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The Influence of Vitamin D Derivatives on the Expression of Apoptotic Genes in Nasal Polyp Fibroblasts

Ocena wpływu pochodnych witaminy D na ekspresję genów *BCL-2* i *BAX* w fibroblastach polipów nosa

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Abstract

Background. Vitamin D (VD) modulates the transcription of genes involved in a variety of biological processes, such as phosphocalcic metabolism, proliferation, differentiation, angiogenesis, cell division and cell death. The pro-apoptotic and antiproliferative effects of VD derivates, although still not fully clear, could potentially be harnessed therapeutically.
Objectives. The aim of the paper was to analyze the influence of VD derivatives on the expression of apoptosis associated genes in fibroblasts derived from nasal polyps (NP).
Material and Methods. During routine endonasal surgery 16 NP samples were collected from 5 women and 11 men suffering from chronic rhinosinusitis with NP. The expression of *BCL-2* and *BAX* was detected using RT-PCR and, at the protein level, via ELISA in fibroblast cultures treated with either calcitriol (1,25-(OH)2D3) or tacalcitol (1,24-(OH)2D3) at concentrations of 10^{-6}M, 10^{-5}M and 10^{-4}M each. The comparison group consisted of NP fibroblasts not treated with VD derivatives.
Results. Both tacalcitol and calcitriol significantly decreased BCL-2 and BAX expression at a concentration of 10^{-4}M ($p < 0.05$). An insignificant decrease in BCL-2/BAX ratios was noted after treatment with both of the examined VD derivatives, regardless of the drug concentration. The highest pro-apoptotic ratio was noted with tacalcitol at a concentration of 10^{-5}M. The decrease in the ratio was more a result of inhibited BCL-2 expression than of enhanced BAX expression. Concentration-dependent action was not seen for either of the examined substances.
Conclusions. The results imply that the pro-apoptotic properties of VD derivatives may engage the BCL-2 family pathway only to a limited extent. The slightly better efficiency of tacalcitol in reducing the *BCL-2/BAX* mRNA ratio, along with its lower calcemic effects, makes tacalcitol preferable to calcitriol (**Adv Clin Exp Med 2010, 19, 6, 679–684**).

Key words: calcitriol, tacalcitol, nasal polyps, QRT-PCR, immunohistochemistry, ELISA, apoptosis.

Streszczenie

Wprowadzenie. Witamina D, oprócz wpływu na gospodarkę wapniowo-fosforanową, moduluje również ekspresję genów uczestniczących w wielu ważnych procesach komórkowych, takich jak: proliferacja, podział, różnicowanie i śmierć komórki. Nie w pełni poznane proapoptotyczne i antyproliferacyjne właściwości witaminy D mają duży potencjał terapeutyczny.
Cel pracy. Ocena wpływu pochodnych witaminy D (kalcytriolu i takalcytolu) na ekspresję genow związanych z procesem apoptozy w hodowli fibroblastów pochodzących z polipów nosa.
Materiał i metody. Materiał do badania został uzyskany podczas operacji endoskopowej od 16 pacjentów (5 kobiet i 11 mężczyzn) chorujących na przewlekłe zapalenie zatok przynosowych. Ekspresję genów *BCL-2, BAX* oceniono metodą RT-PCR oraz na poziomie białka metodą ELISA w hodowli fibroblastów z dodatkiem kalcytriolu lub takalcytolu w stężeniu 10^{-6}M, 10^{-5}M i 10^{-4}M. Grupę porównawczą stanowiły fibroblasty z polipów nosa nietraktowane pochodnymi witaminy D.

Wyniki. Zarówno kalcytriol, jak i takalcytol w stężeniu 10^{-4}M istotnie zmniejszał ekspresję BCL-2 i BAX. Niezależnie od stężenia żadna z substancji nie obniżyła istotnie statystycznie wartości współczynnika BCL-2/BAX. Współczynnik BCL-2/BAX osiągnął najbardziej proapoptotyczną wartość po dodaniu takalcytolu w stężeniu 10^{-5}M. Obniżenie jego wartości było rezultatem raczej zahamowania ekspresji BCL-2 niż zwiększenia ekspresji BAX.
Wnioski. Wyniki sugerują, że proapoptotyczne działanie witaminy D tylko w ograniczonym zakresie opiera się na rodzinie białek BCL-2. Nieznacznie bardziej korzystny wpływ na stosunek ekspresji *BCL-2* do *BAX* oraz słabszy wpływ na gospodarkę wapniową sprawiają, że takalcytol jest lepszy niż kalcytriol (**Adv Clin Exp Med 2010, 19, 6, 679–684**).

