]	MG-63	10 nM	G1 arrest, induction of p21 (Waf1/Cip1), decreased activity of Rb protein
Wu et al. 2007[19]	SaOS-2	10 nM	G1 arrest, MAPK /AP-1/p21 pathway activation
Atkins et al. 2007[25]	MG-63, SaOS-2, HOS, G-292	10-100 nM	Not studied
Hansen et al. 2001[23]	MG-63, SaOS-2	100 nM	Not studied
Ryhanen et al. 1998[27]	MG-63	100 nM	G1 arrest
Morales et al. 2004[34]	Rat UMR-106	10 nM	G1 arrest[2]

[1]Unless otherwise noted (Canine POS, Barroga et al, Rat UMR-106, Morales et al.), all cells are human osteosarcoma cell lines
[2]No inhibition of proliferation was noted in this study

Mechanisms Involved in 1,25D Effects on Proliferation

The mechanisms by which 1,25D regulates proliferation in osteosarcoma are not fully understood. The progression of the cell cycle from the G1 phase to the S phase is known to be a critical step in the control of cell proliferation (52). Cells that progress into the S phase synthesize new DNA in preparation for cell replication while cells resting in G1 may differentiate. Because the severity of a tumor is inversely correlated with the degree of cell differentiation (53), any effect of 1,25D to arrest cells in the G1 phase could be an important feature of the vitamin.

Several studies in osteosarcoma cells demonstrate that 1,25D exerts its antiproliferative effect by arresting cells in the G1 phase of the cell cycle (20, 24, 27, 28) giving cells the opportunity to terminally differentiate.

In these cells, 1,25D may regulate one or more factors critical for the G1/S transition in the cell cycle (54). The main cyclin-dependent kinases (Cdk) involved in G1 phase are Cdk2, Cdk4 and Cdk6, and potential mediators in G1 phase block are the Cdk inhibitors (CKIs), p21 and p27 (7). Several studies show 1,25D binds to the VDR which functions as a transcription factor to modulate expression of various genes involved in cell cycle progression (9, 45, 55, 56). The specific proteins responsible for the 1,25D mediated G1 arrest are not yet known.

Differentiation of Osteosarcoma Cells by 1,25D

Treatment with 1,25D results in the differentiation of osteosarcoma cells to mature osteoblasts (29, 20, 21, 23-26, 28, 30, 31). Differentiation studies in human osteosarcoma cells report that, 1,25D at concentrations of 10nM-100nM, stimulates the expression of several markers of osteoblastic differentiation including osteocalcin (21-25, 27, 30, 31), alkaline phosphatase (20, 21, 26, 28, 30, 31), and incorporation of calcium into the extracellular matrix (25, 29). Early in osteoblastic differentiation, type I collagen is expressed after exposure to 1,25D (29, 32, 33). **Table 2** summarizes the results of studies of the effects of 1,25D on markers of osteoblastic differentiation.

The ability of 1,25D to enhance the expression of these differentiation markers in osteosarcoma cells could be an important attribute of the vitamin in modulating osteosarcoma. Osteocalcin secretion and mineralization are important late markers of osteoblastic differentiation. Alkaline phosphatase activity and type I collagen production are proportional to the degree of osteogenesis (6). Differentiation of those cells shows a progression away from nonspecific proliferation and towards defined lineage-specific growth and development. The results in the *in vitro* literature suggest that 1,25D promotes terminal differentiation of cancerous cells into normally functioning osteoblasts.

TABLE 2. Osteogenic markers induced or enhanced in osteosarcoma cells after treatment with 1,25D or 25D[1]

Study	Cells	Dose 1,25D	Markers Induced or Enhanced
Atkins et al. 2007[25]	MG-63, SaOS-2, HOS, G-292	10-100 nM 1,25D 10-100 nM 25D	Mineralization Osteocalcin
Barroga et al. 2000[32]	Canine POS	10 nM	Alkaline Phosphatase Osteocalcin Type I Collagen
Hansen et al. 2001[23]	MG-63, SaOS-2	100 nM	Osteocalcin
Li et al. 2008[26]	MG-63	0.01-10 nM	Alkaline Phosphatase
Matsumoto et al. 1998[21]	MG-63	10 nM	Alkaline Phosphatase
Matsumoto et al. 1991[29]	MC3T3-E1	0.3 nM	Alkaline Phosphatase Type I Collagen Mineralization
Nozaki et al. 1999[33]	Canine POS, 53C, 53D, 14A	0.1-1000 nM	Alkaline Phosphatase Osteocalcin Type I Collagen
Rao et al. 2001[30]	SaOS-2	0.1-1000 nM	Alkaline Phosphatase Osteocalcin
Ryhanen et al. 1998[27]	MG-63	0.1-1000 nM	Osteocalcin
Sunita Rao et al. 2002[31]	MG-63	1000 nM	Osteocalcin
Tsuchiya et al. 1993[21]	OST	0.1-1000 nM	Alkaline Phosphatase Osteocalcin
Witsap et al. 2005[22]	Rat UMR-106	1-50 nM	Osteocalcin
Zenmyo et al. 2001[28]	MG-63	1-100 nM	Alkaline Phosphatase Osteocalcin

[1]Unless otherwise noted, all cell lines are human osteosarcoma lines

Apoptosis in Osteosarcoma Cells after Treatment with 1,25D

Apoptosis generates characteristic morphological and biological changes in the cellular life cycle ending in cell death. Apoptosis is an important factor in cell-cycle regulation of all cells (57). It is an important contributor to growth suppression in hyperproliferative states like cancer (35).

It is hypothesized that the anti-proliferative activity of 1,25D may be attributed to 1,25D-induced apoptosis (33, 35). The vitamin D-modulated G1 arrest observed in earlier literature (19, 20, 23, 27, 28, 34) is due to an antiapoptotic effect (34), an antiproliferative effect (24) or a combination of both. *In vitro* studies report 1,25D is capable of inducing apoptosis as confirmed by DNA fragmentation in canine osteosarcoma (33, 35). The results of studies in rodent and human osteosarcoma cells show 1,25D or 25D inhibited (26, 34) or had no effect on apoptosis (22, 23), although studies in canine osteosarcoma cells were performed at higher dosages (**Table 3**).

In the presence of apoptosis-inducing agents, the literature is also contradictory: 1,25D is either protective (23, 34, 58) or fails to protect cells from death (22). It is suggested that this discrepancy is due to cell density (cells per milliliter or total cells/ml) at initiation of the experiment. Evidence suggests that at high density (100% confluence), the majority of cells are killed by 1,25D, whereas at lower density (60-70% confluence), 1,25D exerts anti-apoptotic effects during the initial days of culture, followed by cell death upon prolonged cultivation (22).

TABLE 3. Summary of research on apoptosis in osteosarcoma cells treated with 1,25D or 25D[1]

	Study	OS Cell lines	Vitamin D (1,25D) Dose
Induces Apoptosis	Barroga et al. 1998[35]	Canine POS	1-1000 nM
	Nozaki et al. 1999[33]	Canine POS, 53C, 53D, 14A	0.1-1000 nM
Inhibits Apoptosis	Li et al. 2008[26]	MG-63	1 nM
	Morales et al. 2004[34]	Rat UMR-106	10 nM
Does Not Affect Apoptosis	Hansen et al. 2001[23]	MG-63 and SaOS-2	1-100nM 1,25D 1-100nM 25D
	Witasp et al. 2005[22]	Rat UMR-106 and Human TE-85	1-50 nM

[1]Unless otherwise noted, all cell lines are human osteosarcoma lines

Because of the contradictory evidence found in the literature, signaling pathways involved in the apoptosis regulatory effects of 1,25D need to be identified. A number of markers involved in the process are now known or identified.

The chain of events in apoptosis is highly ordered and includes the sequential activation of different members of a family of proteases called caspases (59). Morales et al. demonstrated that caspase-3, 8 and 9 activities significantly decreased in osteosarcoma cells after treatment with 10nM 1,25D for 2 days (34). This verifies a major role for 1,25D in their observed antiapoptotic effect. Witasp et al. demonstrated that 1,25D failed to activate caspase 3-like enzymes in rat UMR-106 and human TE-85 osteosarcoma lines (22).

Members of the Bcl-2 protein family have the ability to modify the apoptotic pathway by regulating caspase activity. Bcl-2 and BAX are two members of this protein family shown to be involved in apoptosis. Bcl-2 exerts an antiapoptotic effect by inhibition of cytochrome c release, while BAX asserts a pro-apoptotic effect by increasing cytochrome c release (60).

The active form of vitamin D protects normal thyrocytes from undergoing apoptosis by up-regulating Bcl-2 expression, an anti-apoptotic protein (61). Hansen et al. suggest that up-regulation of Bcl-2 may play a central role in the inhibition of apoptosis observed after 1,25D treatment in osteosarcoma (23). Morales et al. showed increased expression of Bcl-2 and reduced levels of BAX in human osteosarcoma cells after treatment of 10nM 1,25D for 24h (34). In breast cancer cells, 1,25D induces apoptosis resulting from the down-regulation of the anti-apoptotic bcl-2 protein (18). Down-regulation of bcl-2 has yet to be illustrated in osteosarcoma cells. It is obvious 1,25D regulates the bcl-2 family proteins. It is unclear what mechanism ultimately affects cell apoptosis. Apoptosis by 1,25D in osteosarcoma cell lines may involve additional signaling pathways but these are unknown.

The Effects of 25D in Osteosarcoma

There is little information available in the literature comparing the effects of 25D versus 1,25D treatment in osteosarcoma cells. In 2002, Gurlek et al. found osteoblast-like cells respond transcriptionally to physiological concentrations (100nM) of 25D by conversion of 25D to 1,25D (7). Atkins et al. further investigated the effects of 25D on cellular processes in a variety of osteosarcoma cell lines. Consistent with 25D's conversion to 1,25D, exposure to 25D at 100nM inhibited cellular growth to the same extent as 1,25D at a pharmacological level (1nM). Messenger RNA expression of osteocalcin and mineralization increased (25).

Gaps in Current Research and Targets for Future Directions

In some osteosarcoma cells 1,25D exerts an antiproliferative effect and is a potent prodifferentiation agent. In human osteosarcoma cells, 1,25D inhibits growth in a time- and dose-dependent manner and stimulates the expression of osteocalcin and alkaline phosphatase and mineralization in human osteosarcoma cells.

There remain significant unknowns and contradictions in current literature on 1,25D and osteosarcoma. The effective dosages of 1,25D in osteosarcoma cells are not well summarized and mechanisms involved in these actions remain unclear. The research on the role of 1,25D in modulating apoptosis of cancer cells is contradictory at present. The potential of 25D to metabolize and exhibit

similar effects to 1,25D is not adequately studied and merits further research. The current study examines the dose response of 1,25D and 25D in modulating proliferation, differentiation and apoptosis in a comprehensive manner.

