EFFECTS OF VITAMIN D TREATMENT ON INFLAMMATORY AND NON-INFLAMMATORY BREAST CANCER CELL LINES

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Bachelor of Science in Biological Sciences with Distinction.

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ABSTRACT

Vitamin D is a known regulator of breast cancer cell proliferation, apoptosis, migration, invasion and differentiation in vitro. Recent epidemiological studies have suggested a preventative role for vitamin D in breast cancer development. These data, in addition to other previous studies, have led to increased research examining the possible therapeutic effects of vitamin D on patients with various forms of breast carcinoma. Furthermore, in comparison to current breast cancer treatment options, vitamin D shows little to no adverse side effects at doses lower than 50,000 IU in adults (1).

This study demonstrates the presence of the vitamin D receptor (VDR) as mRNA in an inflammatory and non-inflammatory breast cancer cell line, respectively SUM-149 and MDA-MB-231. Stimulation of the VDR may influence certain molecular pathways responsible for the effects of vitamin D on MDA-MB-231 and SUM-149 cells. Both breast cancer cell lines showed an increase in protein concentration in response to 24 hours of 1,25-dihydroxyvitamin D₃ exposure; likely mediated by an increase in protein synthesis as opposed to increased cellular proliferation. In addition, treatment with 100 nM 1,25-dihydroxyvitamin D₃ showed a significant decrease in SUM-149 migration (67.8% decrease, p = 0.030), invasion (43.9% decrease, p = 0.015), and emboli size (69.4% decrease, p = 0.018) compared to control groups. Due to the importance of these processes for breast cancer metastasis, vitamin D treatment may have the potential to decrease the rate and incidence of metastasis in breast cancer patients; however, further research is necessary.
Chapter 1
INTRODUCTION

1.1 Sources and Synthesis of 1,25-dihydroxyvitamin D₃

The majority of vitamin D within the body is obtained through exposure to sunlight (2). As shown in Figure 1A, this process begins when ultraviolet B radiation penetrates the skin, where it converts cutaneous 7-dehydrocholesterol to previtamin D₃ (3). Previtamin D₃ is then spontaneously converted to vitamin D₃, a form of vitamin D known as cholecalciferol. In addition to sunlight, vitamin D can be obtained naturally through the consumption of a small number of foods. For example, vitamin D₃ is found in fish, beef liver, cheese and egg yolks (4). However, most of the vitamin D present in the American diet comes from fortified foods (5).

Vitamin D obtained through diet or exposure to sunlight is originally inert and must be activated within the body. Two consecutive hydroxylation reactions are involved in vitamin D₃ activation, as shown in Figure 1B (6). The first occurs in the liver and converts vitamin D₃ to 25-hydroxyvitamin D₃, also known as calcidiol, through hydroxylation at the C-25 position. The next reaction occurs mainly in the kidney and is catalyzed by renal 25-hydroxyvitamin D₃ 1α-hydroxylase (7). In this reaction, 25-hydroxyvitamin D₃ is converted into a biologically active form of vitamin D, 1,25-dihydroxyvitamin D₃, also known as calcitriol.
Daily vitamin D intake varies widely between countries and age groups, depending on the types of food readily available. Vitamin D intoxication in adults has only been reported at very high doses, greater than 50,000 IU (1).

1.2 1,25-dihydroxyvitamin D₃ Signaling within the Cell

The traditional vitamin D signaling pathway begins with the activation of the nuclear vitamin D receptor (VDR) by 1,25-dihydroxyvitamin D₃. The VDR forms
dimer complexes with other VDRs or retinoid-x receptors (RXR) (8). The dimer complexes then bind to DNA sequences, known as vitamin D response elements (VDREs), found in the promoter region of target genes. Binding of the VDREs facilitates transcriptional activation of various genes through histone acetylation and increased RNA-polymerase II binding (9, 10). Transcriptional repression is mediated through histone deacetylation leading to chromatin remodeling. The resulting cellular response usually occurs several hours after the initial stimulation, following the time consuming transcription and translation of the target genes. In contrast, a more recently proposed pathway begins with the activation of a cell-surface receptor termed 1,25D$_3$-MARRS (membrane associated rapid response steroid binding) receptor, independent of the nuclear VDR (11). Binding of 1,25-dihydroxyvitamin D$_3$ to the 1,25D$_3$-MARRS receptor leads to a rapid biological response, usually within seconds or minutes of stimulation. This mechanism generates a prompt response through its immediate influence on various cellular pathways, separate from gene involvement. The activated 1,25D$_3$-MARRS receptor regulates voltage-gated calcium channels, chloride channels, protein kinase C activity, and MAP (mitogen-activated protein) kinases (12).

