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IDENTIFICATION OF NOVEL MEDIATORS OF VITAMIN D SIGNALING AND 1,25(OH)$_2$D$_3$ RESISTANCE IN MAMMARY CELLS

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Abstract

Since the discovery of the vitamin D receptor (VDR) in mammary cells, the role of the vitamin D signaling pathway in normal glandular function and in breast cancer has been extensively explored. *In vitro* studies have demonstrated that the VDR ligand, 1,25(OH)$_2$D$_3$, modulates key proteins involved in signaling proliferation, differentiation and survival of normal mammary epithelial cells. Anti-proliferative and pro-differentiating effects of 1,25(OH)$_2$D$_3$ have also been observed in VDR positive breast cancer cells, indicating that transformation *per se* does not abolish vitamin D signaling. However, many breast cancer cell lines are less sensitive to 1,25(OH)$_2$D$_3$ than normal mammary epithelial cells. Reduced sensitivity to 1,25(OH)$_2$D$_3$ has been linked to alterations in vitamin D metabolizing enzymes as well as down regulation of VDR expression or function. In this report, we describe results from a proteomics screening approach used to search for proteins involved in dictating sensitivity or resistance to vitamin D mediated apoptosis in breast cancer cells. Several proteins not previously linked to 1,25(OH)$_2$D$_3$ signaling were identified with this approach, and a distinct subset of proteins was linked to 1,25(OH)$_2$D$_3$ resistance. Follow-up studies to determine the relevance of these proteins to vitamin D signaling in general are in progress.

Keywords

1; 25-dihydroxyvitamin D; VDR; apoptosis; mammary

Overview: cell regulatory functions of 1,25(OH)$_2$D$_3$

Since the discovery of the VDR in mammary cells, the role of the vitamin D signaling pathway in normal glandular function and in breast cancer has been extensively explored. In most VDR positive epithelial cells, 1,25(OH)$_2$D$_3$ mediates anti-proliferative effects, and it may subsequently trigger differentiation or apoptosis. Expression profiling of breast, prostate, colon and squamous carcinoma cells by microarray technology has identified 1,25(OH)$_2$D$_3$ responsive gene clusters involved in regulation of cell cycle, differentiation, cell adhesion and immune responses [1–4], indicating a diverse and broad range of VDR target genes potentially involved in cell regulation. Anti-proliferative effects of 1,25(OH)$_2$D$_3$ have been linked to alterations in key cell cycle regulators which lead to de-phosphorylation of the retinoblastoma protein [5,6] and arrest of cells in G_0/G_1. The cyclin dependent kinase inhibitors p21 and/or p27 are genomic targets of the 1,25(OH)$_2$D$_3$ – VDR complex in many cell types [7,8]. 1,25 (OH)$_2$D$_3$ also blocks mitogenic signaling, including that of estrogen, EGF and IGF-1, and up-regulates growth inhibitors such as TGFβ [9].

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Induction of apoptosis by $1,25(OH)_2D_3$

Our laboratory was the first to report the activation of apoptosis by $1,25(OH)_2D_3$ [10], and the requirement of the VDR for this process [11]. Since our initial reports in MCF-7 breast cancer cells, cells derived from prostate cancer, squamous carcinoma, glioma and others have been shown to undergo apoptosis in response to $1,25(OH)_2D_3$ [8,12–15]. The relevance of vitamin D mediated apoptosis *in vivo* has been confirmed in the context of normal mammary gland development and tumor biology. Mice lacking VDR exhibited delayed apoptosis in mammary epithelial tissue during post-lactational involution [16], indicating a role for vitamin D signaling during physiological apoptosis. Treatment of tumor bearing mice with low-calcemic vitamin D analogs induced tumor regression via activation of apoptosis [5,17,18], indicating the potential of vitamin D based drugs for manipulation of the cell death pathway in transformed cells.

These findings underscore the importance of dissecting the cellular mechanisms of VDR mediated apoptosis. In breast cancer cells, $1,25(OH)_2D_3$ induced apoptosis involves generation of oxidative stress, dissipation of the mitochondrial membrane potential and cytochrome c release [5,10,19], features of the intrinsic (mitochondrial) pathway of apoptosis. These effects of $1,25(OH)_2D_3$ involve redistribution of the pro-apoptotic protein Bax from cytosol to mitochondria [5,19,20], and can be prevented by overexpression of the anti-apoptotic protein Bcl-2 [21]. Downstream events involved in dismantling of the cell during $1,25(OH)_2D_3$ induced apoptosis are mediated via several protease pathways, including caspases [15,19,22], μ-calpain [23] and cathepsins [6,24,25]. Collectively, these studies indicate that a wide variety of different signaling pathways, apoptotic regulatory proteins and proteases may contribute to $1,25(OH)_2D_3$ mediated apoptosis depending on the specific cell type and/or context.