CHAPTER 3 METHODS

The purpose of this study was to investigate the dose response of vitamin D on the processes of cellular differentiation, proliferation and apoptosis in human osteosarcoma cell lines, SaOS-2 and 143B. Concentrations of 1-1000nM 1,25D and 25D were tested versus ethanol controls in osteosarcoma cells. A variety of assays, immunocytochemical and staining techniques were used to examine changes in cell differentiation, proliferation and apoptosis in these cells.

Setting

All activities took place at the University of Kansas Medical Center (KU Medical Center) in the Bone Research Lab, Department of Pathology and Laboratory Medicine and the Department of Dietetics and Nutrition.

Ethics

This study qualifies as exempt from Human Subjects Approval as the cell lines used are considered *secondary data,* or data collected prior to the research for a purpose other than the proposed research. The cells used in this study are publicly available and contain no identifying markers.

Materials

Cell Culture

Human osteosarcoma cell lines, SaOS-2 and 143B (American Type Culture Collection; Manassas, VA) were used in this study. SaOS-2 and 143B cells are tumorigenic with an osteoblast-like phenotype. SaOS-2 is p53 null and metastatic (62), while 143B is ras gene transformed and highly metastatic (66).

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℃ in a humidified (95% air: 5% CO_2) incubator. Culture medium was changed 3 times per week.

Agents

Osteosarcoma cells were cultured in the presence of 1,25D or 25D at concentrations of 1nM, 10nM, 100nM or 1000nM or appropriate ethanol controls. Vitamin D stocks (1,25D and 25D) were purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). Stock solutions were prepared in ethanol and frozen at -20ºC until use.

Data Collection and Procedures

Cell Counting and Viability

Thirty thousand (3×10^4) cells were seeded in 6-well plates. Cells were trypsinized and quantified with a Bright-Line Hemocytometer (Sigma-Aldrich, St Louis, MO) using trypan blue exclusion assay after 96h of proliferation. Trypan blue dye is taken up by non-viable cells whose cell membrane is disrupted.

Fifty microliter cell suspension was removed and diluted 1:10 with 0.4% Trypan blue. Suspension was mixed thoroughly and incubated at room temperature for 5 minutes. Approximately 1 µl of suspension was placed in a hemocytometer and the number of viable (unstained) and nonviable (stained) cells in the corner quadrants were counted (n=3).

The average number of cells in the four quadrants (N) was determined and the cells per ml were determined using the formula:

Cells per ml= N (average number of cells) x 2500 x 10 (dilution factor)

Cell Proliferation MTS –based Assay

Cell proliferation and viability was determined using the Cell Titer 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI; Cat# TB245). 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-carboxymethoxyphenyl)-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 96h according to manufacturer's instructions. Plates were read on a microplate reader at 450nm instead of 490nm due to filter availability. The absorbance spectrum of the formazan product shows an absorbance maximum at 490nm but data can be recorded at wavelengths between 450-540nm.

Ki67 Immunocytochemistry

Thirty thousand cells (3×10^4) were seeded in 6-well plates. After 96h, cells were washed 1X with Hank's Balanced Saline Solution (HBSS) and fixed at room temperature with 4% paraformaldehyde for 30 minutes. Cells were washed with Tris-buffered saline – Tween, scraped into centrifuge tubes, and centrifuged for 5 minutes. The cell pellet was embedded into a paraffin block. Paraffin blocks were sectioned at 4 µm, mounted on Superfrost + slides and baked in a 65℃ oven for one hour. The sections were processed fo r Ki67 immunocytochemistry on a Dakocytomation Autostainer according to the manufacturer's protocol (Dakocytomation, Carpinteria, CA). Antibody to Ki67

(clone MIB-1) was purchased from Dakocytomation, and Ki-67 expression was detected using peroxidase conjugated secondary antibody and a peroxidase substrate (3, 5-diaminobenzidine).

Flow Cytometry Analysis

Three hundred thousand cells (3×10^5) were plated in T-25 flasks. Cell culture medium was removed at subconfluence (96-120h); cells were trypsinized, resuspended in HBSS and stored in ethanol at 4℃ until further use. The day of flow cytometry analysis, cells ($\geq 10^6$) were pelleted and resuspended in Propidium Iodide (50 µg/ml). Cells were incubated in the dark at 37℃ for 45 min after the addition of 100 µl of 1mg/ml RNase. Samples were analyzed on BD LSR II equipment in the Flow Cytometry Core Laboratory at KU Medical Center (Department of Microbiology, Molecular Genetics and Immunology).

Alkaline Phosphatase 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 48h. Cell lysate was

collected and alkaline phosphatase (ALP) activity in cell cultures was determined using colorimetric assay (AnaSpec, Inc. San Jose, CA; Cat#71230). The manufacturer's protocol was followed except in the detection step, 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.

Osteocalcin Enzyme Immunoassay (ELISA)

The human osteocalcin enzyme immunoassay (ELISA) is an analytical method using enzyme-linked antibodies as reagents to quantitate osteocalcin secretion on a microtiter plate. Bound enzyme labeled antibody is measured through a chromogenic reaction. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which is proportional to osteocalcin concentration.

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). After 10-12 days, cells were maintained in serum-free media for 48h. Cell lysate was collected and secretion of osteocalcin was determined by enzyme-linked immunoassay (ELISA; Invitrogen, Inc. Camarillo, CA; Cat#KAQ1381) according to manufacturer's protocol. Osteocalcin in each sample was determined from the standard curve.

Osteocalcin Immunocytochemistry

Eight thousand (143B) or 16,000 (SaOS-2) cells were seeded in chamber slides with 700 µL medium per well. Cells adhered overnight and ascorbic acid (50 µg /ml) was added the following day. Beta glycerophosphate (5mM) was added upon confluence and cells grew an additional 5 days. After 14 days, cells were washed with HBSS and fixed with paraformaldehyde (4%). Cells were washed twice with phosphate-buffered saline (PBS). Cells were treated with 0.3 % Triton X-100 in PBS for 15 minutes. The permeabilized cells were washed three times with PBS followed by incubating with 0.3 % H_2O_2 for 15 minutes to block endogenous peroxidase activity. Cells were washed with PBS prior to incubating with 1.5 % blocking serum for 1h (to reduce non-specific binding of primary antibody), followed by a 3h incubation with primary antibody. Polyclonal goat anti-human osteocalcin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and osteocalcin immunocytochemistry was performed according to the manufacturer's instructions.

In vitro Mineralization

Staining by the von-Kossa method is used for demonstrating calcium or calcium salt deposits. Cells are treated with silver nitrate solution. The calcium is reduced by UV light, replaced with silver deposits. The bone mineral is blackened by the deposition of silver and the osteoid is counterstained with Fast Red dye (Sigma-Aldrich, St Louis, MO).

Eight thousand (143B) or 16,000 (SaOS-2) cells were seeded in chamber slides with 700 µL medium per well. Cells adhered overnight and ascorbic acid (50 µg /ml) was added the following day. Beta glycerophosphate (5mM) was added upon confluence and cells grew an additional 5 days. After 14 days, cells were washed with HBSS and fixed with paraformaldehyde (4%). Cells were washed with PBS twice. Mineralization through incorporation of calcium into the extracellular matrix was determined by von-Kossa staining per standard protocol (63) with minor adaptations.

Standard protocol changes included: after adding 50% $AgNO_3$ dropwise to each specimen, slides were incubated at room temperature for 10 minutes. The unbound $AgNO_3$ was removed by washing the slides twice with distilled H_2O. Slides were then incubated under UV light for 60 minutes. The original protocol was changed to avoid non-specific binding of silver nitrate.

Apoptosis Detection Using Terminal Deoxynucleotide Transferase dUTP Nick End Labeling (TUNEL)

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) is a technique for detection of apoptotic cells. Fragmentation of genomic DNA during apoptosis generates strand breaks, which can be identified by labeling free 3’ OH ends with modified nucleotides in an enzymatic reaction.

Cells are treated to produce membrane permeability to the reagents and enzymes necessary to label the DNA fragments. After cellular uptake of the reagents, the enzyme TACS TdT catalyzes the addition of labeled bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) to 3′ OH ends of the multimers.

Eight thousand (143B) or 16,000 (SaOS-2) cells were plated in chamber slides. Cell culture medium was removed after 15 days and the cells were fixed with paraformaldehyde (4%). Detection and quantification of apoptosis was determined by TUNEL at 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; Cat# AH1001). Manufacturer instructions were followed apart from the Detection Step, where slides were incubated in DAB solution (DAB, H_2O_2\ Urea in tap H_2O) for 30 minutes instead of 15 minutes. This step was modified to increase contrast in staining.

Analysis of Data

Excel software (Microsoft Corp., Redmond, WA, USA) was used for all statistical analysis. Means, standard deviations and standard error of the mean were calculated for all data. Paired Student's t-Tests were used to analyze differences in experimental and control data. A p value <0.05 was considered statistically significant.

CHAPTER 4 RESULTS

The objectives of the current study were to determine the dose-response and identify the effects of vitamin D (25D and 1,25D) on cellular processes in osteosarcoma cells. The effects of 25D and 1,25D (1-1000nM) on proliferation, differentiation and apoptosis in human osteosarcoma cell lines SaOS-2 and 143B were examined.

The human osteosarcoma cell lines SaOS-2 and 143B were used in this study (**Figure 2**). Both lines are tumorigenic. The source of the SaOS-2 cell line is an 11-year-old Caucasian girl (64). It is p53 null and metastatic (62). SaOS-2 cells have a high proliferative capacity and are known to possess high affinity receptors for 1,25D (65). The 143B cell line is a Ki-ras oncogene transformation of the HOS osteosarcoma cell line (66) and is highly metastatic.

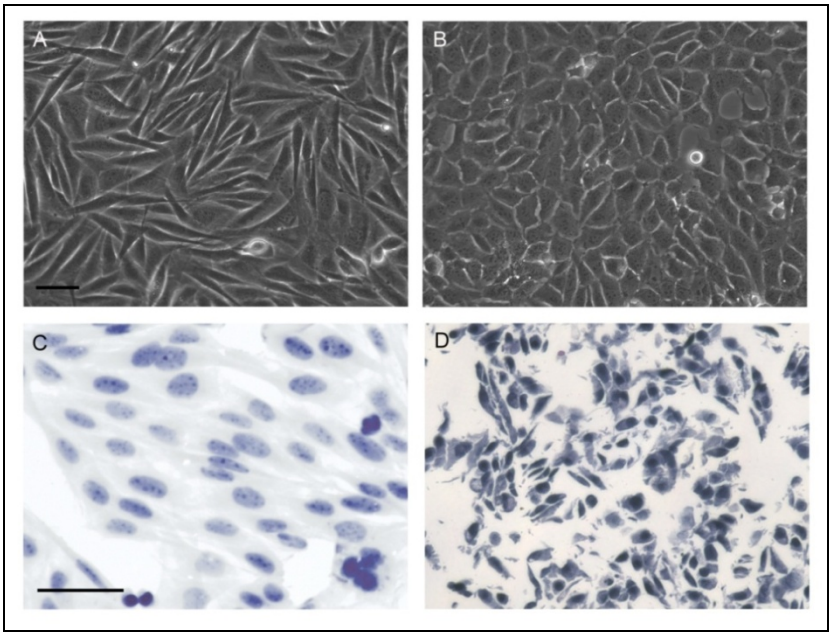


FIGURE 2. (A-B) Phase-contrast photomicrographs of (A) SaOS-2 and (B) 143B cells at confluence in culture. (C-D) Photomicrographs showing Hematoxylin Staining of (C) SaOS-2 and (D) 143B cells (Bar= 50 microns).