Previous studies have demonstrated the anti-proliferative effects of 1,25-dihydroxyvitamin D$_3$ on MCF-7 non-IBC breast cancer cells through various mechanisms. Arrest in the G1 phase of the cell cycle in response to 1,25-dihydroxyvitamin D$_3$ has been associated with alterations in the expression of cell cycle regulators, such as increased cyclin-dependent kinase (CDK) inhibitors (13) (14). For example, increased expression of p21, a CDK inhibitor, at the mRNA and protein level is associated with cell cycle arrest in the G1 stage in response to 1,25-
dihydroxyvitamin D$_3$ treatment (15). Induction of apoptosis in MCF-7 cells after 48 hours of exposure to 100 nM 1,25-dihydroxyvitamin D$_3$ has been demonstrated through morphological assessment of the cell line (16). Furthermore, 1,25-dihydroxyvitamin D$_3$ induces apoptosis in MCF-7 and SUM-159 cells through the regulation of Bcl-2 family proteins. One member of this protein family is Bcl-2, an anti-apoptotic protein often overexpressed in cancerous cells. Bcl-2 is down-regulated in MCF-7 and SUM-159 breast cancer cell lines in response to 1,25-dihydroxyvitamin D$_3$ treatment (17). In SUM-159 cells the reduction in Bcl-2 is also associated with an increase in Bax, a pro-apoptotic BH-123 protein belonging to the Bcl-2 protein family. BH-123 proteins form channels in the outer mitochondrial membrane, allowing the release of cytochrome c from the intermembrane space of the mitochondria (18). Cytochrome c is involved in the activation of procaspase-9, which causes the subsequent induction of apoptosis. Bcl-2 proteins function through the binding and inhibition of BH-123 proteins, therefore reducing apoptosis in many cancerous cells in the absence of 1,25-dihydroxyvitamin D$_3$ stimulation. Modulation of growth factor signaling may also be responsible for the decrease in proliferation seen in MCF-7 cells. Specifically, 1,25-dihydroxyvitamin D$_3$ has been shown to block the mitogenic effects of insulin-like growth factor I (IGF-I) through the down-regulation of its receptor, leading to a decrease in proliferation and an increase in apoptosis (19, 20).

Several mechanisms may be responsible for the decrease in invasion of MDA-MB-231 cells in response to 1,25-dihydroxyvitamin D$_3$ treatment as demonstrated by Mørk Hansen and colleagues (21). Reduced invasion in MDA-MB-231 cells is associated with decreased activity of the metalloproteinase MMP-9 and two serine proteases, urokinase-type plasminogen activator (uPA) and tissue
plasminogen activator (tPA). These effects are likely modulated by an increase in PA inhibitor 1 and MMP inhibitor 1 (22). In addition, the anti-invasive effects of 1,25-dihydroxyvitamin D₃ on MDA-MB-231 cells may be due to its inhibition of tenasin-C, an extracellular matrix protein that promotes proliferation, invasion, and angiogenesis, and is often up-regulated in cells during tumorigenesis (23). Furthermore, inhibition of angiogenesis in response to 1,25-dihydroxyvitamin D₃, as demonstrated by chick embryo chorioallantonic membrane assays, may also contribute to the decrease in invasion seen in MDA-MB-231 cells (24). A decrease in angiogenesis in response to 1,25-dihydroxyvitamin D₃ has also been demonstrated in vivo, using a mouse model (25).

1.3 SUM-149 Human Inflammatory Breast Cancer Cell Line

The SUM-149 human inflammatory breast cancer (IBC) cell line was obtained from an inflammatory ductal carcinoma tumor and provided by Dr. Steve Ethier, the cell line originator. SUM-149 cells are of the basal B breast carcinoma subtype, which is associated with younger patient age, aggressive clinical course, development of distance metastasis, poor prognosis, and high mortality rate (26). SUM-149 cells are negative for the estrogen receptor (ER) and progesterone receptor (PR).

The SUM-149 cell line is composed of different subpopulations of cells. When characterized based on cell surface expression of CD44 and CD24, the SUM-149 cell line contains two distinct populations. The population composed of CD44⁺/CD24⁻ cells exhibits a basal cellular phenotype, while the population of CD44⁻/CD24⁺ cells exhibits features similar to luminal epithelial cells (27). CD44⁺/CD24⁻ cells are associated with an increased tumorigenic potential in vivo.
Furthermore, a recent study demonstrated that a subpopulation of SUM-149 cells expressing the enzyme aldehyde dehydrogenase 1 (ALDH1) is highly associated with increased tumorigenicity and significantly enriched for CD44+/CD24- cells (28). All SUM-149 subpopulations were adherent when grown as a monolayer.

A previous study conducted by van Golen and colleagues demonstrated that the overexpression of RhoC GTPase and loss of expression of the LIBC (lost in inflammatory breast cancer) gene in SUM-149 cells are highly correlated (p < 0.0095 and p < 0.0013, respectively) with the IBC phenotype (29).

1.4 MDA-MB-231 Human Breast Cancer Cell Line

The MDA-MB-231 human breast cancer cell line was obtained from the American Type Culture Collection (No. HTB-26) after isolation from a pleural effusion of a 51 year old white female breast adenocarcinoma patient (30). This well characterized, invasive cell line was originally established by MD Anderson Cancer Center in Houston, Texas. MDA-MB-231 cells are negative for the estrogen and progesterone receptors and are of the basal B breast carcinoma subtype. MDA-MB-231 cells have been shown to migrate significant distances in scratch assays and invade across a modified Boyden chamber in 24 hours (31).

