J. Basic. Appl. Sci. Res., 3(2)526-531, 2013
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ISSN 2090-4304
Journal of Basic and Applied Scientific Research
www.textroad.com

Impact of Calcitriol on Human Squamous and Transitional Bladder Carcinoma Cell Lines

Mahmoud M. Gabr[1], Mohamed A. Taha*[2] and Mahmoud M. Zakaria[3]

Urology and Nephrology Center, Mansoura University Mansoura, Egypt

ABSTRACT

Although the effect of vitamin D (calcitriol) on the risk of different malignant cells has been widely studied, few studies were examined its real effect on human bladder cancer cells. Our aim was to investigate the impact of calcitriol on human bladder squamous and transitional carcinoma cell lines.
Bladder cancer cell lines (SCaBER and T24) and the normal transitional epithelial cells were treated with 40µM, 60µM and 80µM of calcitriol for 2 days. The viability of cells was determined by trypan blue stain. Apoptosis of normal and tumor cells were assessed by Giemsa, acridine orange and propidium iodide stains, whereas the total DNA fragments resulting from apoptotic DNA fragmentation was determined by DNA laddering assay. The results were reported as mean ± Standard deviation (SD). The P value of < 0.05 shows a significant result, while P value of > 0.05 indicates a non significant result.
Growth inhibition of cancer cells were enhanced by calcitriol. At 40µM concentration, the cancer cell lines were inhibited by 18% after 24 hours and by 53% after 48 hours. With increasing the concentrations of calcitriol, the morphological changes, the growth inhibition and apoptosis of cancer cells were augmented reached to more than 98% after 24 hours with 80µM of calcitriol. The normal epithelial cells were tolerant to all concentrations. According to the DNA laddering assay, no ladder was detected in tumor cells in complete media, while with 80µM of calcitriol the DNA ladder in tumor cells were clearer and more than in normal epithelial cells.
In conclusion, calcitriol induced not only growth inhibition but also apoptosis in the two different types of bladder cancer cell lines. Calcitriol showed promise as a chemopreventive agent for preventing and treating bladder cancer.
KEY WORDS: Vitamin D, Calcitriol, Bladder, Cancer, Apoptosis, Cell lines.

INTRODUCTION

Bladder cancer is a complex and multifactorial disease and a very common genitourinary malignancy [1]. Similar to other solid tumor, bladder cancer developed through a series of genetic changes such as chromosomal alterations and loss of cell cycle regulation that lead to tumor progression [2]. Calcitriol or 1, 25-dihydroxycholecalciferol (vitamin D3) is the active form of Vitamin D. It is synthesized in the skin by conversion of 7-dehydrocholesterol (provitamin D3) to previtamin D3 by the action of ultraviolet light. Previtamin D3 is then hydroxylated at the 25 and 1 carbon position in the liver and kidney, respectively, to convert it into its physiologically active form. Because the body can synthesize vitamin D, it has been reclassified as a steroid hormone that is important in calcium metabolism [3].

Calcitriol has been shown to inhibit proliferation and induce apoptosis in human bladder cancer cells in vitro and may have therapeutic potential in bladder cancer [4]. Calcitriol also, has antiproliferative and differentiating effects on a variety of human tissues and tumors [5], such as human prostate cancer cells in culture and sublines of the rat tumor in vitro and in vivo [6], as well as it enhances the antitumor activity of gemcitabine and cisplatin in bladder cancer cells in vitro and in vivo [7].

In animal studies vitamin D3 reduces the risk of invasive breast and prostate cancer and suggests a role for chemoprevention [8]. Calcitriol also decreases metastases from subcutaneously injected prostate tumors [9]. Another study showed that vitamin D3 has an antitumor effect on renal carcinoma in mice [10].

Calcitriol mediates gene expression *via* binding to the nuclear vitamin D receptor (VDR) in target tissue, causing up-regulation of gene transcription and translation of specific proteins. It has been shown to exert antiproliferative effects in variety of human cancers including breast, colon and prostate cancers, through interaction with the VDR [11].

Epidemiological studies suggested that lower levels of vitamin D3 may play an important role in the development of bladder cancer; low vitamin D serum levels, low vitamin D intake, and decreased UV light activation of vitamin D in the skin are associated with an increased risk of this tumor [12].