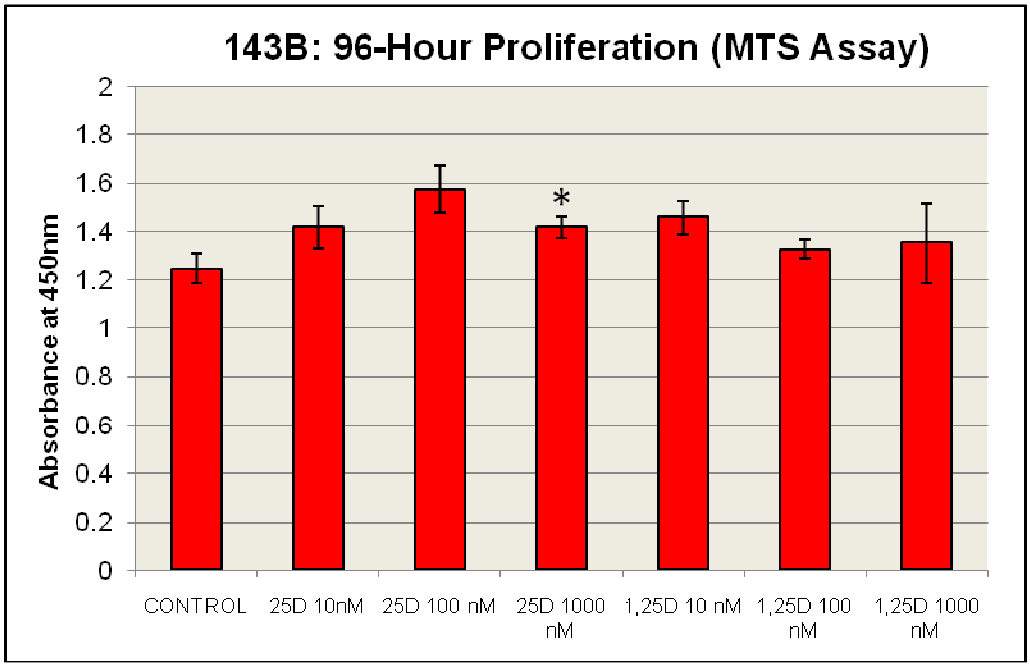
Cellular Proliferation

In order to investigate the effects of 1,25D and 25D on proliferation of osteosarcoma cells, the cells were cultured for 4 days in the presence of different concentrations of 1,25D or 25D.

Cell Proliferation MTS –based Assay

Proliferation was increased significantly (p value < 0.05; Student's t-Test) in 143B cells exposed to 1000nM 25D versus control. At other concentrations, neither 1,25D nor 25D had any effect on proliferation. Neither 1,25D nor 25D had any significant effects on proliferation in SaOS-2 cells (**Figure 3**).

(A)



(B)

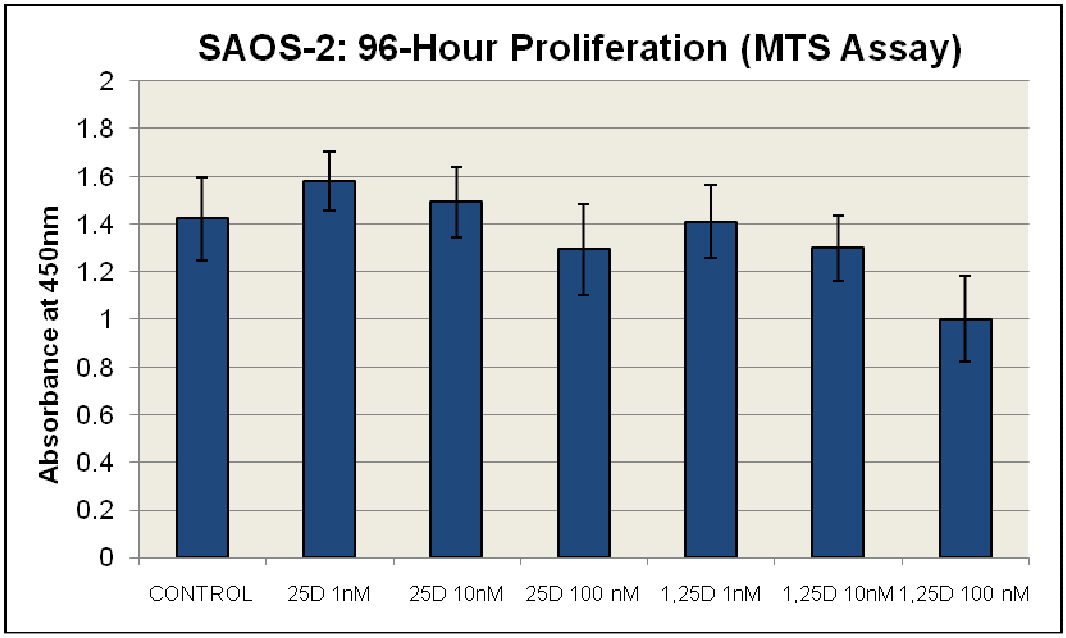


FIGURE 3. Effects of 1,25D and 25D on cell proliferation in (A) 143B and (B) SaOS-2 cells. Cells were cultured in 96-well plates and proliferation measured by MTS-based assay after 96h. Each data point represents the mean ± SEM (n=6). (*Statistical significance, *p* value < 0.05; Student's t-Test)

Ki67 Immunocytochemistry

Ki67 (a marker of cellular proliferation) immunocytochemistry results mirrored those of MTS-based assay, with little or no effect in proliferation observed in SaOS-2 or 143B cells after treatment with 1,25D or 25D (**Figure 4**).

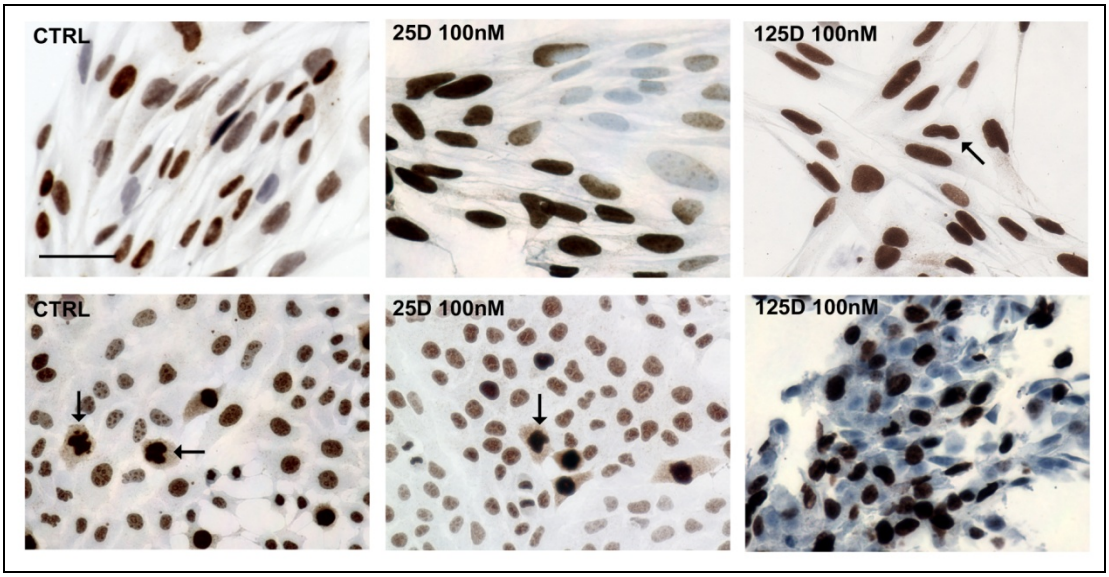
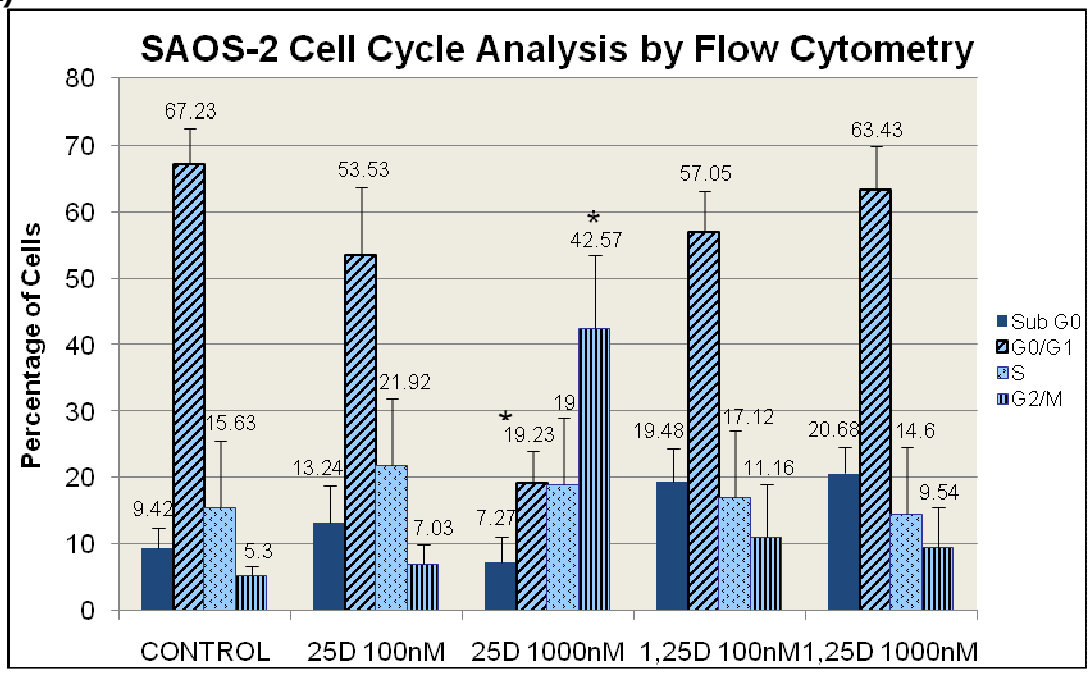


FIGURE 4. Photomicrographs of Ki67 immunostaining (a proliferation marker) after 96h of incubation: (Top Row) SaOS-2 control, SaOS-2 100nM 25D and SaOS-2 100nM 1,25D (Base Row) 143B control, 143B 100nM 25D and 143B 100nM 1,25D. Brown Peroxidase staining indicates Ki67 expression. Arrows indicate an actively dividing cell where Ki67 is maximal (Bar= 50 microns).