Models of resistance to $1,25(OH)_2D_3$ mediated growth regulation

Although it is clear that the VDR is required for breast cancer cell responsiveness to vitamin D compounds [11] a number of breast cancer cell lines that express VDR fail to respond to the anti-proliferative effects of $1,25(OH)_2D_3$. Data from mammary cell lines suggest that oncogenic transformation with SV40 or ras inhibits VDR signaling and induces resistance to the growth inhibitory effects of $1,25(OH)_2D_3$ [26,27], supporting the concept that breast cancer progression may be facilitated by deregulation of the vitamin D pathway. Reduced sensitivity to $1,25(OH)_2D_3$ has been linked to alterations in vitamin D metabolizing enzymes as well as down regulation of VDR expression or function. In an effort to understand the basis for vitamin D insensitivity, we selected resistant sub-clones by continuous culture of MCF-7 cells in 100nM $1,25(OH)_2D_3$ [28]. The resulting MCF-7^{DRES} cells express wild type VDR at lower levels that the parental MCF-7 cells, and do not undergo growth arrest or apoptosis in response to $1,25(OH)_2D_3$. MCF-7^{DRES} cells are selectively resistant to $1,25(OH)_2D_3$ and its structural analogs, and respond to other anti-proliferative agents [28–30]. Similar results were subsequently reported in an independently derived $1,25(OH)_2D_3$ resistant MCF-7 sub-clone labeled MCF-7/VDR [31]. Collectively, these MCF-7 sub-clones with selective $1,25(OH)_2D_3$ resistance provide an excellent model for probing the mechanisms underlying vitamin D resistance.

Identification of novel mediators of vitamin D signaling and $1,25(OH)_2D_3$ resistance in mammary cells

In recent studies, we used a proteomic screening approach (BD Powerblot, BD Biosciences, San Jose, CA) to identify additional mediators of vitamin D signaling and $1,25(OH)_2D_3$ resistance in breast cancer cells. With this approach, we identified ten proteins (out of 270 proteins evaluated) that were differentially expressed between MCF-7 cells treated for 72 hours

with 100nM $1,25(OH)_2D_3$ and vehicle treated MCF-7 cells. As shown in Table 1, eight of these proteins (SHC, Stat6, cyclin A, cyclin D3, Rho-GDI, ILK, GSK3b and Grim 19) were significantly down regulated by $1,25(OH)_2D_3$, whereas only two proteins (PTEN, Cathepsin D) were significantly up-regulated by $1,25(OH)_2D_3$. The functions of most of the altered proteins are consistent with the anti-proliferative and pro-apoptotic effects of $1,25(OH)_2D_3$ in the MCF-7 cell line. These include the down regulated proteins cyclin A and cyclin D3 (which drive cell cycle progression), ILK and Stat6 (which promote survival), SHC and GSK3b (proteins implicated in signal transduction cascades), and Rho-DGI (a regulator of ras signaling that is cleaved by caspases during apoptosis). Up-regulation of the lysosomal protease cathepsin D is consistent with our previous observations that $1,25(OH)_2D_3$ and its analog EB1089 mediate lysosomal activation and up-regulate cathepsin B expression during apoptosis of MCF-7 cells [6,24]. Furthermore, EB1089 has recently been shown to induce autophagy, a form of apoptosis characterized by increased cathepsin activity, in MCF-7 cells [25]. Up-regulation of PTEN, an inhibitor of the AKT pathway, by $1,25(OH)_2D_3$ in MCF-7 cells is consistent with up-regulation of this tumor suppressor protein during vitamin D analog induced terminal differentiation of leukemic cells [32].

Grim-19, a protein down-regulated by $1,25(OH)_2D_3$ in our initial screen, was of special interest since it had previously been linked to apoptosis induced by the combination of interferon β (IFN) and retinoic acid (RA) [33]. Grim-19 was significantly down regulated in MCF-7 cells treated with $1,25(OH)_2D_3$ for 72 hours (Figure 1A). It is important to note that $1,25(OH)_2D_3$ did not alter Grim-19 mRNA expression (data not shown), and therefore would not have been identified as a VDR target using genomic approaches. In MCF-7 cells, Grim-19 was re-distributed from mitochondria to nuclear bodies during apoptosis induced by $1,25(OH)_2D_3$ (not shown). Expression of Grim-19 did not differ between MCF-7 and MCF-7^{DRES} cells, which were selected for resistance to $1,25(OH)_2D_3$ (Figure 1B). Furthermore, there was no effect of $1,25(OH)_2D_3$ on Grim-19 in MCF-7^{DRES} cells, but its expression was reduced in association with apoptosis triggered by IFN/RA in these cells. Surprisingly, Grim-19 was not down-regulated during apoptosis induced through cell surface TNFα death receptors (not shown). These data suggest that $1,25(OH)_2D_3$ induced apoptosis is mechanistically distinct from that induced by TNFα, but mechanistically similar to that induced by the IFN/RA combination. These findings are worthy of follow-up considering the known cross-talk between retinoid receptors and VDR as well as the multiple effects of $1,25(OH)_2D_3$ on cytokine signaling that have emerged from studies in other systems.