***Corresponding Author:** Mohamed Ahmed Taha, PhD Medical analysis fellow, Urology & Nephrology Center Mansoura University, Mansoura – Egypt. Mobile: (+2) (01144117779). E-mail: mohataha3@hotmail.com

There is also evidence that dietary supplementation with vitamin D can reduce the incidence of colon cancer [13].

In a recent study, Mondul and his coworkers observed that there is no association between vitamin D status and risk of bladder cancer [14]. Others demonstrated that higher circulating vitamin D concentration may associate with increased risk of aggressive prostate cancer disease [15].

Due to these controversies and the fewer studies that have examined the association between calcitriol and bladder cancer, we constructed this study to investigate the real effect of calcitriol on human bladder squamous and transitional carcinoma cell lines.

MATERIALS AND METHODS

The bladder cancer cell lines used were derived from transitional cell carcinoma (T24) and squamous cell carcinoma (SCaBER). The cell lines were maintained in a complete medium which consists of Eagle's minimum essential medium (MEM) [Sigma, Chemical Co., St Lous, USA], 100U/mL penicillin, 100U/mL streptomycin, 50U/mL gentamicin, 2 mmol/L L-glutamin, 1 mmol/L sodium pyruvate and 10 % heat inactivated fetal calf serum at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. Vitamin D3 (calcitriol) was dissolved in absolute ethanol as a stock solution of 0.1 mmol and stored at 4°C.

Tumor cells were harvested by overlaying the monolayer with a solution of 0.05% trypsin and 0.5 mmol/L ethylene diamine tetra-acetic acid (EDTA) and then resuspended in a complete medium. The cells were plated in 24 - well plates at a concentration of 10^6 cells per well and grown for 24 hours. Normal transitional epithelial cells were isolated from a pig bladder. The bladder was cut into halves and the smooth muscle layer was removed with scissors. The epithelial layer attached to the lamina propria was cut with scissors into rectangles and treated with bacterial normal protease. The epithelial layer was digested in a trypsin solution and the resulted cell suspension was filtered and cultured in 24-well plates (10^6 cells per well) containing complete media. Normal and Tumor cells were then incubated with 40µM, 60µM, 80µM of calcitriol in duplicate for 2 days, while control cultures contained absolute ethanol (solvent).

1- Viability of cells:

Viability test: After 24 and 48 hours of incubation, the dislodged cells in each well were collected and the adhered cells were trypsinized. Triplicate counts for each well were determined, by trypan blue exclusion using a hemocytometer. The mean count of dislodged and adhered cells, from duplicate wells was determined. This experiment was performed in triplicate and the mean plus or minus standard deviation was calculated.

2- Morphology of cells:

Light microscopy: Tumor cells were cultured in Leighton's tubes, each containing a glass cover slip. At confluent state, 40µM, 60µM, 80µM of calcitriol were added and incubated for 24 hours and 48 hours. Control cultures contained absolute ethanol. Subsequently, the medium was decanted and 1.5mL of 95% ethanol was added for 10 minutes to fix the cells. The cover slips were then removed and stained with haematoxylin for 5 minutes followed by eosin for 3 minutes.

3- Apoptosis of cells:

We analyzed the apoptotic effect of calcitriol by Giemsa stain, acridine orange stain, propidium iodide stain and DNA laddering assay:

I- Giemsa stain: Cells were removed from each culture, fixed in ethanol, harvested on slides and slides were stained with May Grunwald Giemsa and examined by oil immersion light microscope. For assessment of the percentage of cells showing morphology of apoptosis 500 cells per slide were examined for each case at different times (zero, 24h., 48h.). Cells were considered apoptotic if they exhibited the highly characteristic morphological features of chromatin aggregation, nuclear pyknosis and cytoplasmic vaculation. The apoptotic cell percentage at different times was calculated.

II- Acridin orange stain: One drop of cell suspension was added to one drop acridin orange (10 µg/mL PBS), mixed gently on a slide, and immediately examined with fluorescent microscope. Green fluorescence was detected between 500 and 535 nm. Cells exhibit bright fluorescent condensed nuclei (intact or fragmented) were interpreted as apoptotic cells and expressed as a percentage of the total number of viable cells , which exhibited a green , diffusely stained intact nucleus.