Słowa kluczowe: kalcytriol, takalcytol, polipy nosa, QRT-PCR, immunohistochemia, ELISA, apoptoza.

Rhinosinusitis with nasal polyps is a chronic upper respiratory tract disease which has a strong impact on the patients' quality of life. Due to their complex and unclear etiology, nasal polyposis (NP) presents difficult challenges. Despite up-to-date pharmacological treatment, in most cases the outcomes are unsatisfactory and recurrences require surgery [1]. At present, steroids in a long-term topical and oral forms are the primary in the therapy for NP. Due to wellknown side effects related to steroid intake, this therapeutic option is often rejected. All of these factors indicate the need for the investigation of new agents suitable for treating NP.

Experimental trials based on various cellular mechanisms have involved many new substances, but there is still a lack of cause-related therapy [2]. Previously the authors' our research group successfully evaluated vitamin D (VD) derivatives as a potential treatment in NP [3].

The classic role of VD – ie, regulation of calcium and phosphorous homeostasis – is achieved by conversion to 1,25(OH)2D3, one of the most active metabolites of VD. The biological effects of VD derivatives are mediated through a specific nuclear receptor, a phosphoprotein, which functions as a transcriptional factor [4]. Through the binding motifs in the promoter regions of target genes, VD is involved in the modulation of the transcription of more than 60 genes [5]. The target genes regulate a variety of biological processes, such as phosphocalcic metabolism, proliferation, differentiation, angiogenesis, cell division and cell death [6]. Vitamin D compounds have also been reported to up-regulate the levels of several apoptosis-associated proteins. The effects of VD derivatives on NP have been previously evaluated previously by the authors *in vitro*. We have demonstrated that treating NP fibroblasts with tacalcitol reduces cell proliferation and promotes apoptotic cell death [3]. Apoptosis is an inherent, energy-dependent process present that takes place within cells, whereby a distinct series of biochemical and molecular events leads to cell death. Although inflammatory processes play a central role in the pathogenesis of NP, growing evidence indicates that aberrations in apoptosis cause some secondary changes, such as epithelial hyperplasia and tissue remodelling, which may potentiate the development of polyps [7].

The most of the target genes relevant to the pro-apoptotic actions of Vitamin D receptor ligands have not yet been identified. This can partially be explained by the complexity of programmed cell death. Although numerous signals participate in the induction of apoptosis, an alteration in the levels of the BCL-2 family members is a critical feature of that process [8]. The action of VD in different cell lines involves different sets of genes, which emphasizes the need to undertake studies into of various cell models. The pro-apoptotic properties of VD derivatives have so far not been deeply analyzed in rhinosinusitis with NP.

The current study investigated whether BAX, a pro-apoptotic protein, and BCL-2, an antiapoptotic protein, are involved in the apoptotic-related mechanism of Vitamin D derivatives (calcitriol 1,25-(OH)2D3 and tacalcitol 1,24-(OH)2D3) in nasal fibroblast cultures.

Material and Methods

The Subjects

Sixteen patients (11 males and 5 females) with NP, treated surgically at the Department of Otolaryngology at Wroclaw Medical University, Poland, were included in the study. All the subjects met the diagnostic criteria for chronic rhinosinusitis as established by the Task Force on Rhinosinusitis (AAO-HNS), and the study was approved by the Local Ethical Committee of Wroclaw Medical University. The patients' ages ranged from 44 to 71 years (mean: 52.6 years).

The extent of the disease was assessed by CT and also endoscopically. The patients had been free of any medication for at least two weeks before surgery, and had bilateral polyps in the nasal cavities on endoscopic examination. The presence of comorbidity or any prior smoking history were also excluded. The subjects underwent functional endoscopic sinus sur-

gery under general anesthesia, with subsequent tissue sampling from the anterior ethmoid.

The nasal polyp specimens were immediately placed in sample tubes containing 1 ml phosphate buffered saline (PBS) and were frozen directly at –70°C to await further investigations. A part of each sample was fixed in 10% buffered neutral formalin, processed routinely and embedded in paraffin wax for subsequent immunohistochemical examination to establish a diagnosis and to exclude other pathologies.