Cell Cycle Analysis

In SaOS-2 cells treated with 25D at 1000nM, cell cycle analysis revealed a significant increase (p value < 0.05; Student's t-Test) in the percentage of cells in the G2/M phase. There were no significant effects on cell cycle distribution in 143B cells with 25D or 1,25D treatment.

(A)



(B)

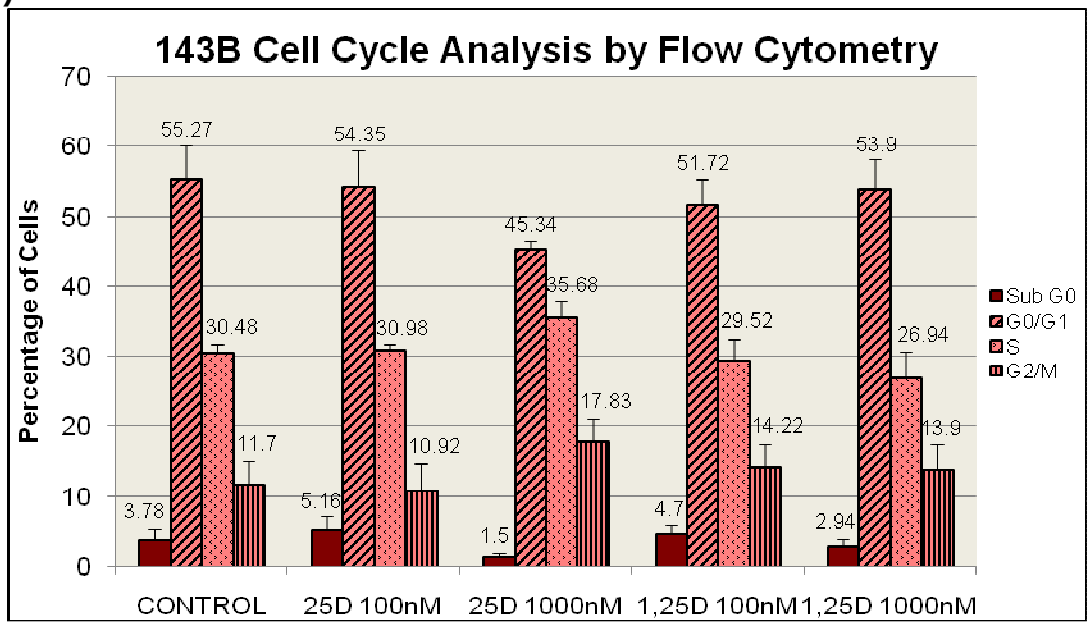


FIGURE 5. Effects of 1,25D and 25D on cell cycle distribution as analyzed by flow cytometry in (A) SaOS-2 cells and (B) 143B cells. Cells were cultured in T-25 flasks and cell cycle distribution was analyzed at subconfluence (96-120h). All data points are means ± SEM (n=3). (*Statistical significance, *p* value <0.05; Student's t-Test)

Cellular Differentiation

A series of experiments measuring markers of osteoblastic differentiation were performed to analyze the response of SaOS-2 and 143B cells to 25D and 1,25D.

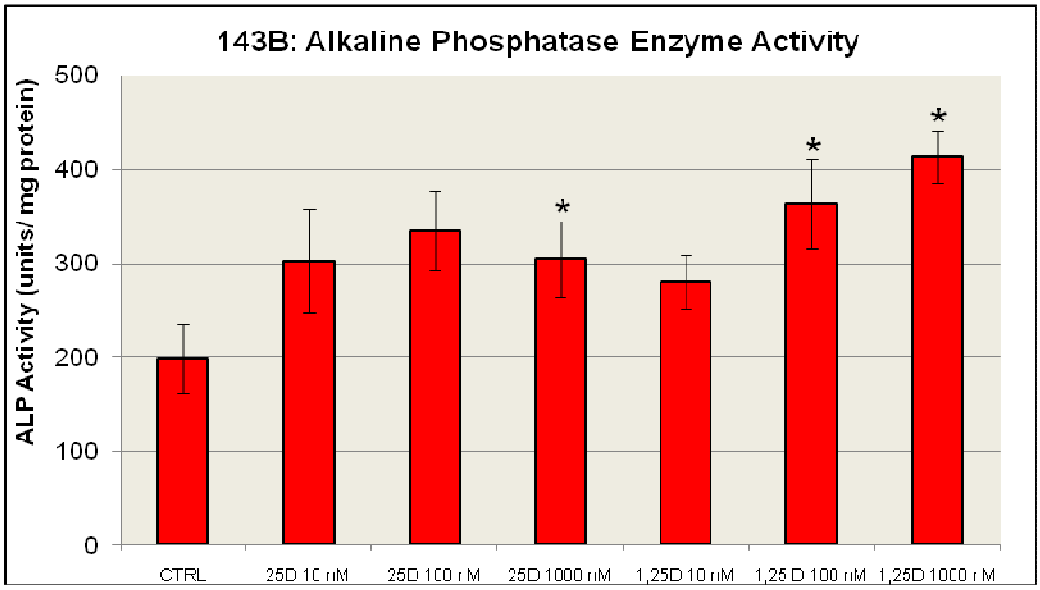
Alkaline Phosphatase Enzyme Activity

Alkaline phosphatase (ALP), an early marker of osteoblastic differentiation, activity was measured via colorimetric assay. In 143B cells treated with 1,25D, ALP activity was significantly increased dose-dependently ($p < 0.05$) from 100nM versus control. In SaOS-2 cells, neither 1,25D nor 25 D significantly modified ALP activity versus control (**Figure 6**).

Osteocalcin Secretion

Osteocalcin (a late marker of osteoblastic differentiation) secretion was measured using ELISA and immunocytochemistry. Neither technique was able to adequately detect osteocalcin secretion in either SaOS-2 nor 143B cells (data not shown). The ELISA technique was successful in recombinant protein osteocalcin (not native) based on the standard curve (**Figure 7**). It is unclear whether a) osteocalcin expression (native form) was out of the detectable range in SaOS-2 and 143B cells or b) SaOS-2 and 143B cancer cells do not express osteocalcin *in vitro*. Currently experiments are ongoing to measure OCN mRNA expression in SaOS-2 and 143B by real time PCR.

(A)



(B)

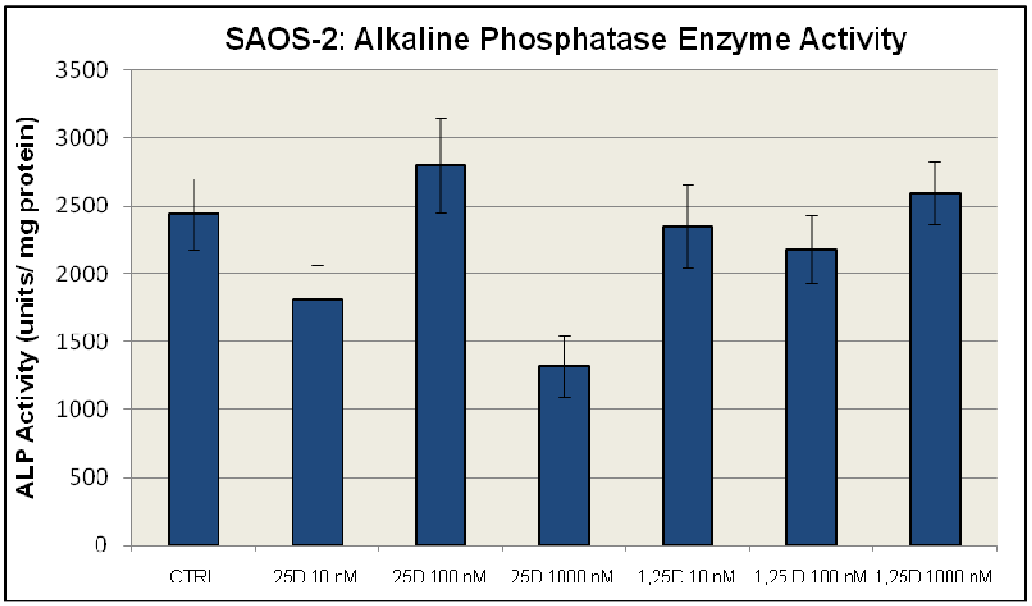


FIGURE 6. Effects of 1,25D and 25D on osteogenic differentiation in (A) 143B cells and (B) SaOS-2 cells. Cells were cultured in 24-well plates in a differentiating medium for 12-14 days. Para-Nitrophenyl Phosphate (pNPP) was used to quantify ALP activity in cell cultures. All data points are means ± SEM. (*Statistical significance, $p < 0.05$; Student's t-Test)

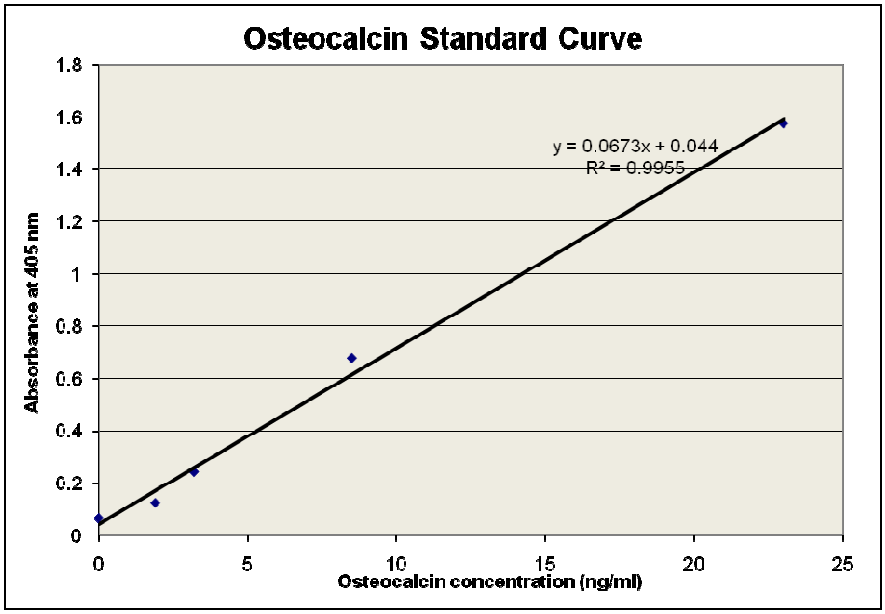


FIGURE 7. Osteocalcin standard curve as determined by ELISA (generated from 0, 1.9, 3.2, 8.5 and 23 ng/ml standards).