III- Propidium iodide stain: Cells were stained with Hoechst 33342 and propidium iodide and visualized using fluorescent microscope. A minimum of 200 cells were counted per sample and were classified as follows:

1- Live cells (normal nuclei, blue chromatin with organized structure).
2- Membrane intact apoptotic cells (bright blue chromatin that is highly condensed, marginated or fragmented).

3- Membrane permeable apoptotic cells (bright red chromatin, highly condensed or fragmented). The extent of apoptosis was calculated as the percentage of total apoptotic cells divided by total number of cells counted.

IV- DNA laddering assay: According to Ausubel [16], total DNA was extracted with 0.5mL saturated phenol followed by 0.5mL chloroform and isoamyl alcohol (24:1) before centrifugation at 7000 g at 4°C. DNA in the supernatant was precipitated by adding 0.1 mL of 3M sodium acetate (pH 5.2) and 2 mL of ice-cold absolute ethanol. The samples were mixed by vortexing and placed in crushed dry ice for 5 minutes. Then, centrifugation at 9500 g for 5 min. For washing the samples, 1 mL of 70% ethanol was added and centrifuged. The pellets were air dried prior to suspending in 0.1mL TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). RNA was eliminated by RNase digestion (0.1mg / mL RNase, incubated at room temperature for 1h). The DNA was electro-phoresed using 1.8% agarose gel and visualized by ethidium bromide staining.

4- Statistical method:

Data entry and analysis were performed using the Statistical Package for Social Sciences version 13.0 (SPSS, Inc., Chicago, IL, USA). The results were reported as mean ± Standard deviation (SD). The P value of < 0.05 shows a significant result, while P value of > 0.05 indicates a non significant result.

RESULTS

The bladder cancer cell lines treated with 40µM calcitriol were inhibited by 18% after 24 hours and by 53% after 48 hours. Increasing the concentration of calcitriol resulted in a significant increase of growth inhibition. At 60µM, calcitriol inhibited the growth of both cell lines by 55% after 24 hours and more than 95% after 48 hours of treatment. At 80 µM both cell lines were inhibited by more than 98% after 24 hours of treatment. Normal epithelial cells were tolerant to all concentrations of calcitriol. The viability of normal epithelial cells treated with media only or with calcitriol was not comparable due to the convergence of the proportion of cells and showed no significant differences (P= 0.13). On the other word, the viability of cancer cell lines was significantly decreased as compared to the normal epithelial cells at different calcitriol concentrations during 24 and 48 hours (Table 1).

Table 1: The effect of vitamin D (cacitriol) on viability of normal and tumor cells.

Medium	Normal epithelial cells		SCaBER cells				T24 cells			
	Via±SD 24hrs.	Via±SD 48hrs.	Via±SD 24hrs.s.	P_1	Via±SD 48hrs.	P_2	Via±SD 24hrs.	P_1	Via±SD 48hrs.	P_2
Complete media	96±1.90	80±2.16	100±0.38	*<0.01*	100±0.38	*<0.001*	100±0.00	*<0.001*	99±0.79	*<0.001*
Solvent	94±1.95	81±2.04	98±1.07	*<0.01*	97±1.11	*<0.001*	96±1.16	*0.08*	96±2.16	*<0.001*
40µM Calcitriol	93±1.41	85±1.62	82±2.16	*<0.001*	47±2.65	*<0.001*	81±3.06	*<0.001*	46±4	*<0.001*
60µM Calcitriol	91±1.63	82±1.63	45±4.31	*<0.001*	4±1.35	*<0.001*	43±4.72	*<0.001*	3±2.16	*<0.001*
80µM Calcitriol	92±1.41	79±1.92	0.5±0.79	*<0.001*	0	*<0.001*	1±1.07	*<0.001*	0	*<0.001*

Normal epithelial cells 10^6 cells/well – Tumor cells 10^6cells/well.
Via= viability%
P_1 = viability of cancer cells vs normal epithelial cells at 24 hours.
P_2 = viability of cancer cells vs normal epithelial cells at 48 hours.

Morphological changes were induced by 80µM after 24 hours of treatment and were in concordance with agent effects on cell growth (Figure 1).