Cell Cultures

The tissue was milled mechanically and then washed in a solution containing 0.25% tripsin and EDTA. The suspended cells were cultured on 96-well plates, beginning with a concentration of 5×10^3 cells per well with RPMI 1640 medium (Gibko) supplemented with penicillin (1000 u/ml, Sigma), streptomycin (10 mg/ml, Sigma) and enriched with 10% fetal bovine serum (FBS, Gibko). The culture was kept in an incubator (ASSAB) at 37°C within a 5% CO_2 atmosphere and 100% humidity. After the fourth to sixth passages, bacterial LPS (Sigma) was added to the final concentration of 10 ng/ml and cultures were continued for 24 h. Then the medium was replaced by a nutrient medium with calcitriol (1,25-(OH)2D3) or tacalcitol (1,24-(OH)2D3) (Pharmaceutical Institute, Poland) at the same culture conditions. The final concentrations of calcitriol and tacalcitol were 10^{-6}M, 10^{-5}M and 10^{-4}M. Each concentration was analyzed in two cell cultures and was finalized after 24 h.

The comparison group consisted of fibroblasts derived from the same NP patients, untreated with calcitriol and tacalcitol. Half of each of them was additionally stimulated with bacterial LPS.

After culture, cell viability was evaluated by staining with trypan blue and assessed with light microscopy. Apoptotic cell death was examined with an Annexin-V-FLUOS Kit (Roche Diagnostics, Germany) according the manufacturer's specifications.

The QRT-PCR Method

The total RNA was extracted from the cell cultures with the use of TRIZOLR reagent (Invitrogen, Carlsbad, USA) according to the producer's protocol. The concentration of RNA was determined spectrophotometrically using a GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, USA). The DNA Engine Opticon™ systems (MJ Research, USA) were applied to quantify the amount of mRNA for *BCL-2* and *BAX* genes using the real-time QRT-PCR technique. The reaction mixture consisted of: 25 µl 2x QuantiTect SYBR Green RT-PCR Master Mix (QIAGEN, Valencia, CA, USA), 0.5 µl QuantiTect RT Mix, 0.5 µl forward and reverse primers and 0.1 µg RNA. Primers for the amplification of the genes under examination were designed using Primer Express™ Version 2.0 (PE Applied Biosystems, USA). The sequences of starters used for amplification were as follows: *BF* 5'-TCACCCACACTgTgCCCATCTACgA-3', *BR* 5'-CCAgCggAACCgCTCATTgCCAATgg-3', *GAPDHF* 5'-gAAggTgAAggTCggAgTC-3', *GAPDHR* 5'-gAAgATggTgATgggATTC-3', *BAXF* 5'--CCTgTgCACCAAggTgCCggAACT-3', *BAXR* 5'--CCACCCTggTCTTggATCCAgCCC-3', *BCL-2F* 5'-TTgTggCCTTCTTTgAgTTCggTg-3', and *BCL-2R* 5'-ggTgCCggTTCAggTACATCAgTCA-3'. The thermal conditions for one-step RT-PCR were as follows: reversed transcription at 50°C for 30 minutes, 95°C for 15 minutes and then 45 cycles of amplification at 94°C for 15 seconds, at 53.3°C for 30 seconds, and at 72°C for 30 seconds. The transcription activity of β-actin and glyceraldehyde-3--phosphate dehydrogenase (*GAPDH*) used as endogenous controls was evaluated in each sample. The RT-PCR specificity was assessed on the basis of the melting temperature for each amplimer. A standard curve was drawn for the commercially accessible patterns of β-actin copies using β-actin Control Reagent Kit (Applied Biosystems, USA) to calculate the number of mRNA copies of the genes tested. Qualitative results were recalculated per 1 µg of the total RNA (c/µg).

ELISA of BAX and BCL-2 Proteins

The Human BAX TiterZyme EIA kit (Assay Designs Inc., USA) and BCL-2 ELISA kit (Bender MedSystems GmbH, Austria) were used to determine the expression of BAX and BCL-2 proteins in the fibroblast cells. Briefly, after 24h incubation with or without the VD derivatives, the cells were pelleted and lysed. The remaining steps were carried out according to the instructions supplied by the manufacturers. The final color intensity was proportional to the amount of tested protein in the sample. The absorbance was read at 492 nm on an automatic plate reader (UVM-340, Biogenet) and the protein (BAX or BCL-2) concentration was determined by interpolating from the standard curve. Relative expression of the protein was calculated from the ratio of absorbance of the test sample to that of the comparison group.

Statistical Analysis

The statistical analysis was performed using the Statistica 5.0 software (Statsoft, Poland). All values were expressed as means ± SE. To check the

normality of the distribution, the Shapiro-Wilk test was performed. In cases of normal distribution Student's t-test was performed; otherwise the Mann-Whitney U test was used. Correlations were calculated using Spearman's rank order test. The level of confidence was established at $p < 0.05$.

