

5-2009

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Honors Project Thesis

The Effects of 1,25-dihydroxyvitamin D on HIF-1 α

Expression In Breast Epithelial Cells Containing the ErbB2

Oncogene

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Purpose

There is evidence that the active form of vitamin D, 1,25 dihydroxyvitamin D (1,25(OH)₂D), may lead to reduced tumor formation by inhibiting vascularization, and thus feeding the tumor (1). Vascularization is controlled in part by the release of proteins which can be stimulated by low oxygen (hypoxia) to increase the transcriptional activity of hypoxia inducible factor 1 α (HIF-1 α) (2). HIF-1 α controls the level of an important protein, vascular endothelial growth factor (VEGF), which stimulates vascularization (2). The level of HIF-1 α protein level is regulated both transcriptionally and by protein stability through the activity of prolyl hydroxylase (PHD) (2). The purpose of this study is to determine if 1,25(OH)₂D alters the mRNA expression of HIF-1 α in untransformed (normal) cells and cells that contain the ErbB2 oncogene, a mutated gene commonly found in cancers.

Literature Review

Breast cancer is the most prevalent cancer affecting women. Around the globe each year there are approximately 1,115,000 cases and 410,000 deaths due to breast cancer (3). In 2006, there were 215,000 cases and 41,000 deaths resulting from breast cancer (3). The estimated statistics for diagnosis is 192,370 and 40,170 for deaths in 2009 (4,5).

Vitamin D can be obtained from the diet or from the sunlight acting on the skin. The sunlight acting on the skin is responsible for 90-95% of humans' vitamin D requirement (6). When skin is exposed to sunlight, the UV rays stimulate the photolytic conversion of 7-dehydrocholesterol to previtamin D₃ in the dermis and epidermis. Previtamin D₃ is isomerized by body temperature rapidly to vitamin D₃ (6). Once Vitamin D enters the body it is hydroxylated on carbon 25, resulting in 25-hydroxyvitmain D (25(OH)D) in the liver. This is the

major circulating form of vitamin D (6). The 25-hydroxyvitamin D is then hydroxylated at the 1 carbon to form 1,25-dihydroxyvitamin D ($1,25(\text{OH})_2\text{D}$) in the kidney (7). This is the biologically active form of vitamin D. This metabolite regulates cellular growth as well as calcium, phosphorus, and bone metabolism (6).

The relationship of the intake of vitamin D and breast cancer prevention is a well studied area. For example, a study by Knight, et al., showed that there was a reduced risk for breast cancer associated with cod liver use and increasing milk consumption, both dietary sources of vitamin D (8). In addition, there was a reduced risk for breast cancer in adulthood associated with higher sun exposure from ages 10 to 19 (8). The researchers stated that exposure earlier in life, particularly during breast development, may be a critical period to decrease risk for breast cancer development (8). A study involving postmenopausal women found that the association of high vitamin D intake with breast cancer was the strongest in the first 5 years after the dietary assessment and then the vitamin D intake declined (9). The researchers showed that vitamin D intake of greater than 800 IU/day was associated with a small decrease in risk (9).

Exposure to the sunlight may decrease the risk for breast cancer by increasing the production of vitamin D_3 in the skin, which would result in a higher circulating level of $25(\text{OH})\text{D}$ (3, 6, 7, 8). The $25(\text{OH})\text{D}$ can be metabolized in breast tissue to $1,25(\text{OH})_2\text{D}$ which may increase the cellular level of $1,25(\text{OH})_2\text{D}$ (6). An increase in $1,25(\text{OH})_2\text{D}$ may help maintain cellular growth and prevent the cells from becoming malignant (6).

