1,25(OH)₂D₃ Initially Reduces TGFβ Activity in PC-3 Prostate Cancer Cells

Masters Thesis in Biomedicine C10, 30 ECTS

(2008-01-14 – 2008-06-01)
The vitamin D metabolite 1,25(OH)₂D₃ 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)₂D₃ 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. In this experiment the activating effects of 1,25(OH)₂D₃ on TGFβ in prostate cancer cells, as well as two other important proteins downstream in this cascade, Smad2 and 3, were investigated. PC-3 cells were incubated for 3, 5, 10, 30 and 60 minutes as well as 38 hours both together with 1,25(OH)₂D₃ of the concentrations 10⁻¹⁰ and 10⁻⁷ M and without. As the downstream cascade protein JNK is a known activator of Smad2/3, this procedure was also repeated with a JNK inhibitor. An ELISA assay scanning for activated TGFβ was then performed on supernatants from the cells treated without JNK inhibitor. In addition, a Western Blot scanning for activated Smad2 and 3 was performed on supernatants from all groups of treatment. The analysis of the result values showed that 10⁻¹⁰ M 1,25(OH)₂D₃ significantly lowered the content of active TGFβ in PC-3 cells within 3 and 5 minutes. Unfortunately the Western Blot was unsuccessful and needs therefore be repeated.
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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 testosterone 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 black people run a much greater risk of developing the disease than white people. Increasing age is another risk factor for this type of cancer (Nystrand, 2005). It may be that differences in vitamin D levels account for 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. Third, 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\(_3\) and the TGF\(\beta\) signalling pathway in prostate cancer

Transforming growth factor \(\beta\), TGF\(\beta\), 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 TGF\(\beta\) dimer, the latency-associated protein (LAP) and the latent TGF\(\beta\) binding protein (LTBP). TGF\(\beta\)-beta signalling is initiated by proteolytic cleavage of LTBP resulting in release of the latent TGF\(\beta\) complex from the extracellular matrix. The protein is activated by dissociation of LAP from the mature TGF\(\beta\) (Taipale \textit{et al.}, 1998), then it influences the prostate cells in an autocrine and paracrine manner (Kelly \& Yin, 2008).

TGF\(\beta\) triggers a signalling cascade through interaction with two transmembrane serine/threonine kinase receptors, T\(\beta\)R1 and T\(\beta\)R2. The main intracellular mediators of these receptors are a family of proteins known as Smads (small mothers against decapentaplegic). The protein first binds to T\(\beta\)R2 which in turn recruits T\(\beta\)R1 to form a ligand-receptor heteromeric complex consisting of two T\(\beta\)R2s and two T\(\beta\)R1s. A constitutively active kinase in the cytoplasmic domain of T\(\beta\)R2 then activates T\(\beta\)R1 at a juxtamembrane site. The activated T\(\beta\)R1, 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 \textit{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).

TGF\(\beta\) 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 shown to be an additional activator of Smad2 and 3 by means of phosphorylation (Mori \textit{et al.}, 2004).

A study from 2004 showed that in prostate cancer cells, 1,25(OH)\(_2\)D\(_3\) increases both production, signalling and receptor levels of TGF\(\beta\), in turn inhibiting cell growth (Murthy \& Weigel, 2004). The study was of long-term effects (1-6 days) and apparently involved the cytosolic/nuclear VDR. However, 1,25(OH)\(_2\)D\(_3\) mediated elicitation of the SPK/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 \textit{et al.}, 2007). Also, in last-mentioned study the involvement of another vitamin D receptor was suggested, the protein disulfide-isomerase A3 precursor, PDIA3 (also called 1,25-MARRS).

