1,25(OH)$_2$D$_3$ and Initial Regulation of Smad2/3 Activity in PC-3 Prostate Cancer Cells

Masters Thesis in Biomedicine, Advanced Level, 30 ECTS

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The vitamin D metabolite 1,25(OH)$_2$D$_3$ has long been known to inhibit growth of prostate cancer cells and this mainly through a VDR-mediated pathway controlling target gene expression, resulting in cell cycle arrest, apoptosis and differentiation. Another major way in which 1,25(OH)$_2$D$_3$ inhibits cell growth in prostate cancer is via membrane-initiated steroid signalling, which triggers activation of signal cascades upon steroid binding to a receptor complex, leading to induction of genes regulating cell growth, proliferation and apoptosis. The main prostate cancer inhibiting membrane-initiated route is the TGFβ signalling pathway, elicited by the protein TGFβ. Two other important proteins downstream in this cascade are Smad2 and Smad3. In this study the early effects of 1,25(OH)$_2$D$_3$ on activated Smad2/3 levels in PC-3 prostate cancer cells were examined. PC-3 cells were incubated for 3, 5, 10, 30 and 60 minutes as well as 38 hours both together with 1,25(OH)$_2$D$_3$ of the concentrations 10$^{-10}$ and 10$^{-7}$ M and without. Western Blots were then performed on supernatants from the cells treated followed by treatment of the membranes with primary antibodies against phosphorylated Smad2/3 C-terminal linker regions, alkaline phosphatase conjugated secondary antibodies and finally visualization with BCIP/ NBT tablets. As the downstream cascade protein JNK is a proposed activator of Smad2/3, this procedure was also repeated with a JNK inhibitor. This is a follow-up to an earlier study which examined the influence of 1,25(OH)$_2$D$_3$ on TGFβ levels using the same doses and time points and which found that 1,25(OH)$_2$D$_3$ initially lowered the level of active TGFβ, then increased it. The results of this study indicated a 1,25(OH)$_2$D$_3$ mediated induction of the same pattern in the levels of active Smad2 and 3, both with and without JNK inhibitor. The results did not indicate that 1,25(OH)$_2$D$_3$ activates the Smad2/3 C-terminal linker region via the JNK pathway.
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1 Introduction

1.1 Prostate cancer and vitamin D

Cancer of the prostate is the most common form of male cancer; each year around half a million cases are diagnosed, worldwide. The symptoms of prostate cancer mainly consist of various urination difficulties and usually do not show until the tumor has spread outside the prostate capsule. The main medical treatments of this disease are prostatectomy, radiation therapy and testosteron ablation. The side-effects of these treatments are rather severe, the most common being impotence, incontinence and hot flushes. Because of this, more effective treatments with less side-effects are required (Nystrand, 2005).

Prostate cancer is much more common in Western countries than in for instance Asia, and in the USA African Americans run a much greater risk of developing the disease than Caucasians. Increasing age is another risk factor for this type of cancer (Nystrand, 2005). It may be that differences in vitamin D levels account for the mentioned observations. Firstly, it is a fact that Japanese men consume larger amounts of fatty fish, the main dietary source of vitamin D, than do Western men (Zhao & Feldman, 2001). Secondly, light skin compared to dark contains less melanin, a compound in the skin inhibiting synthesis of vitamin D. Thirdly, as men age their serum vitamin D levels decrease as the efficiency of vitamin D synthesis decreases with age (Holick, 2005). These suggestions are also supported by research showing that vitamin D has anti-proliferative effects on prostate cancer cells (Chen et al, 2000; Zhao & Feldman, 2001; Holick, 2006).

The active form of vitamin D is called 1,25(OH)$_2$D$_3$ and functions like a hormone in the body. It is, together with the parathyroid hormone, a major regulator of mineral homeostasis and bone metabolism. 1,25(OH)$_2$D$_3$ aids intestinal calcium absorption and is important for prevention of diseases such as rickets and osteomalacia (Zhao & Feldman, 2001).