The vitamin D receptor, VDR, and $1,25(\text{OH})_2\text{D}$ affect the proliferation, differentiation and apoptosis in mammary glands (10). Thus, there is a possibility that optimal vitamin D status may protect against mammary epithelial cell transformation with ultimate development of

tumors (10). Preclinical studies in animals show that vitamin D compounds can reduce breast cancer development (10). There is also human data which show that both vitamin D status and genetic variations in the VDR may affect one's breast cancer risk (10). Thus, vitamin D may be an effective chemopreventive agent against the development of breast cancer; however, it is not clear how vitamin D may inhibit tumor development (1). Mechanisms proposed include the regulation of blood vessel supply to the tumor regulated by low oxygen status, or hypoxia. Hypoxia is the major pathophysiologic condition that regulates angiogenesis (1). The adaptive changes in response to hypoxia can lead toward treatment resistance to both radio and chemotherapy (11). Hypoxia-inducible factor-1 (HIF-1) is a transcription factor that is a master regulator of oxygen homeostasis and it mediates increased angiogenesis in response to hypoxia (2). It is composed of HIF-1 α and HIF-1 β . HIF-1 α protein present in the cell regulates the formation and transcriptional activity of HIF-1 (2). The expression of HIF-1 α , at least in part, is controlled by the cellular oxygen tension. HIF-1 α is very unstable under normoxic condition having a half-life of less than 10 minutes (2). HIF-1 α protein level is regulated at three levels; increase in transcription, mRNA stability and through protein degradation (2). Protein degradation is controlled primarily by prolyl hydroxylase (PHD). PHD hydroxylates HIF-1 α and thus targets the protein for degradation, reducing the amount of HIF-1 α protein (2). HIF-1 aids in tumor cell survival under hypoxic conditions through the expression of gene products that promote anaerobic ATP synthesis (glucose transporter, a series of glycolytic enzyme, and cell survival factors) (2). HIF-1 also induces the expression of vascular endothelial growth factor (VEGF), a critical angiogenic factor which promotes blood vessel formation to supply oxygen and nutrients to cells for growth, including tumors (2).

There is an increasing amount of evidence that shows that HIF-1 contributes to tumor progression and metastasis (2). Studies have shown that the expression levels of HIF-1 α in biopsies of various tumors correlate with tumor aggressiveness, vascularity, treatment failure, and mortality (2). Another study showed similar results finding that high HIF-1 α expression was associated with poor overall survival and high risk for metastasis (11). One study showed that preneoplastic lesions in breast, colon, and prostate overexpressed HIF-1 α , but there was no increase in benign tumors in the breast and uterus (12). The same study also showed that HIF-1 α overexpression was noted in only 29% of primary breast cancers, but was noted in 69% of breast cancer metastases (12). The data from this study suggests that overexpression of HIF-1 α can occur early in carcinogenesis even before histological evidence of angiogenesis or invasion (12).

There are multiple steps involved before a tumor develops and becomes metastatic. The stages are initiation, promotion, conversion and progression (13). It is important to study these stages because nutritional components may inhibit any of them. Since nutrients are readily available and low risk it is likely a good preventative tool. It is important to study how nutrients affect the early stages of 'normal' cells, initiation, and promotion to see if it will be effective at the later stages, but at the same time not harmful in 'normal' cells. The ErbB2 oncogene has been associated with larger tumors in a few studies (14). The main mechanism of ErbB2 activation involves amplification. This has always been associated with overexpression (14). The HER2 receptor tyrosine kinase encoded by ErbB2 occurs in approximately 1/3 of breast tumors and is associated with increased tumor grade, chemotherapy resistance, and decreased rates of patient survival (15). Understanding how vitamin D regulates cellular metabolism in

cells containing the mutated ErbB2 gene will contribute to identifying if vitamin D will be an effective chemopreventive agent in an early stage of cancer development.