Results

The findings show that both calcitriol and tacalcitol influence BCL-2 and BAX expression.

Calcitriol administered at a concentration of 10^{-4}M affected the expression of both *BCL-2* and *BAX* genes significantly compared to the comparison group ($p < 0.05$; Table 1). At a concentration of 10^{-4}M, tacalcitol decreased *BAX* gene expression ($p < 0.05$). At concentrations of 10^{-4}M and 10^{-5}M, tacalcitol also significantly lowered *BCL-2* gene expression ($p < 0.05$). The remaining dilutions of both VD derivates had no effect on BCL-2 and BAX expression.

At the protein level, significant BAX decrease was observed after treatment with calcitriol at a concentration of 10^{-4}M ($p < 0.05$). Tacalcitol significantly affected both BAX and BCL-2 protein level at a concentration of 10^{-4}M compared to the comparison group ($p < 0.05$). The rest of the solutions did not influence protein levels.

Analysis of a single pro- or anti-apototic agent seems to be insufficient, since the decisive factor in activating programmed cell death is the ratio between BCL-2 and BAX expression; a lower ratio is favorable for apoptosis. In the present study, a slight decrease in the *BCL-2/BAX* mRNA ratio was noted after treatment with both of the examined VD derivatives, independently of the drug concentration. There were no statistical differences compared to the comparison group. The highest pro-apoptotic ratio was noted with tacalcitol at a concentration of 10^{-5}M.

At the protein level, the BCL-2/BAX ratio showed a significant increase after treatment with both calcitriol and tacalcitol at a concentration of 10^{-4}M ($p < 0.05$). The remaining concentrations barely influenced BCL-2/BAX protein ratios compared to the comparison group.

No concentration-dependent action of the VD derivatives was observed in the fibroblast cultures.

Discussion

In the context of the potential pro-apoptotic properties of VD, the present study was undertaken to elucidate the mechanism of its influence on fibroblasts derived from NP. The results obtained provide additional information for understanding the biological behavior and pathogenesis of NP.

Although there are multiple pathways leading to apoptosis, most of them are ultimately regulated by the BCL-2 family of proteins. The BCL-2 proteins are regarded as important regulators of the apoptotic process, since they include at least 15 proteins that can be divided into two subclasses that either promote apoptosis (BAX, BCL-XS, BAD, BIK and BAK) or suppress it (BCL-2, BCL-XL and MCL-1) [9]. These proteins can form heterodimers, and the ratio of apoptosis promoters to apoptosis suppressors is one determinant of cellular response. Therefore, the ratio between BCL-2/BAX ratio is thought to be a decisive factor in activating cell death, with a lower ratio favoring active cell death [10].

The BCL-2 family governs mitochondrial membrane permeability, either promoting or suppressing the release of apoptogenic proteins from these organelles. Among the mitochondrial pro-

Table 1. The influence of tacalcitol and calcitriol at different dilutions on BCL-2 and BAX expression (means ± SE). Comparison group (C); comparison group stimulated with LPS (C-LPS); mRNA copy number per 1 μg of total RNA; † $p < 0.05$

Tabela 1. Wpływ różnych stężeń takalcytolu i kalcytriolu na ekspresję BCL-2 i BAX (średnia ± OS). Grupa porównawcza (C); grupa porównawcza po stymulacji LPS (C-LPS); liczba kopii mRNA na 1 μg całkowitego RNA; † $p < 0{,}05$

	Comparison group (Grupa porównawcza)		Calcitriol concentration [M] (Stężenie kalcytriolu)			Tacalcitol concentration [M] (Stężenie takalcytolu)		
	C	C-LPS	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-4}	1×10^{-5}	1×10^{-6}
BAX protein	5705 ± 269	5981 ± 2109	761 ± 300†	6173 ± 1686	6195 ± 1165	290 ± 380†	5980 ± 1209	6155 ± 499
BAX mRNA copy number	94261 ± 6507	84819 ± 18442	36458 ± 13480†	76373 ± 13226	76395 ± 13446	36215 ± 13893†	68461 ± 7612	88666 ± 19157
BCL-2 protein	2.33 ± 1.65	2.43 ± 1.6	1.27 ± 0.47	2.14 ± 2.15	2.23 ± 1.59	0.72 ± 0.54	1.66 ± 1.0	1.69 ± 1.18
BCL-2 mRNA copy number	168694 ± 22804	183335 ± 13035	48635 ± 18773†	77983 ± 13836	119947 ± 23493	49396 ± 10928†	54295 ± 11259†	141775 ± 22403