The main cellular receptor for 1,25(OH)$_2$D$_3$ is a cytosolic/nuclear receptor called the vitamin D receptor (VDR). The genes regulated upon binding with the VDR include genes important for calcium metabolism such as osteocalcin, osteopontin, 24-hydroxylase and calbindin (Haussler et al., 1998) but also genes involved in cellular proliferation and differentiation such as c-myc, c-fos, p21, p27 and Hox A10 (Freedman, 1999). Expressing VDR, the prostate, especially the tumorous prostate (Krill et al., 2001), is a target organ for vitamin D and 1,25(OH)$_2$D$_3$ has long been known to inhibit growth of prostate cancer cells. This has been ascribed to a VDR-mediated, nuclear-initiated signalling controlling target gene expression, resulting in cell cycle arrest, apoptosis and differentiation (Lou et al., 2004).

However, it has been found that another major way in which 1,25(OH)$_2$D$_3$ inhibits cell growth in prostate cancer is via membrane-initiated steroid signalling (Murthy & Weigel, 2004; Larsson et al, 2007). Membrane-initiated steroid signalling triggers activation of signal cascades upon steroid binding to the receptor complex, leading to induction of genes regulating cell growth, proliferation and apoptosis (Norman et al, 2004).

1.2 1,25(OH)$_2$D$_3$, the TGFβ signalling pathway and Smad proteins in prostate cancer

Transforming growth factor β, TGFβ, is a signalling protein widespread among mammalian tissues. In the prostate, it regulates many critical cellular functions, particularly growth arrest, differentiation and apoptosis (Danielpour, 2005). The protein is secreted from cells in
complexes composed of three proteins, the mature TGFβ dimer, the latency-associated protein (LAP) and the latent TGFβ binding protein (LTBP). TGFβ signalling is initiated by proteolytic cleavage of LTBP resulting in release of the latent TGFβ complex from the extracellular matrix. The protein is activated by dissociation of LAP from the mature TGFβ (Taipale et al, 1998) and then it influences the prostate cells in an autocrine and paracrine manner (Kelly & Yin, 2008).

TGFβ triggers a signalling cascade through interaction with two transmembrane serine/threonine kinase receptors, TβR1 and TβR2. The main intracellular mediators of these receptors are a family of proteins known as Smads (small mothers against decapentaplegic). The TGFβ protein first binds to TβR2, which in turn recruits TβR1 to form a ligand-receptor heteromeric complex consisting of two TβR2s and two TβR1s. A constitutively active kinase in the cytoplasmic domain of TβR2 then activates TβR1 at a juxtamembrane site. The activated TβR1, with the help of a couple of proteins named SARA and Hrs/Hgr, recruits and activates Smad2 and 3 by means of phosphorylation (Danielpour, 2005). SARA is present in an early endosome which, through clathrin-mediated endocytosis, internalizes the receptor complex (Runyan et al, 2004). Once activated Smad2 and 3 homodimerize, they then enter the nucleus either with or without a third Smad, Smad4. The phosphorylated complex then binds transcription promoters/cofactors and causes the transcription of DNA (Danielpour, 2005).

TGFβ has also been described to initiate other pathways such as the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway. The mitogen-activated protein kinase (MAPK) JNK, a mediator in this cascade, has been proposed to be an additional activator of Smad2 and 3 by means of phosphorylation (Mori et al, 2004).

In a study from 2004 it was shown that in prostate cancer cells, 1,25(OH)2D3 increases both production, signalling and receptor levels of TGFβ, in turn inhibiting cell growth (Murthy & Weigel, 2004). The study was of long-term effects (1-6 days) and only referred to the cytosolic/nuclear VDR as a potential hormone receptor. However, 1,25(OH)2D3 mediated elicitation of the SAPK/JNK branch of this pathway has been detected already at the prenuclear stage; activation of JNK in the cascade has been shown as early as within 10 minutes of treatment (Larsson et al, 2007). Also, in the last-mentioned study the involvement of another vitamin D receptor was suggested, the protein disulfide-isomerase A3 precursor, PDIA3 (also called 1,25-MARRS).

A study in 2008 examined the early effects (3 minutes – 38 hours) of 1,25(OH)2D3 on the levels of active TGFβ and found that 1,25(OH)2D3 induced a statistically significant initial lowering of the active TGFβ level followed by a statistically significant successive rise of the level with time, a fall and rise which was not observed in their 0.01% EtOH treated controls (Stahel, 2008).

2 Aims and expectations of this Masters Thesis

The main aim of this work was to study the early effects of 1,25(OH)2D3 on activated Smad2/3 levels in PC-3 prostate cancer cells, both with and without the proposed activator JNK. The levels of phosphorylated Smad2/3 were expected to show the same pattern as the levels of active TGFβ after the same treatment, that is, an initial decrease followed by an
increase. Also, this pattern was expected to have a lowered level overall in the samples with JNK inhibitor.