Vascular endothelial growth factor (VEGF) is known as proangiogenic factor (16). Angiogenesis is known as the formation of new blood vessels from preexisting vasculature (16). Angiogenesis is essential in normal development, growth and maintenance of bone structure, reproduction, and wound healing (16). More evidence is starting to show that angiogenesis plays an imperative role in the progression of cancer (16). It is important to study VEGF because HIF-1 α controls the levels of this growth factor (2). Since 1,25(OH)₂D affects multiple cellular signaling pathways in multiple cell types, it has been hypothesized that it may have an influence on angiogenesis (16). Studies have shown that 1,25(OH)₂D influences angiogenesis in a variety of target tissues by regulating 1 or more components of the angiogenic process (16). A study was done by Levine and Teegarden investigating the effects of 1,25(OH)₂D on angiogenesis in normal cells versus cancerous cells (16). The researchers found evidence that 1,25(OH)₂D altered the angiogenic phenotype of human umbilical vein endothelial cells after being treated with 1,25(OH)₂D-treated C3H10T1/2 mouse fibroblasts and their Harvey ras-oncogene transfected counterparts (16). When MCF10A AND MCF10A*ras* cells (a human breast epithelial cell model for multistage carcinogenesis) were studied, the researchers found similar effects on VEGF release (16). Other Studies show VEGF is significantly induced after exposure to hypoxia due to the increase in HIF-1 α (1). When treated with 1,25(OH)₂D, VEGF was significantly inhibited under normoxic and hypoxic conditions (1).

There is evidence that 1,25(OH)₂D inhibits HIF-1 α expression in tumor cells (17). However, it is not known if 1,25(OH)₂D has an impact on HIF-1 α gene expression in normal or

in cells which are models for multistage carcinogenesis such as those containing the ErbB2 oncogene, a common genetic mutation found in breast cancer, under normoxic or hypoxic conditions. The purpose of this study is to determine if 1,25(OH)₂D alters the mRNA expression of HIF-1 α in untransformed (normal) cells and cells that contain the ErbB2 oncogene, a mutated gene commonly found in cancers. The hypothesis is that 1,25(OH)₂D treatment of normal breast cells in a hypoxic environment will increase HIF-1 α expression. In contrast, 1,25(OH)₂D treatment of ErbB2 oncogene containing cells in a hypoxic condition will decrease the expression of HIF-1 α .

Methods

Human mammary epithelial MCF10A and MCF10A cells transfected with the ErbB2 oncogene were used in these experiments. The MCF10A cell line is derived from benign, or untransformed, breast tissue. This is a good model for normal breast tissue and cancer progression. The cells were maintained in MEGM (Mammary Epithelial Growth Medium, Serum-free), supplemented with 100 ng/ml cholera toxin and 1% penicillin-strep at 37 C with 5% CO₂. The cells were treated with the vehicle (ethanol) and 10 nM of 1,25(OH)₂D in culture medium for 24 hours. For the hypoxic conditions the cells were treated with 100 μ M of cobalt chloride 2 hours before harvest. The RNA was isolated by Trizol as per manufacturer's instructions. The c-DNA was prepared by using the oligoDT and random hexamer primers using the Omniscript kit from Qiagen. Brilliant SYBR green QPCR Master Mix (Stratagene, Cedar Creek, TX) in at least duplicate using a thermocycler (Mx3000P, Stratagene) was used to check for gene expression. The final n minimum for each point was 6. Gene expression levels were determined by the Δ CT method using GAPDH for control.

Primers

VEGF: CAAGATCCGCAGACGTGTAA
TCACATCTGCAAGTACGTTTCG

HIF-1a: TGCTGAAGACACAGAAGCAAA
AAAGCGAAGTCCTCAAAGC

PHD: CTTTGACCGGTTGCTCATT
TAGGCGGCTGTGATACAGGT

GAPDH: TCACCATCTTCCAGGAGCG
CTGCTTCACCACCTTCTTGA

Statistical Analysis

Statistical analysis was determined by using ANOVA. A p value of less than 0.05 was considered to be significant.

Results

To determine if $1,25(\text{OH})_2\text{D}$ regulates HIF-1 α mRNA expression, untransformed and ErbB2 containing cells were treated with vehicle or $1,25(\text{OH})_2\text{D}$ for 24 hours and mRNA levels determined. $1,25(\text{OH})_2\text{D}$ significantly reduced HIF-1 α mRNA abundance in both cell lines under normoxic conditions but not under hypoxic conditions (Figure 1).