Previous studies have explored the effects of 1,25-dihydroxyvitamin D₃ on the MDA-MB-231 cell line. The vitamin D receptor (VDR) was shown to reside in MDA-MB-231 cells, but the level of VDR mRNA tended to be lower than in other breast cancer cell lines (32). The MDA-MB-231 cell line had little to no growth inhibition when treated with up to 1 mM of 1,25-dihydroxyvitamin D₃ (32). When treated for 4 days with varying concentrations of 1,25-dihydroxyvitamin D₃, MDA-MB-231 cells showed a dose-dependent decrease in invasion (21). The process of
migration was also inhibited after 4 days of treatment with 1,25-dihydroxyvitamin D₃. Past research indicated no significant effect of 1,25-dihydroxyvitamin D₃ on the invasive and migratory potential of the MDA-MB-231 cells in response to shorter periods of exposure.

1.5 Inflammatory Breast Cancer

Inflammatory breast cancer (IBC) is the most lethal form of locally advanced breast cancer due to its rapid progression and extremely high metastatic potential (33). IBC is highly angiogenic, promoting the growth of new blood vessels, and highly angioinvasive, with a propensity to invade the surrounding lymphatic vessels.

Primary IBC occurs de novo, or as the first occurrence of breast cancer. Secondary IBC is the inflammatory recurrence of a non-inflammatory primary breast cancer (33). Primary IBC accounts for 1-6% of all breast cancers in the United States. It affects African Americans at higher rates than Caucasians and other ethnic groups (34). The mean age of patients presenting with IBC is approximately 52 years, similar to the age of patients with other forms of breast cancer (35). The survival rate for IBC patients is about 50% behind that of non-IBC patients (36). Estimated five- and ten-year disease-free survival rates are respectively 35% and 20% (37).

The clinical symptoms associated with IBC include redness, warmth, swelling and tenderness of the breast tissue, resembling an acute inflammation (33). In addition, the texture of the skin over the affected breast often resembles the skin of an orange. Despite its name, the symptoms associated with IBC are not due to an immunoreaction, but rather result from the invasion of malignant cells into the dermal lymphatic vessels of the breast, forming tumor emboli. The emboli block the
lymphatic vessels, causing the inflamed and swollen appearance characteristic of IBC. Furthermore, it is the invasive tumor emboli that disseminate through the body and often form distant metastases in the lung, liver, and bone (38). The metastasis of the cancerous cells to these organs, followed by a subsequent disruption in their functioning, is commonly the cause of death for inflammatory breast cancer patients.

Inflammatory breast cancer metastasis requires the completion of several cellular processes. Initially, epithelial-derived inflammatory breast cancer cells must invade the epithelial basement membrane and migrate into the underlying stroma of the breast. Next, the cancerous cells must invade the lymphatics, or much less commonly, the vasculature. The mechanism through which IBC cells invade the lymphatics and vasculature is currently unclear and controversial. Some studies support the active migration of SUM-149 cells through the surrounding tissue towards lymph or blood vessels. This process begins when the IBC cells undergo the epithelial-mesenchymal transition (EMT), characterized by the loss of adherens junctions through the repression of E-cadherin expression (39). The cancerous cells break away from cell to cell adhesions and become motile, moving through the surrounding stroma and eventually invading the target vessel. Most lymphatic vessels do not contain a basement membrane, and therefore invasion only requires passage through the epithelial layer. The main problem with the active migration hypothesis is the tendency of IBC cells to overexpress E-cadherin. Supporters of this mechanism suggest a fluctuating expression of E-cadherin, decreasing prior to invasion (40). In contrast, other studies support the passive invasion of IBC. In this process, tumorigenic cells secrete differentiation factors, which stimulate vasculogenesis around the IBC cells, while maintaining strong cell to cell attachments (41). This results in the
formation of tumor emboli within the de novo vessel. After disseminating through the lymphatics or vasculature the cancerous cells must adhere to the endothelium, extravasate into the surrounding tissue, and reestablish as a secondary tumor.

To confirm a clinical diagnosis of IBC, a skin biopsy is usually performed to verify the presence of tumor emboli invading the dermal lymphatic vessels of the breast (33). However, the pathological findings do not always match the clinical diagnosis. An absence of tumor emboli in the dermal lymphatics may be due to sampling heterogeneity in a patient with IBC or may indicate a possible misdiagnosis. For instance, the clinical symptoms characteristic of IBC are similar to those associated with acute mastitis, an inflammation of the breast tissue due to infection.

IBC is often diagnosed in the later stages of its progression. At the time of diagnosis almost all IBC patients have regional lymph node involvement and approximately 30% of patients have gross distant metastasis (37). Some patients may initially be misdiagnosed with mastitis, as discussed previously, prolonging the correct diagnosis and treatment. In addition, during earlier stages of IBC a mass in the breast tissue is not usually palpable due to the tendency of IBC cells to grow in sheets, making the symptoms less recognizable. Furthermore, the onset of IBC is extremely rapid, with usually less than six months separating the first symptoms and the diagnosis of a stage III-B or IV tumor (35).