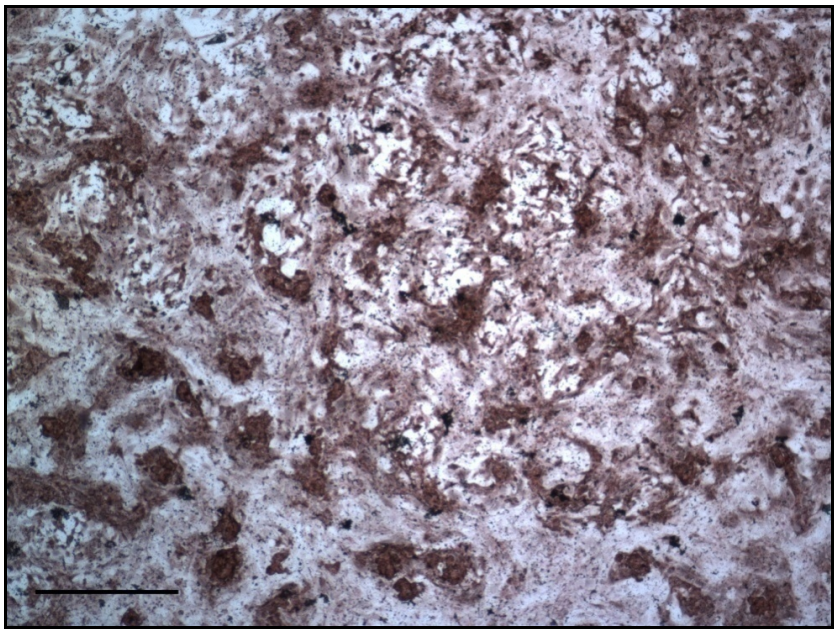
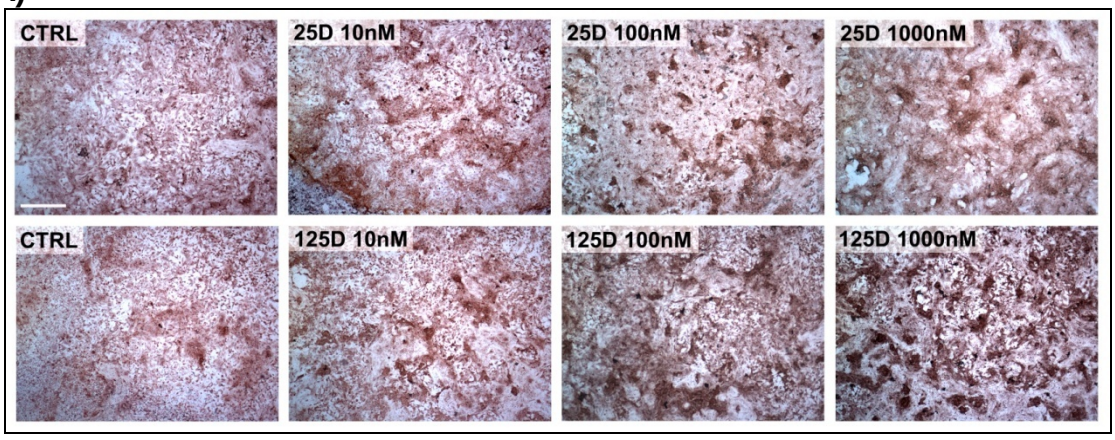


FIGURE 8. Slide showing von-Kossa staining of mineralization nodules in SaOS-2 cells treated with 1000nM 1,25D. Cells were plated in chamber slides and incubated for 12 days in differentiating medium and 1,25D or 25D. Black staining indicates mineralization through incorporation of calcium into the extracellular matrix (Bar= 500 microns).

(A)



(B)

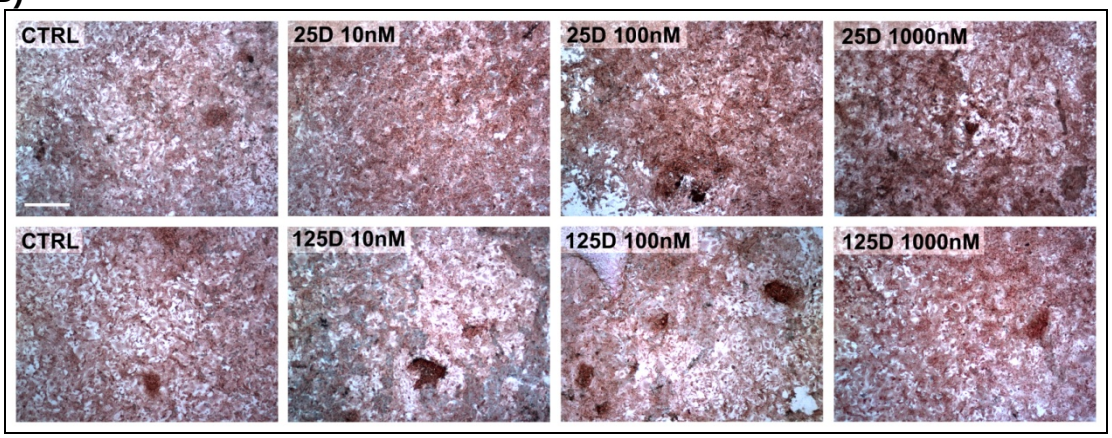


FIGURE 9. Slides showing von-Kossa staining of (A) SaOS-2 and (B) 143B cells. Cells were plated in chamber slides and incubated for 12 days in differentiating medium and indicated concentrations of 1,25D or 25D. Black staining indicates mineralization through incorporation of calcium into the extracellular matrix (Bar= 500 microns).

Apoptosis

Both 25D and 1,25D had a limited effect on the process of apoptosis in SaOS-2 and 143B cells (**Figure 10**) as evidenced by TUNEL staining. A significant induction of DNA fragmentation (*p* value < 0.05; Student's t-Test) was

observed with 25D at 1000nM in both cell lines but no other significant changes were observed after 15 days of treatment (**Figure 11**).

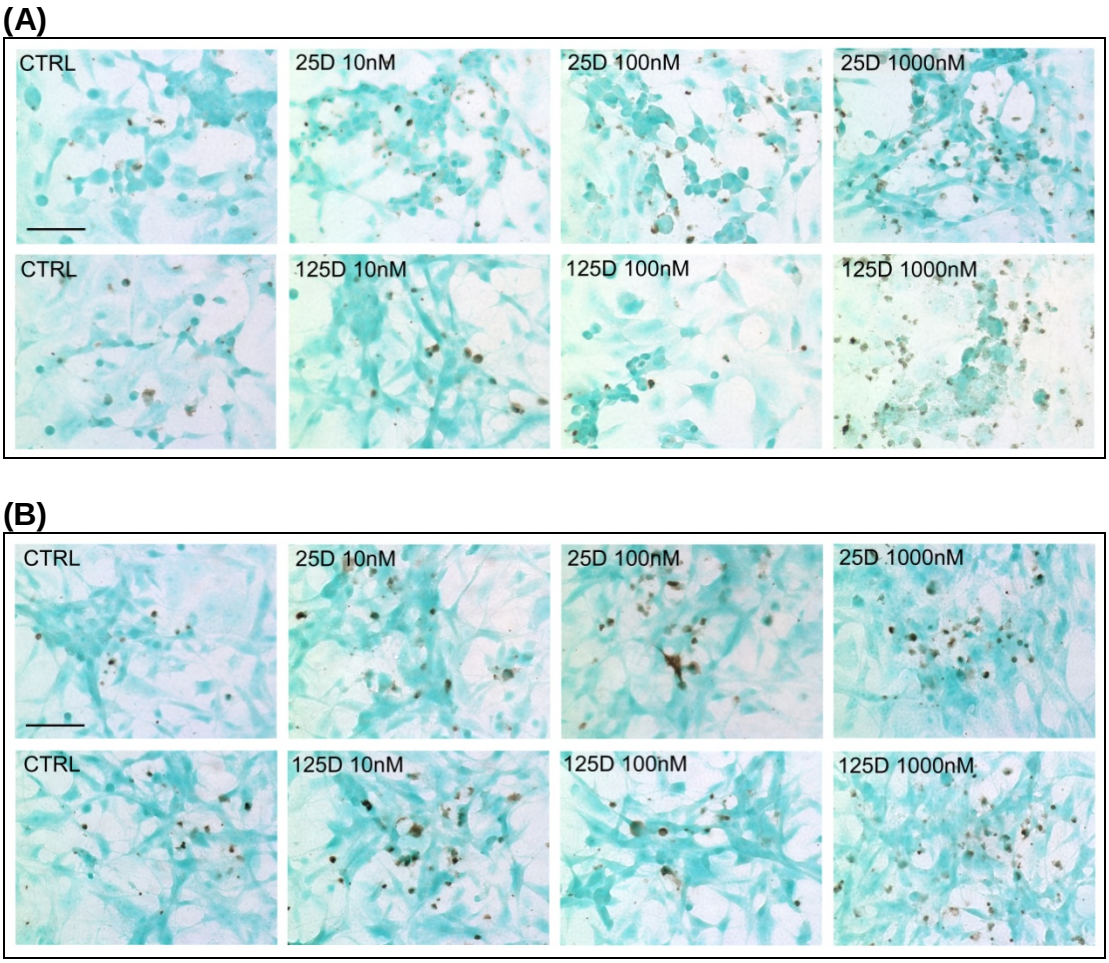
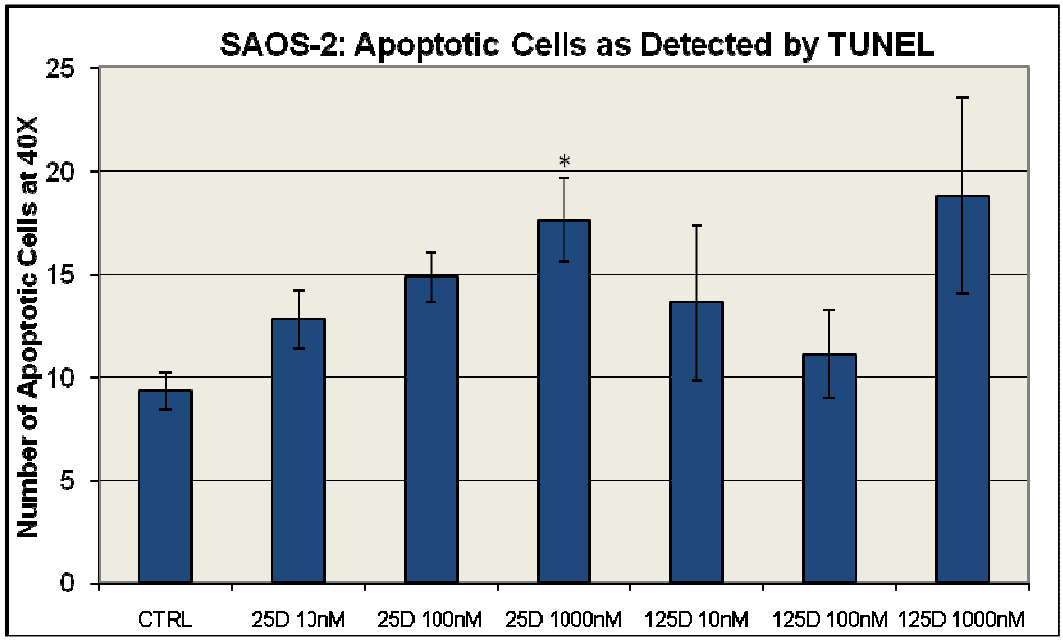


FIGURE 10. TUNEL staining in (A) SaOS-2 and (B) 143B cells. Cells were plated in chamber slides and incubated with indicated 1,25D or 25D concentrations for 15 days. Peroxidase staining indicates an apoptotic cell (Bar= 50 microns).