teins released into the cytosol during apoptosis is Cyt-c, which binds and activates Apaf-1, an oligomeric protein that activates the cell death protease, pro-caspase-9 [11, 12]. It has been shown that 1,25-dihydroxyvitamin D3 evokes apoptosis by down-regulating antiapoptotic proteins, such as BCL-2, BCL-XL and MCL-1 [13], and by up-regulating pro-apoptotic molecules, such as BAX [14]. Although the mechanism of VD analog induction of apoptosis is uncertain, a recent study has indicated its pro-apoptotic action in various cellular models. In colorectal adenoma VD treatment has been associated with the induction of the pro-apoptotic gene *BAK* and a reduction in *BCL-2* [15].

Calcitriol has previously been proved previously to induce apoptosis, associated with a simultaneous decrease in BCL-2 expression in the K562 chronic myeloid leukemia cell line and and prostate cancer [16, 17]. Incubating acute promyelocytic leukemia cells with KH1060 and other 20-epi vitamin D3 analogs caused a decrease in BCL-2 that was paralleled by an increase in the BAX protein [18]. Apoptosis induced by 1,25-(OH)2D3 was blocked by an overexpression of BCL-2, suggesting that BCL-2 acts downstream of calcitriol to inhibit apoptotic cell death [19]. VD analogues have been tested in tissue culture and animal models of retinoblastoma for their use as anti-tumor drugs with a significant reduction in the tumor size [20]. Topically applied vitamin VD has proved effective in the treatment of psoriasis, and 1,25-dihydroxyVD3 has been shown to induce apoptosis in cultured human keratinocytes [21]. In MCF-7 cells, the BCL-2/BAX protein ratio was decreased by treatment with 1,25-dihydroxyVD3, suggesting alterations in the heterodimerization of these proteins, which may promote apoptosis [22].

Enhanced cell proliferation is one important mechanism of nasal polyp growth. Thus, suppression of NP fibroblast proliferation and additionally as well as induction of apoptosis is an expected and advantageous effect of the action of new medication intended to be applied for use in NP treatment. Kimura et al. [23] showed that induction of apoptosis by steroid treatment in human nasal fibroblast cultures is enhanced by the transfer of the exogenous *BAX* gene. Thus, exogenous BAX protein expression by gene transfer might be useful for the treatment of NP.

Our previous study demonstrated that active VD analogs show antiproliferative properties and enhanced programmed cell death in fibroblast cultures [3]. The current study has revealed that at certain dilutions calcitriol and tacalcitol may down-regulate the expression of both BCL-2 and BAX, but did not prove that BCL-2 and BAX may play an important role in the mechanism by which VD derivatives regulate NP cell apoptosis. The most obvious (albeit insignificant) decrease in the BCL-2/BAX ratio – which is favorable for apoptotic death – was seen after the application of tacalcitol at a concentration of 10^{-5}M. However, none of the dilutions of calcitriol or tacalcitol was sufficient to shift the BCL-2/BAX ratio favorably compared to the comparison group. The decrease in the BCL-2/BAX ratio was the outcome of inhibited BCL-2 expression rather than enhanced BAX expression.

The authors concluded that the results of this study imply that the pro-apoptotic properties of VD derivatives may engage the BCL-2 family pathway only to a limited extent. The mechanism responsible for decreased apoptosis in NP fibroblasts after treatment with VD is still unclear. It can be stressed, however, that apoptosis is not a universal response to calcitriol treatment. Consequently, studies of breast cancer cell lines have shown that calcitriol or calcitriol analogs cause extensive apoptosis in some lines [24] but not in others [25]. Prostate cancer cell line LNCaP did not become apoptotic after 1,25-(OH)2D3 treatment even though the treatment caused cell growth arrest [26].

It is known that different molecules are involved in the cascade of events that characterizes the apoptotic process. Thus, the induction of programmed cell death in NP may also be independent of BCL-2 and BAX function.

The slightly higher efficiency of tacalcitol in reducing the *BCL-2/BAX* mRNA ratio, along with the higher antiproliferative effect demonstrated in our previous study and its lower calcemic effects, suggest that tacalcitol has an advantage of over calcitriol.

The antiproliferative effect of VD derivatives could potentially be harnessed therapeutically as a supplementary method in rhinosinusitis, targeting not only the fibroblasts but also a wide range of inflammatory cells like the eosinophils and T lymphocytes that infiltrate polyps. That is why further basic studies must be conducted for a better understanding of the mechanism of vitamin D action in nasal polyposis.

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