PC-3 is a commonly used cell line in cancer research, which was derived in the late 1970s from a human prostatic adenocarcinoma metastatic to bone (Kaighn et al., 1979).

Discovering the details of the cancer growth inhibiting mechanism behind vitamin D is important as it means progress in the search for new and less maiming treatments of prostate cancer. Included in the aim of this study was to form a lead in that search. This is a follow-up to the 2008 study investigating the early effects of 1,25(OH)₂D₃ on activated TGFβ levels in PC-3 (Stahel, 2008).

3 Materials and methods

3.1 Cell culturing

Human prostate cancer cells from the cell line PC-3 (ECACC, Salisbury, UK) were used for this experiment. They were grown in monolayers in cell culturing medium: RPMI 1640, supplemented with 2 mM Glutamine, 10 mM Hepes, 1 mM Na-Pyruvate, 10% Fetal Bovine Serum and 100 U/ml Penicillin-Streptomycin. The culture was kept at 37°C in a humidified atmosphere with 5% CO₂.

3.2 Treatment with 1,25(OH)₂D₃ with and without JNK inhibitor followed by Western Blot analysis of activated Smad2/3

Cell lysates were prepared from monolayers of PC-3 cells cultured on 24 well plates (TPP, Switzerland) and treated for 3, 5, 10, 30 and 60 minutes as well as 38 hours with 0.01% EtOH or 1,25(OH)₂D₃, 10⁻¹⁰ or 10⁻⁷ M at 37°C and 5% CO₂. This procedure was then repeated but with the addition of 20 µM JNK inhibitor (SP600125, Sigma) to each well. The lysates were then resolved on 10% SDS-PAGE gels and the gels blotted to nitrocellulose membranes (Amersham).

The membranes were incubated with goat polyclonal antibodies to phosphorylated Smad2/3 C-terminal linker regions, 2 µg/ml (Ser 423/425, Santa Cruz Biotechnology), followed by incubations with alkaline phosphatase conjugated rabbit antigoat antibodies, 0.08 µg/ml (Santa Cruz Biotechnology). The Smad2/3 bands were then visualized using 5-Bromo-4-chloro-3-indoly phosphate / Nitro blue tetrazolium (BCIP/NBT) buffered substrate tablets (Sigma), which detect alkaline phosphatase activity through coloration (Sigma-Aldrich, 2009).
4 Results

4.1 Early effects of 1,25(OH)₂D₃ with and without JNK inhibitor on activated Smad2/3 levels

The blotted and visualized membranes all showed stretches of bands of protein at the molecular weight standard (MW) level of approximately 50 kDa although they were partly obstructed by demarcated light areas. These bands were identified as phosphorylated Smad2 and 3 since the molecular weights of these proteins are 58 kDa and 50 kDa, respectively (Abcam, 2009). See Figures 1, 2, 3 and 4.

Figure 1: Membrane 1. PC-3 cells were treated with vitamin D for different time periods, then levels of phosphorylated Smad2/3 in cell lysates were analyzed by Western Blot, phospho-Smad2/3 antibody incubation and BCIP/NBT visualization. Treatments were with 1,25(OH)₂D₃ 10⁻⁷ M for 3 min (2), 5 min (3), 10 min (4), 30 min (5), 60 min (6) and 38 h (7), and with 1,25(OH)₂D₃ 10⁻¹⁰ M for 3 min (8), 5 min (9) and 10 min (10).
Figure 2: Membrane 2. PC-3 cells were treated with vitamin D and with EtOH (control) for different time periods, then levels of phosphorylated Smad2/3 in cell lysates were analyzed by Western Blot, phospho-Smad2/3 antibody incubation and BCIP/NBT visualization. Treatments were with 1,25(OH)$_2$D$_3$ $10^{-10}$ M for 30 min (1), 60 min (3) and 38 h (4), and with EtOH 0.01% for 3 min (5), 5 min (6), 10 min (7), 30 min (8), 60 min (9) and 38 h (10).
Figure 3: Membrane 3. PC-3 cells were treated with vitamin D + JNK inhibitor for different time periods, then levels of phosphorylated Smad2/3 in cell lysates were analyzed by Western Blot, phospho-Smad2/3 antibody incubation and BCIP/NBT visualization. Treatments were with 1,25(OH)\textsubscript{2}D\textsubscript{3} $10^{-7}$ M + JNK inhibitor 20 µM for 3 min (1), 5 min (2), 10 min (4), 30 min (5), 60 min (6) and 38 h (7), and with 1,25(OH)\textsubscript{2}D\textsubscript{3} $10^{-10}$ M + JNK inhibitor 20 µM for 3 min (8), 5 min (9) and 10 min (10).
Membrane 4. PC-3 cells were treated with vitamin D + JNK inhibitor and with EtOH + JNK inhibitor (control) for different time periods, then levels of phosphorylated Smad2/3 in cell lysates were analyzed by Western Blot, phospho-Smad2/3 antibody incubation and BCIP/NBT visualization. Treatments were with 1,25(OH)\textsubscript{2}D\textsubscript{3} 10\textsuperscript{-7} M + JNK inhibitor 20 µM for 30 min (1), 60 min (2) and 38 h (3), and with EtOH 0.01% + JNK inhibitor 20 µM for 3 min (5), 5 min (6), 10 min (7), 30 min (8), 60 min (9) and 38 h (10).