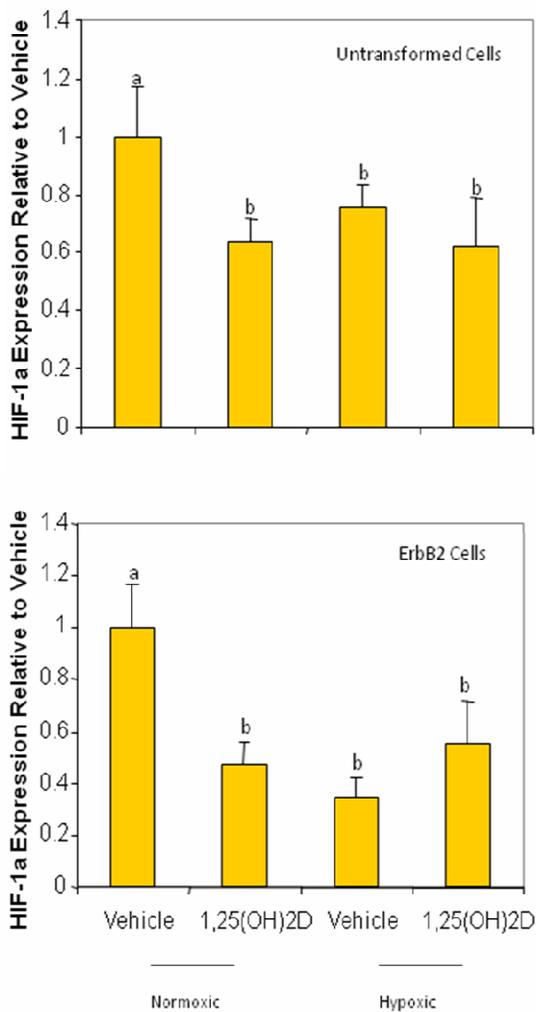


Figure 1: Effects of $1,25(\text{OH})_2\text{D}$ on the expression of HIF-1 α under normal and hypoxic conditions. Cells were treated for 24 hours with vehicle or $1,25(\text{OH})_2\text{D}$, and cells harvested for determination of HIF-1 α mRNA abundance using RT-PCR. Bars with different letters indicate significant difference.

The effect of 1,25(OH)₂D on PHD mRNA abundance, the enzyme involved with stability of HIF-1 α stability and whose activity is regulated by hypoxia, was tested. Following treatment with vehicle or 1,25(OH)₂D, the PHD mRNA abundance was significantly reduced only under normoxic conditions and only in the ErbB2 cell line as shown in Figure 2.

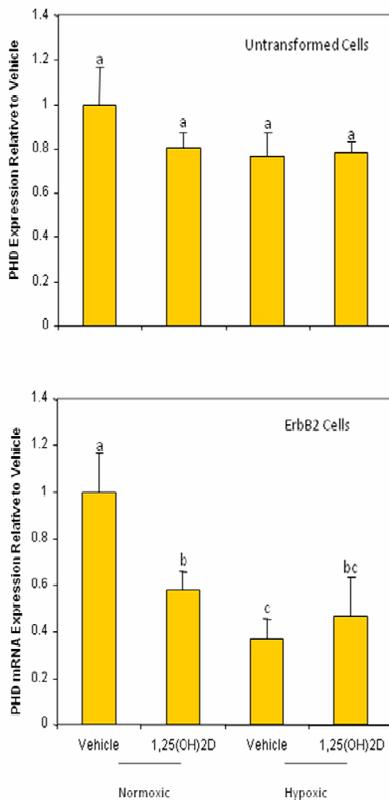


Figure 2: The effects of 1,25(OH)₂D on the expression of PHD mRNA expression under normal and hypoxic conditions. Cells were treated for 24 hours with vehicle of 1,25(OH)₂D, and cells harvested for determination of HIF-1 α mRNA abundance using RT-PCR. Bars with different letters indicate significant difference.

To determine if the changes in the HIF-1 α protein expression affected the downstream activity of this transcriptional factor, the effect of 1,25(OH)₂D treatment of a well described transcriptional target of HIF-1 α , vascular endothelial growth factor (VEGF), was tested. Treatment with 1,25(OH)₂D reduced VEGF mRNA expression only under normoxic conditions and only in the ErbB2 cell line.