The goal of this study was to research the possible therapeutic effects of 1,25-dihydroxyvitamin D$_3$ on inflammatory and non-inflammatory breast cancer cell lines in vitro. Vitamin D may become a potential non-toxic treatment option for various forms of breast carcinoma, in addition to its current preventative role.
Chapter 2
EXPERIMENTAL METHODS

2.1 Cell Culture and Experimental Preparation

The MDA-MB-231 cell line was obtained from ATCC (no. HTB-26), and the SUM-149 cell line was received from Dr. Steve Ethier, the cell line originator. MDA-MB-231 human breast cancer cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, CellGro) supplemented with 5% fetal bovine serum (FBS, HyClone), 1% penicillin-streptomycin solution (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, CellGro), 1% L-glutamine (200 mM solution, CellGro), and 0.2% insulin stock solution (0.75 mg/mL insulin in PBS, Sigma-Aldrich). SUM-149 human inflammatory breast cancer cells were maintained in Ham’s F-12 Medium (CellGro) supplemented with 5% fetal bovine serum, 1% penicillin-streptomycin solution, 1% antibiotic-antimycotic solution (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin, CellGro), 1% L-glutamine, 0.1% hydrocortisone stock solution (1 mg/mL, Sigma-Aldrich) and 1% insulin-transferrin-selenium stock solution (1 mg/mL insulin, 1 mg/mL transferrin, 3.4 µM sodium selenite, ScienCell). Both cell lines were incubated in a humidified atmosphere maintained at 5% CO2 and 37°C.

Prior to all experimental procedures, both MDA-MB-231 and SUM-149 cell lines were grown to approximately 75% confluence in a T-75 flask. Cells were detached from the bottom of the flask using trypsin (0.25% trypsin in HBSS, CellGro). The cell suspension was transferred to a 15 mL tube and media with 5% serum was
added to the cell suspension. The cells were centrifuged at 2,500 rpm for 5 minutes to form a pellet. The supernatant was removed and the cell pellet was re-suspended in the appropriate volume of media. The concentration of cells in the solution was determined using a hemacytometer, and the cell suspension was diluted to obtain the necessary cell concentration for each procedure.

2.2 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Prior to RT-PCR, RNA was extracted from the MDA-MB-231 and SUM-149 cell lines. Each of the following steps was completed with both cell lines. 1 mL of Trizol Reagent (Invitrogen) was added to a cell pellet, spun down from a confluent t-75 flask of cells. 0.2 mL of chloroform (Fisher Scientific) was added and the mixture was shaken for 15 seconds and then incubated at room temperature for 3 minutes. The tube was centrifuged at 12,000 G for 15 minutes and the top clear aqueous phase was transferred to new tubes. 0.5 mL isopropyl (Fisher Scientific) was added to the clear aqueous phase and the new tube was incubated at room temperature for 10 minutes. The tube was centrifuged at 12,000 G for 10 minutes. After, the supernatant was removed and 1.5 mL of 75% ethanol (Fisher Scientific) was added. The tube was mixed and then centrifuged at 7,500 G for 5 minutes. The supernatant was removed and the remaining pellet was dried. 40 µL RNAase-free water (Invitrogen) was added to the dry pellet and the concentration and quality of the RNA was measured.

Complementary DNA (cDNA) was then made using the RNA from the previous steps and the components of the SuperScript 2 First-Strand Synthesis System for RT-PCR (Invitrogen). The following components were combined in a small eppendorf tube for each cell line: 5 µg RNA, 1 µL of 50 µM oligo(dT) primer, 1 µL of
10 mM dNTP, and diethylpyrocarbonate-treated water to total 10 µL. The mixture was incubated at 65° C for 5 minutes, then placed on ice for 1-2 minutes. The cDNA synthesis mix was prepared by combining the following components for 1 reaction: 2 µL of 10X RT Buffer, 4 µL of 25 mM MgCl₂, 2 µL of 0.1 M DTT, and 1 µL of RNAase Out. 9 µL of cDNA synthesis mix was added to the small eppendorf tube containing the RNA mixture, and it was incubated at 42° C for 2 minutes. Next, 1 µL of SuperScript 2 RT was added and the mixture was incubated at 42° C for 50 minutes. The reactions were terminated at 70° C for 15 minutes and then chilled on ice for 1-2 minutes. Lastly, 1 µL of RNAase H was added and the tube was incubated for 20 minutes at 37° C.

To begin the RT-PCR, the following was combined in a PCR tube for each cell line: 1 µL of the forward primer, 1 µL of the reverse primer, 1 µL cDNA, and 7 µL GoTaq Green Master Mix (Promega). The GoTaq Green Master Mix was made using the components of the GoTaq Hot Start Polymerase kit (Promega) and contained the following: 10 µL of 5X Green GoTaq Flexi Buffer, 4 µL of 25 mM MgCl₂, 1 µL dNTP mix, 0.25 µL GoTaq Hot Start Polymerase, and 23.75 µL nuclease free water. The base sequence of the forward primer for the VDR was 5’-ATGGCCATCTGCATCGTCTC-3’ and the base sequence of the reverse primer for the VDR was 5’-GCACCGCCACAGGCTGTCCTA-3’. The positive control contained the β-actin forward primer (5’-TGTGATGGTGGAATGGGTCAG-3’) and reverse primer (5’-TTTGATGTCACGCAGCAGATTCC-3’); the negative control contained no primer. The PCR tubes were centrifuged and loaded into the PCR machine. The PCR program had a denaturation temperature of 94° C, an annealing temperature of 57.5° C, an extension temperature of 72° C, and repeated for 35 cycles.
After the PCR program ended, a 1000 base pair ladder (500 µg/mL, New England BioLabs) and the finished samples were loaded into an agarose gel and run at 100 V for 30-40 minutes. Pictures were taken of the gel using the computer program Alphamager v5.5.