As expected, the Smad2/3 double bands which were treated with 1,25(OH)\textsubscript{2}D\textsubscript{3} 10\textsuperscript{-7} M showed a tendency toward decrease in color intensity initially followed by an increase in color intensity with time, while their 0.01% EtOH treated controls did not (barring some demarcated light areas). Membrane close-ups of this are shown in Figure 5.

Figure 4: Membrane 4. PC-3 cells were treated with vitamin D + JNK inhibitor and with EtOH + JNK inhibitor (control) for different time periods, then levels of phosphorylated Smad2/3 in cell lysates were analyzed by Western Blot, phospho-Smad2/3 antibody incubation and BCIP/NBT visualization. Treatments were with 1,25(OH)\textsubscript{2}D\textsubscript{3} 10\textsuperscript{-7} M + JNK inhibitor 20 µM for 30 min (1), 60 min (2) and 38 h (3), and with EtOH 0.01% + JNK inhibitor 20 µM for 3 min (5), 5 min (6), 10 min (7), 30 min (8), 60 min (9) and 38 h (10).

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Figure 5: Close-ups of chosen parts of membranes 1 and 2. PC-3 cells were treated with vitamin D and with EtOH (control) for different time periods, then levels of phosphorylated Smad2/3 in cell lysates were analyzed by Western Blot, phospho-Smad2/3 antibody incubation and BCIP/NBT visualization. Treatments were with 1,25(OH)\textsubscript{2}D\textsubscript{3} 10\textsuperscript{-7} M for 3 min, 5 min, 10 min, 30 min, 60 min and 38 h (left), and with EtOH 0.01% for same time periods (right).
Similarly, the latter half of the Smad2/3 double bands which were treated with $1,25(\text{OH})_2\text{D}_3$ $10^{-10}$ M + 20 μM JNK inhibitor indicated an initial decrease in color intensity followed by increase in color intensity with time, while their 0.01% EtOH + 20 μM JNK inhibitor treated controls did not. Membrane close-ups of this are shown in Figure 6.

Figure 6: Close-ups of chosen parts of membrane 4. PC-3 cells were treated with vitamin D + JNK inhibitor and with EtOH + JNK inhibitor (control), then levels of phosphorylated Smad2/3 in cell lysates were analyzed by Western Blot, phospho-Smad2/3 antibody incubation and BCIP/NBT visualization. Treatments were with $1,25(\text{OH})_2\text{D}_3$ $10^{-10}$ M + JNK inhibitor 20 μM for 30 min, 60 min and 38 h (left), and with EtOH 0.01% + JNK inhibitor 20 μM for same time periods (right).
Contrary to expected, the Smad2/3 bands of the membranes with JNK inhibitor treated samples did not have weaker color intensities overall than did the corresponding bands on the membranes with samples without JNK inhibitor treatment (barring demarcated light areas). This observation was most marked in the dose- and time point corresponding membranes 2 and 4. Membrane close-ups of this are shown in Figure 7.