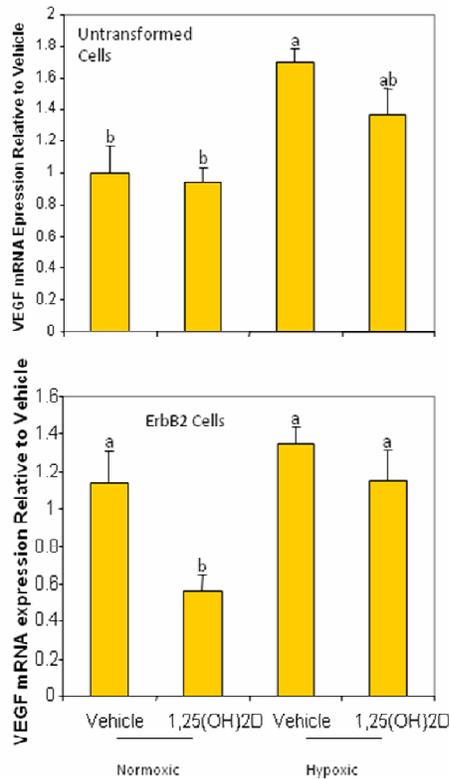


Figure 3: The effects of 1,25(OH)₂D on the expression of VEGF mRNA under normal and hypoxic conditions. Cells were treated for 24 hours with vehicle of 1,25(OH)₂D, and cells harvested for determination of HIF-1 α mRNA abundance using RT-PCR. Bars with different letters indicate significant difference.

Discussion

Hypoxia in the untransformed LXS cells increased VEGF, reduced HIF-1 α and did not change PHD mRNA expression. Hypoxia in the ErbB2-containing cells lead to no change in VEGF and a reduction in PHD, but a similar reduction in HIF-1 α mRNA expression as in LXS cells. In both the untransformed and the ErbB2-containing cells, 1,25(OH)₂D did not alter VEGF expression under hypoxic conditions. Under normoxic conditions, 1,25(OH)₂D did not alter the expression of VEGF in untransformed cells, but VEGF expression in ErbB2

cells was reduced. In contrast to the hypothesis, 1,25(OH)₂D significantly reduced the expression of HIF-1 α in the normoxic condition but there was no difference under hypoxic conditions, and the results were similar in the ErbB2-containing cells. There was no difference in PHD expression with 1,25(OH)₂D treatment under both conditions in the untransformed cells. However, under normoxic conditions, 1,25(OH)₂D treatment reduced the expression of PHD in the ErbB2-containing cell line. These results suggest that HIF-1 α is regulated similarly in the untransformed and ErbB2-containing cells under both normoxic and hypoxic conditions, but PHD regulation differs in the cell lines under normoxic conditions.

Thus, overall, 1,25(OH)₂D treatment did not change mRNA abundance of any of the genes tested, HIF-1 α , PHD or VEGF under hypoxic condition in either untransformed or ErbB2 containing cells. Although 1,25(OH)₂D treatment reduced HIF-1 α mRNA abundance under normoxic conditions in untransformed cells, there was no effect on its target gene, VEGF mRNA. In contrast, in ErbB2 containing cells, 1,25(OH)₂D not only reduced mRNA abundance of HIF-1 α but also of its target gene, VEGF. These results are consistent with down regulation of HIF-1 α by 1,25(OH)₂D affecting the down regulation of VEGF. In addition, 1,25(OH)₂D treatment reduced PHD mRNA abundance. If the decrease in PHD alters overall activity of this enzyme, this would lead to an increase in the stability of HIF-1 α , which is not consistent with the decrease in VEGF mRNA.

Future studies are required to better understand the regulation of HIF-1 α by 1,25(OH)₂D. Cell culture studies should be done to explore the differential regulation of PHD in untransformed compared to ErbB2 containing cells. Other cell lines should be tested to see if

these results will apply in other cancers. Animal studies should be completed on animals that have tumors containing the ErbB2 oncogene and those who don't. These studies would contribute to a better understanding of what may happen in humans. Ultimately, this information will lead to developing better recommendations for the use of vitamin D as a chemopreventive agent.

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