(A)



(B)

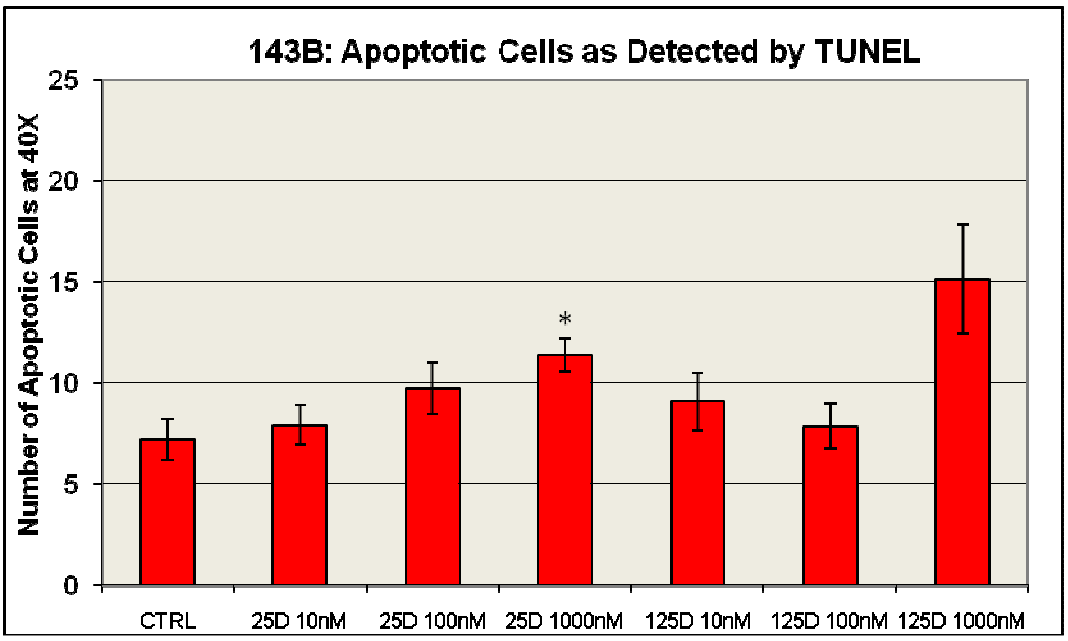


FIGURE 11. Semiquantitative analysis of apoptotic cells after treatment with 1,25D or 25D in (A) SaOS-2 and (B) 143B cells. Cells were cultured on chamber slides and apoptotic cells were identified via TUNEL staining after 15 days. Five sites on each slide were selected at random and apoptotic cells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

Differences in Control Cells

Biological differences between SaOS-2 versus 143B control cells were observed. Cell cycle analysis revealed significantly more ($p< 0.05$; Student's t-Test) SaOS-2 control cells in G0/G1 than 143B and significantly fewer ($p< 0.05$; Student's t-Test) SaOS-2 cells in synthesis phase than 143B. There was a 2-fold increase in SaOS-2 control cells in G2/M than 143B (**Table 4**; **Figure 12**). SaOS-2 control cells had alkaline phosphatase activity levels significantly higher ($p<0.05$; Student's t-Test) than 143B cells (**Table 5**).

TABLE 4. Cell cycle distribution of SaOS-2 versus 143B control cells[1]

Cell Cycle Phase	SaOS-2 Control Cells (%)	143B Control Cells (%)	*P*-value
Sub G0	9.42 ± 3.04[2]	3.78 ± 1.51	0.2167
G0/G1	67.23 ± 5.45	55.27 ± 4.89	0.0181*
S	15.63 ± 2.74	30.48 ± 1.36	0.0029*
G2/M	5.3 ± 1.37	11.7 ± 3.36	0.1458

[1]Cells were cultured in T-25 flasks and cell cycle distribution was analyzed at subconfluence.
[2]Mean percentage ± SEM (all such values).
*Statistical significance, *p* value <0.05; Student's t-Test.

TABLE 5. ALP enzyme activity in control cells[1]

	Mean	SEM	P-value
SaOS-2 Control Cells	2439.51	262.44	
143B Control Cells	198.19	36.54	0.0115*

[1]ALP enzyme activity per milligram protein in SaOS-2 versus 143B control cells. Cells were cultured in 24-well plates in a differentiating medium for 12-14 days. Para-Nitrophenyl Phosphate (pNPP) was used to quantify alkaline phosphatase activity in cell cultures.
*Statistical significance, *p* value < 0.05; Student's t-Test

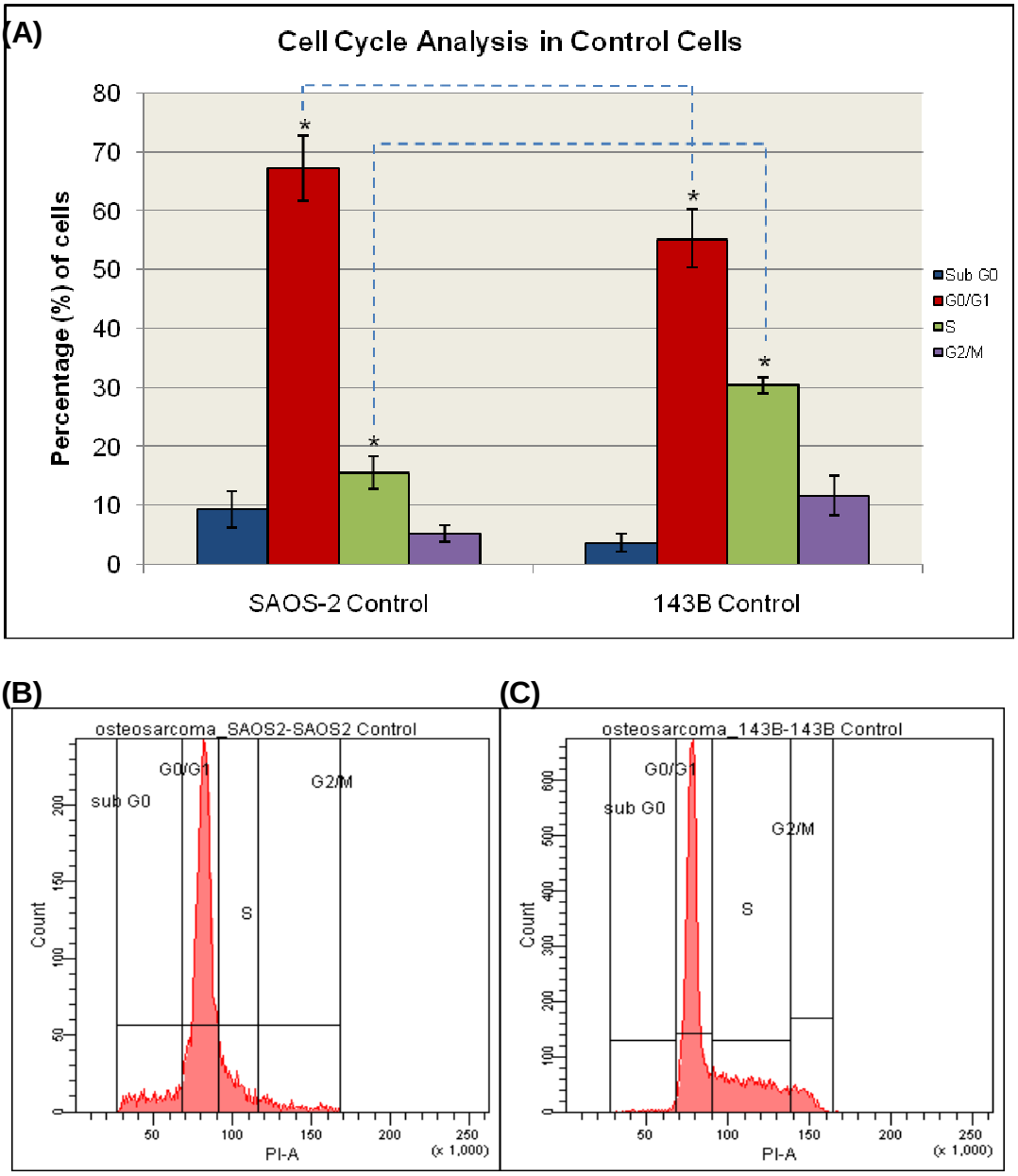


FIGURE 12. (A) Cell cycle distribution as analyzed by flow cytometry in SaOS-2 versus 143B control cells. (B-C) Flow cytometry histograms of (B) SaOS-2 and (C) 143B cells. Cells were cultured in T-25 flasks and cell cycle distribution was analyzed at subconfluence (96-120h). All data points are means ± SEM (*Statistical significance, *p* value <0.05; Student's t-Test).

CHAPTER 5 DISCUSSION

Cellular Proliferation

Treatment with 1,25D did not affect proliferation of either cell line after 96 hours of incubation. Proliferation was increased significantly versus control in 143B cells exposed to 25D at 1000nM (MTS assay). Ki67, a marker of cellular proliferation that is maximal in an actively dividing cell, was detected in this study via immunocytochemistry. Little differences were seen in Ki67 expression between control and treatment cells in either cell line.

The findings showed neither 25D nor 1,25D inhibit cellular proliferation. This finding contradicts the study hypothesis as well as other research. It was expected from previous investigations (11, 19, 20, 22-28) that 25D and 1,25D at concentrations as low as 10nM would inhibit cell growth in a dose dependent manner. The lack of effect on cellular proliferation by 1,25D has been observed in rat osteosarcoma cells (34). SaOS-2 was previously shown to possess high affinity receptors for 1,25D (23, 65), verifying the cells have the ability to respond to 1,25D. Higher concentrations of 1,25D or 25D may be needed to elicit a response from the cells or a different time period may be necessary to inhibit proliferation.