2.3 Proliferation Assay

MDA-MB-231 and SUM-149 cell lines were plated in 6-well culture dishes at a concentration of 1.0 x 10^6 cells per milliliter of media. Each well was then treated with varying concentrations of 1,25-dihydroxyvitamin D₃ (Alexis Biochemicals) including 0 nM, 10 nM and 100 nM. The cells were incubated at 37°C for 24 hours. The media was removed from each well, and the cells were trypsinized and counted under a microscope using a hemocytometer.

2.4 Bicinchoninic Acid (BCA) Protein Analysis

MDA-MB-231 and SUM-149 cell lines were plated in 6-well culture dishes at a concentration of 1.0 x10^6 cells per milliliter of media. Each well was then treated with varying concentrations of 1,25-dihydroxyvitamin D₃ including 0 nM, 10 nM and 100 nM. The cells were incubated at 37°C for 24 hours. The media was removed from the wells, and each well was rinsed twice with PBS (phosphate buffered saline). A lysis mixture was prepared by adding 10 µL of protease inhibitor mix (BD Biosciences) and 10 µL of PMSF (Gold Biotechnology) for every milliliter of lysis buffer added. The lysis buffer contained 0.5 mL of 1 % Triton (Fisher Scientific), 0.1 mL of 1 mM EDTA (Fisher Scientific), and 0.5 mL of 10 mM Tris (Fisher Scientific). 500 µL of the lysis mixture was then added to each well, and allowed to sit for 5 minutes. A cell scraper was used to remove the cells from the bottom of the well, and
the lysis mixture was pipetted into eppendorf tubes. The standards supplied by the BCA Kit (Thermo Scientific Pierce Protein Research Products) were then prepared in the appropriate dilutions. 10 µL of each lysis mixture sample and each prepared standard was pipetted into separate wells of a 96-well plate. The working reagent was prepared by mixing the components labeled A and B of the BCA Kit in a 20:1 ratio. 200 µL of working reagent was then added to each well and the plate was shaken for 30 seconds. The plate was covered in tinfoil and incubated at 37° C for 30 minutes. The absorbance of each well at a wavelength of 570 nm was then determined using a plate reader. The resulting absorbance for each prepared standard was then used to generate a standard-curve. The standard-curve was used to determine the approximate protein concentrations of the experimental samples from each recorded absorbance.

2.5 Scratch Assay

MDA-MB-231 and SUM-149 cell lines were plated in 6-well culture dishes at concentrations of 3.0 x10^5 cells per milliliter of media. The cells were then incubated at 37° C for 24 hours. After incubation, the media was removed from all wells and new media was added. The cells were then treated with 0 nM, 10 nM, or 100 nM 1,25-dihydroxyvitamin D₃. A scratch was made down the middle of each well with a silicon coated pipette tip. Immediately after, 4 pictures were taken of each scratch. The cells were then incubated for 48 hours and final pictures were taken of each scratch. Using the computer program ImageJ, the diameter of the scratch in each picture was calculated. For each well, the average diameter of the initial scratch was compared to the average diameter of the final scratch, and the calculated difference was considered the migration distance of the cells over 48 hours.
2.6 Invasion Assay

Matrigel Invasion Chambers (BD Biosciences), containing 24 wells per plate, were removed from -20° C and allowed to come to room temperature. Serum-free media was made for both cell lines, containing all of the original supplements except FBS. Warm serum-free media was added to the inserts and bottom of the wells and incubated at 37° C for 2 hours. The serum-free media was then removed from both the inserts and the wells. The cells were then suspended in serum-free media at a concentration of 1.0 x 10⁵ and 0.25 mL was pipetted into each insert. Then, 0.25 mL of serum-free media containing 0 nM, 20 nM, 100 nM, or 200 nM 1,25-dihyrdoxyvitamin D₃ was added to each insert. After both components were added, each insert contained a cell concentration of 5.0 x 10⁴ and a 1,25-dihydroxyvitamin D₃ concentration of 0 nM, 10 nM, 50 nM, or 100 nM. Functioning as the chemoattractant for cellular invasion, 0.75 mL of serum-containing media was added to the bottom of each well containing an insert. The invasion chambers were then incubated at 37° C for 22 hours in a humidified tissue culture incubator.