Comparative analysis of protein expression in MCF-7 and MCF-7^{DRES} cells under basal conditions (Table 2) identified 14 differentially expressed proteins (seven up-regulated and seven down-regulated). Proteins up-regulated in MCF-7^{DRES} cells included those with known roles in cytoskeletal dynamics and motility (ACAP2, CapZ2), apoptosis (Bid) and mitogenic signaling (phospho-p38, MEK2), which could reasonably be expected to be the result of continued exposure to $1,25(OH)_2D_3$ and/or the development of resistance. The potential roles of other differentially expressed proteins, such as Nip1 (a nuclear cap binding protein) and Sema 4C (a neuronal membrane receptor), are less obvious, and further studies will be needed to determine their relevance if any, to the phenotype of this sub-clone. Proteins down-regulated in MCF-7^{DRES} cells relative to MCF-7 cells include those involved in induction of apoptosis (caspase 7, caspase 14, Rip2), those driving growth factor signal transduction pathways and/or cell cycle (cdk2, RAS-GAP) and, cytosolic proteins involved in remodeling of focal adhesions and stress fibers (Rho-GDI, ROCK-D1). Although some of these differentially expressed proteins appear to be consistent with the $1,25(OH)_2D_3$ resistant phenotype of these cells (ie, down regulation of caspases, up-regulation of mitogenic signals), others may represent novel effectors of survival in MCF-7^{DRES} cells. Additional work is needed to confirm

differential expression of these proteins and to determine their relevance, if any, to 1,25 $(OH)_2D_3$ resistance.

Summary and Future Directions

These studies have implicated several novel pathways in dictating breast cancer cellular sensitivity and resistance to the anti-proliferative and pro-apoptotic effects of $1,25(OH)_2D_3$. Many of the proteins/pathways identified with our screening approach have now been linked to $1,25(OH)_2D_3$ mediated apoptosis, including cathepsin mediated proteolysis, stat phosphorylation/signaling and Grim-19. Further work will clearly be necessary to clarify the role(s) of the remaining proteins in mediating the cellular actions of $1,25(OH)_2D_3$ and how specific pathways intersect with VDR signaling in mammary cells. Complementary work in animal models, including mice with targeted ablation of the VDR, supports the concept that the vitamin D signaling pathway contributes to control of both proliferation and apoptosis in the mammary gland in vivo. Our pre-clinical studies have been complemented by emerging data from other groups suggesting that human breast cancer may be influenced by VDR genotype and vitamin D status. Collectively, these studies have reinforced the need to further define the regulation and function of the vitamin D pathway at the cellular and molecular level in relation to prevention and treatment of human breast cancer.

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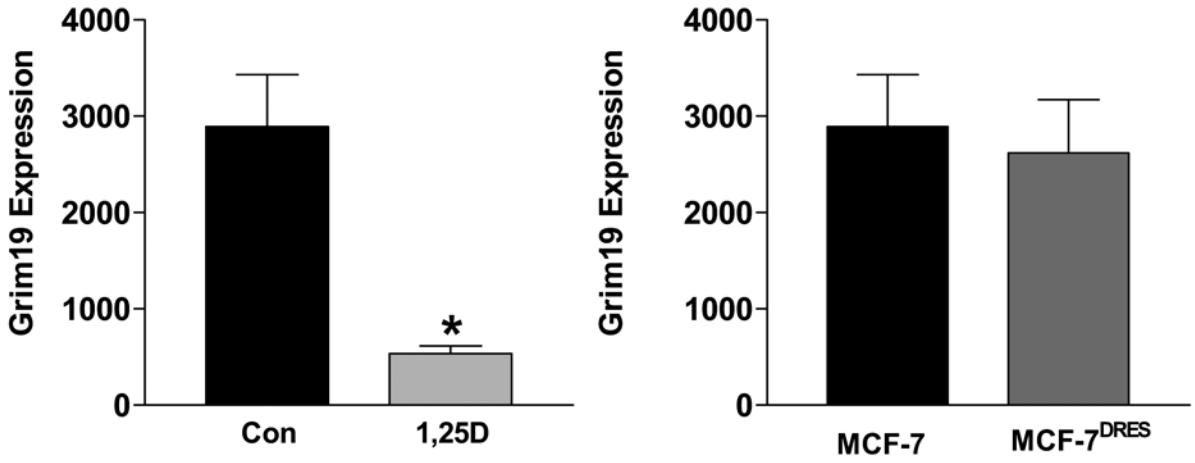