**Figure 7:** Close-ups of membranes 2 and 4. PC-3 cells were treated with $1_25(OH)_2D_3$ $10^{-10}$ M for 30 min, 60 min and 38 h, and with EtOH 0.01% (control) for 3 min, 5 min, 10 min, 30 min, 60 min and 38 h, then levels of phosphorylated Smad2/3 in cell lysates were analyzed by Western Blot, phospho-Smad2/3 antibody incubation and BCIP/NBT visualization (furthest above). PC-3 cells were also treated with $1_25(OH)_2D_3$ $10^{-10}$ M + JNK inhibitor 20 µM and with EtOH 0.01% + JNK inhibitor 20 µM (control) for same time periods, then levels of phosphorylated Smad2/3 in cell lysates were likewise analyzed (below).

5 Discussion and conclusion

The light areas partly obstructing the stretches of Smad2/3 bands on the membranes were likely due to insufficient contact between fluid and membrane during the antibody and/or visualization procedures. However, it is likely that the bands hidden because of this if visualized would have shown a colour intensity similar to the bands which did become visible and to which they bordered. So despite these light areas, two conclusions could be drawn on basis of the results.

Firstly, the visible Smad2 and 3 bands indicated a confirmation of the finding in the prequel of this study, which examined the same doses and time points (Stahel, 2008). As mentioned the finding was that $1_25(OH)_2D_3$ induced a statistically significant successive rise in the level of active TGFβ during the course of the measuring after initially having lowered it; a fall and rise which was not observed in their 0.01% EtOH treated controls. Since active TGFβ when bound to the TGFβ receptor phosphorylates Smad2 and 3 (Danielpour, 2005), it is logical to conclude that the levels of the activated form of these proteins would behave in a manner
which rhymes with this TGFβ level fall and rise after same treatment. That is also what this study indicated and this suggests that 1,25(OH)_{2}D_{3} when added affects the whole TGFβ signalling pathway in this rapid pattern of decrease followed by increase.

As suggested in the 2008 study, it is possible that the explanation to this regulation by 1,25(OH)_{2}D_{3} of the TGFβ pathway could be an effect of the fact that the integral/associated 1,25(OH)_{2}D_{3} membrane receptor PDIA3 has been found to exert enzymatic activity. After binding to the hormone, PDIA3 catalyzes the rearrangement of both intrachain and interchain disulfide bonds in other proteins. The receptor performs this interaction selectively with any protein or protein complex (European Bioinformatics Institute, 2008). It may be that this action of PDIA3 impairs the capacity of the TGFβ receptors to bind TGFβ. Future studies of how the TGFβ content of the extracellular fluid is affected by 1,25(OH)_{2}D_{3} during these time points might shed some light on this. Also as suggested in the 2008 study, it could be that the later induction by 1,25(OH)_{2}D_{3} of TGFβ production, signalling and receptor formation (Murthy & Weigel, 2004; Stahel, 2008) is abundant enough to override the suggested TGFβ receptor impeding effect of the PDIA3 complex allegedly initially lowering the TGFβ activity, and this may account for the following indication of phosphorylated Smad2 and 3 increase observed here.

Secondly, the results in this study at first seemed to contradict the study from 2004 by Mori et al showing that 1,25(OH)_{2}D_{3} phosphorylates Smad2 and 3 via the JNK pathway (Mori et al, 2004). As mentioned the expectation of the present study was that the Smad2/3 bands treated with JNK inhibitor would show a weaker colour intensity overall than those without JNK inhibitor treatment since all phosphorylation by JNK taking place in the latter would have been inhibited in the former. The results did not match this expectation but a probable explanation to this was later found. Mori et al had tested four different linker regions of the Smad2/3 proteins and therefore used four different types of primary antibodies for the pre-visualization procedure. While three of the four Smad bands resulting from this did show a greater colour intensity in the samples without JNK inhibitor in their experiment, the fourth band did not. Incidentally, these were the samples treated with the same type of primary antibody as used in the present study: Ser 423/425, an antibody against phosphorylated Smad2/3 C-terminal linker regions. So in conclusion, in accordance with the study by Mori et al the results of this study did not indicate that 1,25(OH)_{2}D_{3} activates the Smad2/3 C-terminal linker region via the JNK pathway.

Further and more detailed studies of the effects of 1,25(OH)_{2}D_{3} on Smad2 and 3 levels are needed, for instance using Enzyme-Linked ImmunoSorbent Assays (ELISA) which yield more exact results than do Western Blots.
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7 References