After incubation, the media was removed from the inserts and the bottom of the wells. A cotton swab was used to remove the cells from the interior of the insert that did not invade across the membrane. 0.5 mL of crystal violet stain was added to each insert and well and left for 45 minutes before it was removed. The inserts were then washed in water to remove excess stain and allowed to dry for 48 hours. Pictures were then taken of each membrane (bottom of the insert) using a confocal microscope. The invaded cells in each picture were counted using the computer program Volocity (Improvision), and then manually validated to obtain more accurate results.
2.7 Emboli Growth Assay

SUM-149 cells were suspended in media supplemented with 0.5% low melting point agar, analytical grade (Thermo Scientific). The experimental flasks were treated with 1,25-dihydroxyvitamin D₃ at a concentration of 100 nM, and the control flasks were left untreated. The cells were then incubated on an orbital shaker at approximately 40 rpm for 72 hours to allow for emboli formation. Representative pictures were then taken of the formed emboli. The ImageJ computer program was used to calculate the diameter of representative emboli from each flask.
Chapter 3

RESULTS

3.1 VDR Present in MDA-MB-231 and SUM-149 Cells Determined by RT-PCR

RT-PCR was used to determine the presence of mRNA for the nuclear vitamin D receptor (VDR) in MDA-MB-231 and SUM-149 cell lines. While traditional mechanisms attribute the cellular effects of 1,25-dihydroxyvitamin D$_3$ to the activation of the nuclear VDR, recent research has suggested the cell surface receptor 1,25-D$_3$-MARRS (membrane associated, rapid response steroid-binding) protein may be responsible. Although previous studies have not researched the existence of the VDR in SUM-149 cells, current literature suggests the VDR is present in the MDA-MB-231 cell line. mRNA was isolated from MDA-MB-231 and SUM-149 cells, and cDNA was synthesized by reverse transcription from both cell lines. The cDNA was then added to the VDR primers and GoTaq green master mix (Promega) for PCR amplification. The samples were run through a gel and photographed using the Alphamager v5.5 computer program. A band for the VDR was present in MDA-MB-231 and SUM-149 cell lines as shown in Figure 2. The bands were nearly aligned with the 300 base pair marker, consistent with the 307 base pair size of the amplified region of the VDR.
Figure 2. Presence of Nuclear Vitamin D Receptor by RT-PCR. Samples of cDNA from MDA-MB-231 and SUM-149 cell lines. Vitamin D receptor approximately 300 base pairs.

3.2 1,25-dihydroxyvitamin D₃ Treatment of SUM-149 Cells led to Increased Protein Synthesis within 24h.

Protein concentration is a measure of both individual cell size and total cell number. Any changes in protein content can be attributed to either an increase in protein synthesis within individual cells or an increase in cell number. As shown in Figure 3, MDA-MB-231 and SUM-149 cell lines do not exhibit any significant changes in cell number with 1,25-dihydroxyvitamin D₃ treatment. Therefore, changes in protein content are due to protein synthesis. BCA protein analysis was used to determine the protein content of both cell lines. Cells were plated in a 6-well dish at
equal concentrations, and treated with 10 nM or 100 nM of 1,25-dihydroxyvitamin D₃. Control groups were untreated MDA-MB-231 and SUM-149 cells. Following 24 hours of treatment, cells were lysed to allow for protein extraction. BCA protein analysis was then used to determine the concentration of protein within each sample as shown in Figure 4. Treatment with 1,25-dihydroxyvitamin D₃ had a significant effect on MDA-MB-231 and SUM-149 protein concentration as defined by ANOVA (p = 0.010 and 0.008, respectively). Determined by Tukey’s test, MDA-MB-231 cells treated with 10 nM 1,25-dihydroxyvitamin D₃ and SUM-149 cells treated with 100 nM 1,25-dihydroxyvitamin D₃ exhibited a significant increase in protein concentration (respectively 33.5% and 29.0%) compared to the control.
**Figure 3.** *Effect of 1,25-dihydroxyvitamin D$_3$ on Proliferation.* Cells were treated for 24 hours followed by cell counting. The experiment was performed in triplicate. The average cell count for control MDA-MB-231 cells was approximately 74 and the average cell count for control SUM-149 cells was approximately 53. As indicated, 1,25-dihydroxyvitamin D$_3$ treatment had no effect on cell number as determined by ANOVA followed by Tukey’s test. Error bars shown are one standard deviation.
Figure 4.  **Effect of 1,25-dihydroxyvitamin D₃ on Protein Concentration.** Cells were treated with 1,25-dihydroxyvitamin D₃ at time of plating. 24 hours after plating cells were used in BCA protein analysis. The experiment was performed in triplicate. The protein concentrations were compared to the control (0 nM 1,25-dihydroxyvitamin D₃) within each experiment to calculate the percent change. Control MDA-MB-231 cells had a protein concentration of approximately 180 µg/ml and control SUM-149 cells had a protein concentration of approximately 90 µg/ml. Error bars shown are one standard deviation. * Significant compared to control, p < 0.05 as determined by ANOVA followed by Tukey’s test.