Figure 1. Expression of Grim-19 in MCF-7 cells treated with 1,25(OH)$_2$D$_3$ or selected for vitamin D resistance

A. Lysates from MCF-7 cells treated with 100nM 1,25(OH)$_2$D$_3$ or ethanol vehicle for 72 hours were subjected to western blotting with an antibody directed against Grim-19. Western blots were scanned and expression of Grim-19 was normalized for protein loading. Data are expressed as mean ± standard error of triplicates. *$p<0.05$, control vs. 1,25(OH)$_2$D$_3$ treated. B. Comparison of basal Grim-19 expression in MCF-7 cells and MCF-7^{DRES} cells, which were selected for resistance to 100nM 1,25(OH)$_2$D. Data was obtained as described in A.

Table 1

Identification of $1,25(OH)_2D_3$ regulated proteins in MCF-7 cells

MCF-7 cells were treated for 72 hours with either ethanol vehicle control (Con) or 100nM $1,25(OH)_2D_3$. Each sample was analyzed in triplicate for expression of 270 apoptosis related proteins. Relative expression of each protein was normalized to several housekeeping genes. The normalized expression of each of the ten proteins listed was significantly different between vehicle and $1,25(OH)_2D_3$ treated cells ($p<0.05$ by Students t test). Sign of change indicates whether the protein was up or down-regulated by $1,25(OH)_2D_3$ in comparison to control.

MCF-7 cells Con vs $1,25(OH)_2D_3$	Sign of Change	Function/Pathway
Grim19	−	Mitochondrial oxidoreductase involved in electron transport, linked to apoptosis mediated by interferon β and retinoic acid.
Cyclin A	−	Cell cycle regulator that promotes both G1/S and G2/M transitions
Cyclin D3	−	Cell cycle regulator required for G1/S transition
Stat 6	−	Transcription factor activated by cytokine signaling through membrane receptors. Mediates anti-apoptotic activity of IL-4.
GSK-3b	−	Proline-directed serine-threonine kinase with multiple substrates, including β-catenin.
Integrin linked kinase(ILK)	−	Serine-threonine kinase, mediates integrin signaling to cell survival through AKT pathway
Rho-GDI	−	GDP dissociation inhibitor, stimulates signaling through Rho, a GTPase that regulates focal adhesions and stress fibers
SHC	−	Adaptor molecule in ras signal transduction
Cathepsin D	+	Lysosomal protease implicated in caspase independent cell death via autophagy
PTEN	+	Protein/lipid phosphatase that preferentially dephosphorylates phosphoinositides. Negative regulator of AKT.

Table 2

Identification of proteins differentially expressed in MCF-7 versus MCF-7^{DRES} cell

Lysates from MCF-7 and MCF-7^{DRES} cells were analyzed in triplicate for expression of 270 apoptosis related proteins. Relative expression of each protein was normalized to several housekeeping genes. The normalized expression of the fourteen proteins listed were significantly different between MCF-7 and MCF-7^{DRES} cells ($p<0.05$ by Students t test). Sign of change indicates whether the protein was up or down-regulated in MCF-7^{DRES} cells relative to MCF-7 cells.

Protein	Sign of Change	Function/Pathway
ACAP2	+	GTPase-activating protein, activates ARF6, a regulator of cell adhesion and motility
CapZ2	+	F-actin capping protein
Bid	+	Pro-apoptotic Bcl-2 family protein
Nip1	+	Nuclear cap binding protein
Phospho-p38	+	Active form of p38 MAP kinase, mediates stress related transcription/cell cycle control
MEK2	+	Dual specificity protein kinase, activates MAPK1/ERK2 and MAPK2/ERK3
Sema4c	+	Semaphorin, linked to differentiation and development
Caspase 7	−	Apoptotic caspase, activated by caspase 3 or caspase 10
Caspase 14	−	Apoptotic caspase, activated by caspase 8 or caspase 10
Rip2	−	Receptor interacting protein, has apoptosis inducing activity
Cdk2	−	Cyclin dependent kinase, essential for G1/S transition
RAS-GAP	−	GTPase activating protein, down regulates ras signaling
Rho-GDI	−	GDP dissociation inhibitor, stimulates signaling through Rho, a GTPase that regulates focal adhesions and stress fibers
ROCK-D1	−	Serine/threonine kinase, activated by Rho, a GTPase that regulates focal adhesions and stress fibers