The 96-hour incubation was not an appropriate time period to observe 1,25D-mediated proliferation changes in this study. In human myeloblastic leukemic (HL60) cells, several studies reported treatment with 1,25D causes an

initial increase in proliferation followed by an inhibition (61, 67-69). A similar increase in proliferation by 25D was observed in 143B cells in this study. Other incubation times need to be observed to obtain a comprehensive depiction of proliferation in these cells.

VDR status and expression of genes and/or proteins important to tumor progression may be different in these cells based on the failure of 1,25D to inhibit proliferation. The differences in response by SaOS-2 and 143B signify a genetic variation between the lines and a further need for testing of VDR status in both cell lines. VDR status is important as a reduction in proliferation of SaOS-2 cells has only been observed in the presence of a functional VDR (19). There is a positive correlation between numbers of 1,25D receptors and the anti-tumor or differentiating effects (19).

Inhibition of cell proliferation is tightly linked to mechanisms that regulate cell cycle progression. The antiproliferative actions of 1,25D were attributed to cell cycle perturbation (19, 24, 28), namely cell cycle arrest in the G1 phase (19, 20, 24, 28). Cell cycle analysis was performed to investigate potential 1,25D or 25D-mediated cell cycle changes. Inhibition of proliferation was not observed, and only minor changes in cell cycle distribution in either osteosarcoma cell line was detected.

Cell cycle analysis varies with different incubation times. Ryhanen et al. reported 1,25D-mediated effects on regulatory cell cycle proteins taking place between 24 and 72h (24). The cell cycle analyses presented in this study were

tested after 96h incubation. Cell cycle changes might be sustained with transient 1,25D treatment from 24 to 96h, but it cannot be assumed. Wu et al. found that sustained but not transient 1,25D treatment caused a significant reduction of SaOS-2 cell proliferation (19). Both 1,25D and 25D need to be tested in higher concentration or added daily to observe cell cycle effects.

Other studies required serum-reduced medium to observe a persuasive reduction in cell proliferation (22, 28). As little as 0.1% FBS was successfully used in osteosarcoma proliferation studies (28). Ten percent FBS was utilized providing proteins that bind 1,25D or 25D, such as albumin and the vitamin D binding protein (25). Recent literature suggests 1,25D binds to serum proteins in the media and is not transported into the cell (58). The use of serum-reduced media will enhance the bioavailability of 25D and1,25D in culture and should be tested in future studies.

Cellular Differentiation

Osteosarcoma results from the malignant transformation of osteoblasts (21). In the differentiation of osteoblasts, a specific order of gene expression exists. The markers of osteoblastic cells—ALP, mineralization and osteocalcin—were examined to determine degree of differentiation of osteoblast-like cells, SaOS-2 and 143B.

Alkaline Phosphatase Enzyme Activity

ALP plays a significant role in facilitating bone mineralization and is used as an early marker of osteoblast differentiation (32). In this study, 1,25D at higher concentrations significantly increased ALP activity in 143B cells but not in SaOS-2 cells. The stimulation of ALP activity by 1,25D at higher concentration in 143B cells supports the study hypothesis and conforms with research in other human osteosarcoma cells (20, 21, 26, 28-30, 47). A similar increase in ALP activity with 1,25D treatment in SaOS-2 cells has been previously observed (30) and this discrepancy needs further explanation.

Treatment with 1,25D may target increases in ALP activity in less differentiated cells. In this case, SaOS-2 cells are innately more differentiated than 143B cells. SaOS-2 cells had 12-fold higher baseline ALP activity levels than 143B cells in control cells (Table 4), as well as an elevated expression of other osteoblastic markers found in literature (23, 25, 62).

ALP expression has differed *in vitro* and *in vivo*. A study by Dass et al. showed ALP expression in culture in SaOS-2 cells increased by 48% in primary tumors and further increase140% in metastatic lesions in the lungs as determined by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). In the same study, 143B did not express ALP in culture, but expressed ALP to the same level as SaOS-2 in the primary lesions (62).

Osteocalcin

Osteocalcin is one of the most abundant osteoblast-specific proteins whose levels post-proliferatively upregulate during extracellular matrix mineralization (70). Osteocalcin was not detected in either of the cell lines using ELISA nor immunocytochemistry. This was due to either: a) a technical issue in antibody binding or detection of the protein, or b) a lack of expression in the cells. Technically, different dilutions of cell suspension were tested (1:1 – 1:100) using the ELISA method. Incubation periods with antibodies were assessed from 2 hours to overnight but without success.

It was expected that osteocalcin would be expressed in SaOS-2 control cells as demonstrated in previous literature (62). A 1,25D responsive element was identified in the promoter region of the human osteocalcin gene (71) and both 1,25D (26-30, 33-35) and 25D (25) were shown to augment osteocalcin secretion or expression in osteosarcoma cell lines. It was cited that human osteoblasts do not express osteocalcin in a stringent maturation stage-dependent fashion (47). Timing and stage of cell growth play a factor in results and vary between cell lines. Dass and colleagues found no expression of osteocalcin in SaOS-2 or 143B cells in culture, but found it was readily detectable in both SaOS-2 primary and secondary tumors (62). Those studies showing differential expression of osteocalcin between *in vitro* and *in vivo* environment suggest that the expression and stability of this protein are influenced by the tumor microenvironment.

Ryhanen et al. found maximum responses in osteocalcin mRNA levels were reached within 24h (27). The osteocalcin ELISA and immunocytochemistry assessments of protein levels in this study were completed after 15 days of incubation. Osteocalcin may be expressed transcriptionally but not post-transcriptionally in these cells. Based on the reported issues, osteocalcin mRNA is currently being tested at various time points via RT-PCR for further investigation.

In Vitro Mineralization

Both SaOS-2 and 143B cells formed multiple mineralization nodules in culture after 12 days, and nodules appeared to increase at higher concentrations of both 25D and 1,25D as examined by von-Kossa staining. The numbers of nodules were not quantified because of their small size, and were not proven statistically significant. Current *in vitro* mineralization experiments are focused on increasing the extent of mineralization time period to 21 days, allowing the nodules to attain a reasonable size to be readily quantified.

These results are similar to the results of others (25, 29) showing that both 25D (100nM) and 1,25D (0.3-10nM) can induce mineralization of osteosarcoma cells by incorporation of calcium into the extracellular matrix. Collectively, the results suggest that both 25D and 1,25D at higher concentration (100-1000nM) have a direct effect on the bone mineralization process of these cells and regulate the osteogenic function of these cells. The ability of 25D to effectively

promote mineralization *in vitro* implies that serum 25D regulates this activity *in vivo*.

In the present study, both 25D and 1,25D act as a differentiation agents in SaOS-2 and 143B cells. Both 25D and 1,25D help promote differentiation towards a functionally mature osteoblastic bone cell as suggested by experiments in markers of osteoblastic differentiation. Tumor severity is inversely correlated with the degree of cell differentiation (53). These findings show that 25D and 1,25D can decrease the severity of osteosarcoma *in vitro*. Both 1,25D and 25D appear more effective in promoting change in the less differentiated cell line, 143B, than SaOS-2.

The discrepancies between cell lines, 1,25D concentrations and other literature cannot be simply explained. Transcriptional activation by 1,25D is complex, involving heterodimerization of retinoid X-receptors (RXR) and VDR, and multiple co-activator proteins (72). This complexity accounts for many of its gene- and stage- specific effects. The apparent differences in the sensitivity to 1,25D involve variations in VDR status, the phenotypic maturation of the cell or the structure-function relationship between 25D and 1,25D used.

Apoptosis

A biochemical feature of apoptosis is the fragmentation of genomic DNA. Using TUNEL staining to label fragmented DNA, it was demonstrated that 1,25D and 25D had limited effects on apoptosis in SaOS-2 and 143B cells. A

significant induction of DNA fragmentation was observed with 25D at 1000nM in both cell lines but no other significant changes were observed.

The current study demonstrates that 1,25D alone does not affect apoptosis. This finding is in agreement with the results of several studies (22, 23, 34), suggesting that programmed cell death may not contribute to the anticancer properties of 1,25D, and that mechanisms of antineoplastic effects lie elsewhere. Semi-quantification of TUNEL staining revealed 25D can indeed enhance apoptosis in human osteosarcoma cell lines. Apoptosis induction by 25D at high concentration (1000nM) in SaOS-2 and 143B cells is not reported elsewhere. The active form of vitamin D affects numerous signaling pathways and 1,25D-induced cytotoxicity differs among cell types. Investigation of the signaling pathways regulated by 1,25D should be examined, as previous research found a variety of pathways and proteins—Bcl-2, BAX and caspases—involved in apoptosis.

Differences in Control Cells

Both human osteosarcoma lines used in this study are tumorigenic, but not isogenic. Biological differences between SaOS-2 and 143B control cells were observed. SaOS-2 cells are intrinsically more differentiated than 143B as evidenced by significantly enhanced ALP specific activity in control cells. Significantly more 143B cells than SaOS-2 cells in the synthesis phase of the cell cycle suggest a higher rate of replication in these cells.

These inherent differences in the biology of SaOS-2 and 143B osteosarcoma cells modulate response to 1,25D. There may be several underlying reasons. First, the switch to differentiation mode is not rapid for some cells but rather an initial increase in proliferation is observed, as seen in HL60 (61, 67, 68) or U937 (69) cells differentiating in response to 1,25D.

Secondly, the inherent differences in the biology of the two cell lines—the absence of expression of retinoblastoma protein in the SaOS-2 cell line, and ras gene transformation in the 143B cell line—may alter the cells' response to 1,25D. Li et al. indicate species differences should be taken into account in studies of 1,25D effects (26). Because these lines are not isogenic, lack of genetic similarity could be a potential problem in the response to 1,25D or 25D.

VDR status as well as expression of proteins important in tumor progression in these two cell lines is not known. Investigation in the differences in VDR status through immunocytochemistry and with RT-PCR may be necessary to quantify these values for a useful conclusion. VDR content and intracellular levels of vitamin D metabolites are the main determinants of the magnitude of the response to 1,25D therapy by a target tissue (51).

Limitations

An obvious limitation of this study is the *in vitro* methodology. This study overlooks the complexity of the *in vivo* tumor environment where there is interplay between different cell types and signaling pathways which could have

influence on tumor growth and progression. *In vitro* studies are important for gathering preliminary data on the effect of 1,25D on osteosarcoma 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.