3.3 Migration of SUM-149 Cells Decreased 67.8% After 1,25-dihydroxyvitamin D₃ Treatment for 24h.

Scratch assays were used to measure changes in cellular migration in response to 1,25-dihydroxyvitamin D₃ treatment for MDA-MB-231 and SUM-149 cell lines. Breast cancer cells migrate from the initial site of tumor growth into the
surrounding tissue. This is the first step of metastasis, allowing for an excellent process to target in cancer treatment. If the ability of the cancerous cells to migrate into surrounding tissue can be down-regulated, it may decrease the spread of the cancer both locally and throughout the body. Cells were plated at equal concentrations into a 6-well dish and incubated for 24 hours to allow for adherence. The media was then replaced with fresh media containing 10 nM or 100 nM 1,25-dihydroxyvitamin D₃; the control groups were un-treated. Scratches were then made down the middle of each well at the time of treatment, and pictures of each scratch were taken immediately. After 48 hours, pictures were taken again and the migration distances were measured using the ImageJ computer program. Treatment with 1,25-dihydroxyvitamin D₃ had a significant effect on SUM-149 cellular migration as determined by ANOVA (p = 0.030). As shown in Figure 5, SUM-149 cells showed a dose-dependent decrease in migration in response to 1,25-dihydroxyvitamin D₃ treatment. Using Tukey’s test, SUM-149 cells treated with 100 nM 1,25-dihydroxyvitamin D₃ showed a significant decrease in migration (67.8%) compared to control SUM-149 cells. MDA-MB-231 cells did not show any significant change in migration compared to the control in response to 1,25-dihydroxyvitamin D₃.
Figure 5.  **Cellular Migration in Response to 1,25-dihydroxyvitamin D₃.** Scratches were made and cells were treated with 1,25-dihydroxyvitamin D₃ 24 hours after plating. The experiment was performed four times. The migration distance was calculated from the difference in scratch width at 24 hours after plating compared to the scratch width at 72 hours after plating. The migration distances were then compared to the control (0 nM 1,25-dihydroxyvitamin D₃) within each experiment to get the percent change. Control MDA-MB-231 cells migrated approximately 0.1 mm and control SUM-149 cells migrated approximately 0.4 mm. Error bars shown are one standard deviation. * Significant compared to control, p < 0.05 as determined by ANOVA followed by Tukey’s test.

### 3.4 Invasion of SUM-149 Cells Decreased 43.9% upon Calcitriol Treatment for 24h.

Invasion assays were used to measure changes in cellular invasion across a basement membrane in response to 1,25-dihydroxyvitamin D₃ treatment for MDA-MB-231 and SUM-149 cell lines. To migrate from the site of initial tumor formation,
obtain access to the circulatory system, and extravasate into the stroma of distant organs, breast cancer cells must travel through the basement membrane underlying various epithelial and endothelial layers. When the cancerous cells gain this ability, the risk of metastasis greatly increases. Therefore, decreasing the invasive potential of cancerous cells through vitamin D treatment may lead to a decrease in metastasis. Cells were plated into invasion chambers at equal concentrations in serum-free media. At the time of plating, cells were treated with 10 nM, 50 nM or 100 nM of 1,25-dihydroxyvitamin D₃; control groups were un-treated. Media with serum was added to the wells containing invasion chambers, acting as a chemoattractant. Cells were incubated for 24 hours, allowing for cellular invasion across the basement membrane. Media was removed from the chambers and wells, and the invaded cells were stained with crystal violet. Pictures of the invaded cells were taken for data analysis. Treatment with 1,25-dihydroxyvitamin D₃ had a significant effect on SUM-149 cellular invasion as determined by ANOVA (p = 0.015). As shown in Figure 6, SUM-149 cells demonstrated a dose-dependent decrease in invasion in response to 10 nM, 50 nM and 100 nM 1,25-dihydroxyvitamin D₃ treatment, respectively 10.7%, 33.3% and 43.9%. SUM-149 cells treated with 100 nM 1,25-dihydroxyvitamin D₃ showed a significant decrease (43.9%) in invasion compared to the control, determined by Tukey’s test. MDA-MB-231 cells did not show any significant change in invasion compared to the control in response to 1,25-dihydroxyvitamin D₃.
Figure 6.  **Influence of 1,25-dihydroxyvitamin D₃ on Invasion.** Cells were treated with 1,25-dihydroxyvitamin D₃ at time of plating. Invaded cells were stained and counted 24 hours after plating. The experiment was performed four times. Invasion was measured as the percent change in number of invaded cells compared to the control for each experiment. Control MDA-MB-231 cells had approximately 1200 invaded cells and control SUM-149 cells approximately 60 invaded cells. SUM-149 cells treated with 100 nM 1,25-dihydroxyvitamin D₃ show the largest decrease in invasion. Error bars shown are one standard deviation. * Significant compared to control (0 nM 1,25-dihydroxyvitamin D₃), $p < 0.05$ as determined by ANOVA followed by Tukey’s test.

3.5 **SUM-149 Emboli Size Decreased 69.4% upon 1,25-dihydroxyvitamin D₃ Treatment for 72h**

SUM-149 cells were plated in conditions to promote in vitro emboli growth. Inflammatory breast cancer cells are known to form tumor emboli, which
break off from the original tumor site and travel through the lymphatic system. The blockage of dermal lymphatic vessels by tumor emboli causes the red, swollen appearance of the breast tissue, characteristic of inflammatory breast cancer. In addition, the tumor emboli may grow as a metastasis within the lymphatic vessel.