2 Aim of this Masters Thesis

While the later stages (days) of the inhibition of prostate cancer cells by 1,25(OH)\(_2\)D\(_3\) via TGF\(\beta\) and Smad2/3 have been investigated (Murthy \& Weigel, 2004), the early stages (minutes) have not. The aim of this work was to study the early effects of 1,25(OH)\(_2\)D\(_3\) on activated TGF\(\beta\) levels in PC-3 prostate cancer cells as well as levels of activated Smad2/3 during same time points. PC-3 is a commonly used cell line in cancer research, which was derived in the late 1970’s from a human prostatic adenocarcinoma metastatic to bone (Kaighn \textit{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 be a lead in that search.
3 Materials and methods

3.1 Cell culturing and treatment with 1,25(OH)₂D₃

Human prostate cancer cells from the cell line PC-3 (ECACC, Salisbury, UK) were used for this experiment. They were grown in monolayers on 24 well plates (TPP, Switzerland) 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 in 37°C in a humidified atmosphere with 5% CO₂.

The monolayers were then treated in 37°C and 5% CO₂ 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, and lysates were prepared from all groups.

3.3 Activated TGFβ ELISA, absorbance measuring and computer analysis

3.3.1 The TGFβ₁ Emax ImmunoAssay System, MultiSkan EX and GraphPad Prism 4

The ELISA (Enzyme-Linked ImmunoSorbent Assay) kit TGFβ₁ Emax ImmunoAssay System is a sensitive and specific assay for detection of biologically active TGFβ₁ in an antibody sandwich format (Hornbeck, 1994). A flat-bottom 96-well plate is first coated with TGFβ coat monoclonal antibodies, which bind soluble TGFβ₁. The captured TGFβ₁ is then bound by a second specific polyclonal antibody. After washing, the amount of specifically bound polyclonal antibody is detected using a species-specific antibody conjugated with horseradish peroxidase as a tertiary reactant. The unbound conjugate is then removed by washing and following incubation with a chromogenic substrate the absorbance, corresponding to the intensity of color change, is measured with a microplate reader. The amount of TGFβ₁ in the test solution is proportional to the color generated in the oxidation-reduction reaction (Promega, 2006).

In this study a microplate photometer called MultiSkan EX (Thermo Electron Corporation, MA, USA) was used for the testing. The MultiSkan measures absorbance in Arbitrary Absorbance Units, AAU, which can then be transformed into pg/ml.

The computer program GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA) was used for the statistical analysis of the result figures.

3.3.2 Assay, measuring and analysis

The TGFβ ELISA assay and microplate absorbance measuring was carried out according to the TGFβ₁ Emax ImmunoAssay System protocol (Promega, 2006). The sample dilution chosen was 1:15.

The result values from the MultiSkan were statistically analyzed in GraphPad Prism where a number of graphs were drawn on basis of the result figures. The figures used for the graphs were mean values, that is, averages were calculated for each time point for the 4 replicates of each hormone concentration as well as the EtOH controls. The analysis made was a Two-way
ANOVA followed by Bonferroni’s post-hoc test. The significance threshold was set to P<0.05.

3.4 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. This procedure was then repeated but with 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 PVDF membranes (BioRad).

The membranes were then meant to have been incubated with antibodies to phosphorylated Smad2/3 and the protein bands eventually visualized using electrochemiluminescence reagent. However due to the result from a total protein detection of the membranes with Ponceau stain (Sigma) carried out after the blot, this was not performed (see Results).

4 Results

4.1 Early effects of 1,25(OH)2D3 on activated TGFβ levels

The analysis of the result values in GraphPad Prism showed that 1,25(OH)2D3 did have statistically significant effects on activated TGFβ levels in PC-3 cells. The observed effects were most pronounced in the hormone concentration 10^{-10} M and during the time points 3 minutes (P<0.01), 5 minutes (P<0.001) and 30 minutes (P<0.01). (The 3 minute 10^{-7} M value was removed due to a mistake during lysis buffer pipetting leading to an incorrect figure.)

A first, vertical interleaved bar graph drawn based on the results from the GraphPad analysis is shown in Figure 1.
Figure 1: Dose- and time-dependent responses with and without 24,25(OH)\textsubscript{2}D\textsubscript{3} treatment were compared with help of an a Two-way ANOVA followed by Bonferroni’s post-hoc test. An absorbance-based ELISA was used where levels of active TGFβ were measured with a chromogenic substrate.