Limitations in methodology were incurred during the duration of study. Other studies have required serum-reduced medium to observe 1,25D or 25D-mediated effects (22, 28). Ten percent FBS was used in this study, while as little at 0.1% FBS has been successfully used in other osteosarcoma proliferation studies (28). Ten percent FBS may provide proteins that can bind 1,25D or 25D such as vitamin D binding protein and albumin (25). Serum reduced media should be tested in future studies.

In apoptosis experiments, TUNEL alone was used to examine apoptotic cells in culture. In reality, other methods of identifying apoptotic cells—Transmission Electron Microscopy (TEM) or Annexin-V staining—should be used to confirm TUNEL results, examine the characteristic morphological features of apoptosis and exclude necrotic cells.

Implications

It is important to further clarify and summarize the effects of 25D and 1,25D on cellular processes in human osteosarcoma cells. Other potential

therapeutic targets for osteosarcoma need to be explored in detail. The observation that 1,25D at higher concentration increases ALP activity in 143B cells and promotes mineralization in SaOS-2 and 143B cells suggests that 1,25D and 25D may act as a differentiation agents in these cells. These studies should be repeated and quantified for statistical power. The potential use of 25D therapeutically as a pro-apoptotic agent in osteosarcoma is uncertain. The role of 25D in apoptosis of osteosarcoma cells exhibited in this study needs to be confirmed and clarified, including investigations into the pathways involved.

Knowledge gained from this study provides a better insight into understanding the mechanism of action of 1,25D on osteosarcoma cells and may provide the framework for future *in vivo* studies. Given the prevalence of osteosarcoma in children, adolescents 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 1,25D or 25D to promote normal osteogenic differentiation and affect apoptosis in osteosarcoma cells may be useful in the future to suppress the aggressive and malignant properties of osteosarcoma.

The research here has led to the presentation of results at the 2008 American Institute of Cancer Research's (AICR) Annual Conference on Food, Nutrition, Physical Activity and Cancer (Abstract- Appendix A). A manuscript is also in preparation.

Conclusions

This study investigated the effects of 1,25D and 25D on proliferation, differentiation and apoptosis in human osteosarcoma cell lines, SaOS-2 and 143B. Neither 25D nor 1,25D significantly affected proliferation in SaOS-2 or 143B cells. These studies suggest that 1,25D and 25D act as differentiation agents in human osteosarcoma cell lines through activation or upregulation of markers of osteoblastic differentiation, including ALP in 143B cells and mineralization in SaOS-2 and 143B cells. Both 25D and 1,25D has a limited effect on apoptosis in SaOS-2 and 143B cells; 25D at high concentration may increase apoptosis in these cells.

There are inherent differences in the biology of SaOS-2 and 143B osteosarcoma cells. These differences in biology may modulate the cells' response to 1,25D and 25D. These studies need to be repeated with statistical power and validated in primary cultures of osteosarcoma, prior to testing in an animal model.

Suggestions for Future Research

1. **The use of isogenic cell lines to compare the effects of 25D and 1,25D**

   In the current study a metastatic p-53 null (SaOS-2) and highly metastatic, ras-gene transformed (143B) cell line were used to assess differences in

response to 1,25D or 25D. However SaOS-2 and 143B differ genetically and thus cannot be compared. Future studies should use isogenic cell lines to assess how differences in metastatic potential affect response to 1,25D or 25D (i.e. 143B and its parent cell line, HOS).

2. **The use of supra-physiological concentrations of 1,25D or serum-reduced medium**

   The high percentage of serum (i.e. 10% FBS used in this study) used in most *in vitro* studies may be reducing the bioavailability of 1,25D in culture. This should be addressed in the future by decreasing the percentage of FBS gradually and determining the minimal most effective concentration that is sufficient to support the growth of osteosarcoma cells *in vitro*. At higher percentages of serum, supra-physiological concentrations of 25D or 1,25D may be necessary to induce similar effects.

3. **Morphology of apoptotic cells verified by Transmission Electron Microscopy (TEM) and Annexin-V staining**

   Apoptotic cells need to be confirmed with further experiments outside of TUNEL in this study. Analysis of morphology via TEM and Annexin-V staining may exclude a proportion of the cells as necrotic versus apoptotic.

4. **RT-PCR to detect osteoblastic differentiation markers**

   There were issues in detecting osteocalcin secretion in SaOS-2 and 143B cells and varying results in other markers of differentiation. Expression of

markers of osteoblastic differentiation can be detected using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), a highly reputable method of assessing RNA expression. These experiments are currently in progress.

5. **Relationship of VDR status in osteosarcoma cells and 1,25D or 25D effects on tumorigenicity.**

   The VDR statuses of SaOS-2 and 143B cells are unknown and may have an effect on the responses of the cells to 1,25D and 25D. Future research should test the expression of VDR in these cells and the associated response or lack thereof.

6. **Investigation into mechanisms of apoptosis----caspase or BAX mediated?**

   Investigation of the 1,25D-regulated signaling pathways should be studied as a variety of pathways and proteins may be involved in apoptosis. In breast cancer cells, 1,25D induces apoptosis via a novel caspase- and p53 independent pathway that can be inhibited by bcl-2 (18). Conversely, in human osteosarcoma cells 1,25D upregulates bcl-2 and this pathway, protecting the cells from apoptosis (23, 34).

CHAPTER 6 SUMMARY

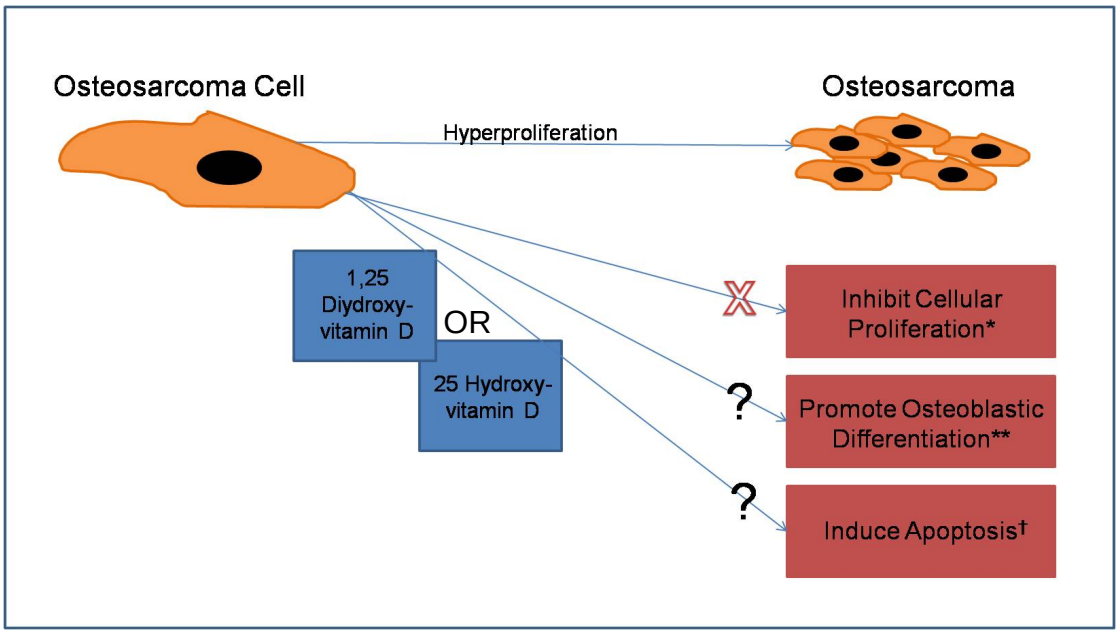


FIGURE 13. Working model of 1,25D and 25D effects on proliferation, differentiation, and apoptosis of osteosarcoma cell lines

*No inhibition of cellular proliferation was observed in the current study. In some cases, proliferation was increased in the presence of 25D.

**In the current study differentiation was measured by alkaline phosphatase enzyme activity, osteocalcin secretion and *in vitro* mineralization. In the presence of 1,25D in higher concentration in 143B, alkaline phosphatase activity was increased. Mineralization was induced dose dependently in both SaOS-2 and 143B cells. Osteocalcin secretion was unable to be measured.

†Apoptosis was induced by 25D at 1000nM in both SaOS-2 and 143B but 1,25D and 25D at other concentrations had no effect on apoptosis.

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APPENDIX

APPENDIX A

American Institute of Cancer Research's (AICR) Annual Conference Abstract

The Dose Response of Vitamin D on Proliferation and Differentiation in Human Osteosarcoma—an *In Vitro* Study

2008 American Institute of Cancer Research's (AICR) Annual Conference on Food Nutrition Physical Activity and Cancer—Poster Presentation

The Dose Response of Vitamin D on Proliferation and Differentiation in Human Osteosarcoma—an *In Vitro* Study

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Keywords: Osteosarcoma, vitamin D, proliferation, differentiation, cell cycle

ABSTRACT

INTRODUCTION: Osteosarcoma is a malignant bone tumor predominantly affecting children and adolescents. Osteosarcoma has a 60-70% survival rate with current treatments; hence there is a need to identify novel therapeutic regimens. The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25D), is being increasingly recognized for its anti-cancer properties. We examined the dose-response of 1,25D and 25-hydroxyvitamin D_3 (25D) on human osteosarcoma cell lines, SaOS-2 (tumorigenic, p53 null, weakly metastatic) and 143B (tumorigenic, ras gene transformed and highly metastatic). We hypothesized that vitamin D would inhibit proliferation and stimulate differentiation of these cells in a dose-dependent manner.

METHODS: Osteosarcoma cell lines, SaOS-2 and 143B, were treated with 1,25D or 25D at concentrations 1-1000nM. Cellular proliferation was measured after exposure to vitamin D using a cell viability assay (MTS-tetrazolium), Ki67 immunocytochemistry and cell cycle analysis by flow cytometry. Osteoblastic differentiation was measured by alkaline phosphatase (ALP) enzyme activity.

RESULTS: In 143B cells, proliferation was increased significantly (p value < 0.05; Student's t-Test) in cells exposed to 25D at 1000nM versus control, but vitamin D at other concentrations had no effect on proliferation or on cell cycle. In cells treated with 1,25D at higher concentrations ALP activity was increased significantly versus control. In SAOS-2 cells, vitamin D did not significantly inhibit proliferation, induce cell cycle changes or modify ALP activity versus control. We also observed biological differences between SAOS-2 vs. 143B control cells. Cell cycle analysis revealed significant differences including more SAOS-2 control

cells in G0/G1 than 143B, and significantly fewer SAOS-2 cells in synthesis phase than 143B. SAOS-2 control cells had alkaline phosphatase activity levels at least 8X (≥) higher than 143B cells.

CONCLUSIONS: These findings show that there are inherent differences in the biology of SAOS-2 and 143B osteosarcoma cells, which might modulate vitamin D response i.e. resisting the antiproliferative actions of vitamin D. These studies need to be validated in primary cultures of osteosarcoma, prior to testing in an animal model.