SUM-149 cells were plated in media supplemented with 0.5% low melting point agar, and treated at the time of plating with 0 nM (control group) or 100 nM 1,25-dihydroxyvitamin D$_3$. As shown in Figure 7, the SUM-149 cells treated with 100 nM 1,25-dihydroxyvitamin D$_3$ for 72 hours formed emboli 69.4% smaller (p = 0.018) than control SUM-149 cells as determined by ANOVA.
**Figure 7.** *Changes in SUM-149 Emboli Growth.* Cells were treated with 1,25-dihydroxyvitamin D₃ at the time of plating. 72 hours after plating the diameter of the emboli were measured. The experiment was performed in triplicate. Error bars shown are one standard deviation. * Significant compared to control (0 nM 1,25-dihydroxyvitamin D₃), p < 0.05 as determined by ANOVA.
Chapter 4

DISCUSSION

4.1 Discussion

As the results show, the VDR is expressed in MDA-MB-231 and SUM-149 breast cancer cell lines. This is consistent with the majority of the literature on MDA-MB-231 cells, which indicate the presence of the VDR in these breast cancer cells (32). Due to the lack of established information on the presence of the VDR in SUM-149 cells, the results from this study are among the first to indicate its existence in the SUM-149 cell line. The decreased migration, invasion and emboli formation in the SUM-149 cell line in response to 1,25-dihydroxyvitamin D$_3$ treatment may be mediated by the VDR; however, additional research is required to determine the specific mechanisms involved.

Stimulation of MDA-MB-231 with 10 nM 1,25-dihydroxyvitamin D$_3$ and SUM-149 cells with 100 nM 1,25-dihydroxyvitamin D$_3$ caused a significant increase in protein concentration compared to the control. This change was not due to an increase in cell number, as shown in Figure 3, suggesting an increase in protein synthesis within each individual cell. This finding is supported by previous research detailing changes in the expression of numerous genes, and therefore protein synthesis, in response to 1,25-dihydroxyvitamin D$_3$ stimulation (42). In addition, it is important to note that the decreases in SUM-149 cellular migration and invasion are not due to increased cell death in response to 1,25-dihydroxyvitamin D$_3$, supported by the results in Figure 3.
Current literature lacks significant information regarding the effects of 1,25-dihydroxyvitamin D$_3$ on SUM-149 cellular migration and invasion. Therefore, the scratch assay results from this study are among the first to demonstrate a significant decrease in migration in SUM-149 cells after 48 hours of exposure to 1,25-dihydroxyvitamin D$_3$. Invasion was also significantly inhibited in the SUM-149 cell line after only 24 hours of treatment with 1,25-dihydroxyvitamin D$_3$. Consistent with the results described in this study, previous research on MDA-MB-231 cells does not indicate any significant changes in migration or invasion after 48 and 24 hours, respectively, in response to 1,25-dihydroxyvitamin D$_3$ treatment. However, past research documenting a decrease in MDA-MB-231 migration and invasion after 4 days of exposure indicates an effect of 1,25-dihydroxyvitamin D$_3$ after longer periods of treatment (21). Overall, the results of this study along with previous research suggest an inhibitory effect of 1,25-dihydroxyvitamin D$_3$ on the migration and invasion of SUM-149 and MDA-MB-231 breast cancer cells.

Conventional mechanisms describing non-inflammatory breast cancer metastasis, and perhaps a form of IBC metastasis, require the disruption of cell to cell adhesions to permit the migration of cancerous cells through the surrounding tissue (39). The migratory cells move towards lymphatic or circulatory vessels, ultimately leading to the invasion of single cells into the vessel. After the cancerous cell travels through the lymphatic or circulatory system to a different location in the body, it must then invade back into the tissue, where it can begin the formation of a secondary tumor. Due to the importance of cancer cell migration and invasion in this mechanism of metastasis, it is suggested that the inhibition of these processes through 1,25-dihydroxyvitamin D$_3$ treatment will decrease the incidence of inflammatory and non-
inflammatory breast cancer metastasis. This decrease in metastatic potential should result in a corresponding decrease of cancer related deaths caused by metastasis, which is currently approximately 90% (39).

Although the mechanism of single cell metastasis may take place in inflammatory breast cancer patients, the major mode of IBC dissemination throughout the body is thought to occur through the formation of tumor emboli. These compact clumps of IBC cells are held together by cell-to-cell adhesions, characterized by an overexpression of E-cadherin. Previous research indicates that the adherens junctions confer a resistance to apoptosis among the cells forming the tumor emboli (43). Due to this dangerous attribute, along with the propensity of IBC cells to metastasize as tumor emboli, the formation of these tightly packed spheres is an excellent chemotherapeutic target. As demonstrated in Figure 7, SUM-149 cells treated with 100 nM 1,25-dihydroxyvitamin D₃ for 72 hours form significantly smaller tumor emboli than those left untreated. As of present, there is no published literature describing the effects of 1,25-dihydroxyvitamin D₃ stimulation on the growth or size of SUM-149 tumor emboli.

In summary, the in vitro results from this research indicate that 1,25-dihydroxyvitamin D₃ has the potential to down-regulate inflammatory and non-inflammatory breast cancer metastasis in vivo. This is accomplished through significantly decreasing the migratory and invasive potential of the breast cancer cells, in addition to decreasing tumor emboli formation in IBC patients. The cellular mechanisms responsible for the effects of 1,25-dihydroxyvitamin D₃ on breast cancer metastasis is still largely unknown and controversial; however, it may be mediated through the VDR, which was shown to exist as mRNA in both cell lines.
REFERENCES