Analyses were also made with error bar category graphs, comparing each time-point within each separate curve. The results showed lack of any statistically significant variation of the slope of the curve for untreated cells (control) while both the curves for the hormone treated (10\textsuperscript{-7} M and 10\textsuperscript{-10} M) cells showed marked statistical variations. The results are shown in Figures 3a, b, c and d.

Significant (P<0.05)/very significant (P<0.01) statistical difference:

\*\*\* from Control
\*\*\* from Vit D 10\textsuperscript{-7} M
Figure 3a: Dose- and time-dependent responses with and without 1,25(OH)_2D_3 treatment were compared with help of a One-way ANOVA followed by Bonferroni’s post-hoc test. An absorbance-based ELISA was used where levels of active TGFβ were measured with a chromogenic substrate. Overview graph.

Figure 3b: Time-dependent responses without any treatment by 1,25(OH)_2D_3 (control). No statistically significant time point differences.
Figure 3c: Time-dependent responses with treatment by $1,25(OH)_2D_3$ of the concentration $10^{-10}$ M.
Figure 3d: Time-dependent responses with treatment by 1,25(OH)₂D₃ of the concentration 10⁻⁷ M.

### 4.2 Early effects of 1,25(OH)₂D₃ on activated Smad2/3 levels

Unfortunately, a total protein detection with Ponceau stain (Sigma) of the membranes from the Western Blot revealed an unsuccessful blot showing no proteins at all.

### 5 Discussion and conclusion

The explanation to the finding in this study that 1,25(OH)₂D₃ lowers the level of activated TGFβ within 30 minutes, with most marked effects around 5 minutes, is not obvious. However, it could be an effect of the fact that the integral/associated 1,25(OH)₂D₃ 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)₂D₃ during these time points might shed some light on this.

As can be seen in Figures 3c and d, both the plots for active TGFβ levels after hormone treatment show statistically significant – even very and extremely significant – changes along
their own slopes. This means that addition of 1,25(OH)₂D₃ first rather sharply lowers the level of active TGFβ and then, again rather sharply, allows the level to rise back up. If the curves were to be continued, keeping the same trend, they would even start rising above the control curve level. This rhymes with the study from 2004 in which was found that long-term treatment with 1,25(OH)₂D₃ increases the production, signalling and receptor levels of TGFβ in PC-3 cells (Murthy & Weigel, 2004). It is possible that this later induction by 1,25(OH)₂D₃ of TGFβ production, signalling and receptor formation is abundant enough to override the suggested TGFβ receptor impeding effect of the PDIA3 complex allegedly initially lowering the TGFβ activity.

Vitamin D is well-known for influencing cells in multifaceted ways, and it has been suggested that rapid and slow effects of the hormone interact in order to efficiently regulate the activity of cells both short-term and long-term (Larsson, 1999; Larsson & Nemere, 2001). It may be an aspect of that pattern in PC-3 cells we see here.

The lack of proteins on the Western Blot membranes was likely due to the fact that because of a mistake, the electrophoresis was run at too high a voltage, causing the proteins to migrate off the gel. The study for detection of activated Smad2 and 3 in short-term 1,25(OH)₂D₃ treated PC-3 cells needs to be repeated. Since Smad2 and 3 are key proteins in the TGFβ signalling pathway it is important to know whether the hormone affects the phosphorylation level of these in order to get a clearer picture of the signalling cascade pattern elicited in prostate cancer cells by the active form of vitamin D.
6 Acknowledgements

Once again I would like to thank my supervisor, Dennis Larsson, for his great kindness, patience and trust when guiding me through my project. I am also very grateful to Jessica Carlsson and Vishal Salunkhe for their good suggestions and invaluable practical help during this study. Finally I would like to thank Aldin Hadzic, Jonathan Holmén, Kajsa Lilja, Anna Karin Roback, Nassrin Wandy, Ghazal Efaizat, Anna Cavalli-Björkman Hellström and Sofia Cavalli-Björkman Hellström for their help and support in lab.

7 References


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