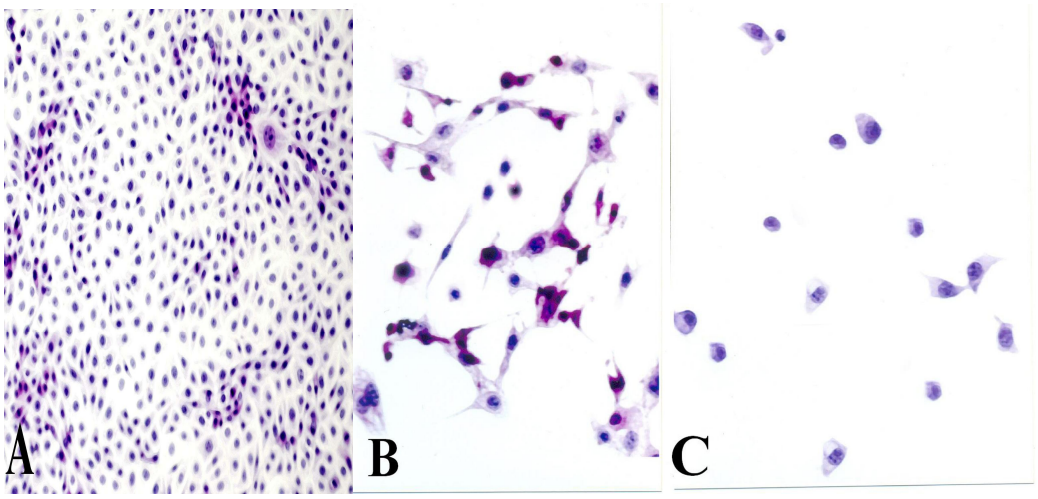

Figure 1: Morphological changes of tumor cells

A. Light photograph showing untreated transitional bladder cancer cells.
B. Light photograph showing transitional bladder cancer cells treated with 60µM calcitriol for 24 hours.
C. Light photograph showing transitional bladder cancer cells treated with 80µM calcitriol for 24 hours.

Giemsa, acridine orange and propidium iodide were used for nuclear staining to look for the ability of calcitriol to induce the morphologic changes that define apoptotic death. Based on these assays, we found that about 24% of normal epithelial cells were subjected to apoptosis with 80µM of calcitriol after 24 hours, however more than 98% of tumor cells with the same concentration of calcitriol demonstrated morphologic changes characteristic of both apoptosis and necrosis after 24 hours (Table 2).

Table 2: Viability percent of normal and tumor cells ± SD after 24 hours incubation with 80µM calcitriol using Giemsa, acridine orange and propidium iodide stains.

Medium	Giemsa stain			Acridine orange stain			Propidium iodide stain		
	N.E.	SCaBER	T24	N.E.	SCaBER	T24	N.E.	SCaBER	T24
Complete media	**67±3.2**	73±3.6 ***P_1<0.05***	74±3.9 ***P_1<0.001***	**68±3.2**	75±2.9 ***P_2<0.01***	75±3.2 ***P_2<0.01***	**69±3.2**	76±2.1 ***P_3<0.05***	75±2.3 ***P_3<0.05***
Solvent	**55±4.1**	69±3.3 ***P_1<0.001***	67±2.9 ***P_1<0.01***	**56±4.2**	70±3.3 ***P_2<0.01***	69±3.4 ***P_2<0.01***	**57±4.8**	70±3 ***P_3<0.01***	70±3.1 ***P_3<0.01***
80µM Calcitriol	**51±2.6**	1±0.9 ***P_1<0.001***	1±0.9 ***P_1<0.001***	**52±2.5**	1±1 ***P_2<0.001***	2±0.9 ***P_2<0.001***	**53±2.7**	1±0.9 ***P_3<0.001***	2±0.7 ***P_3<0.001***

N.E.= Normal epithelial cells.
P_1 = viability of cancer cells vs normal epithelial cells using Giemsa stain.
P_2 = viability of cancer cells vs normal epithelial cells using Acridine orange stain.
P_3 = viability of cancer cells vs normal epithelial cells using propidium iodide stain.

The effect of calcitriol on apoptosis induction was also tested in both normal and bladder cancer cells. Cells were treated by 40µM, 60µM and 80µM of calcitriol for 24 hours and apoptosis was analyzed in floating and attached cells by DNA laddering assay. No ladder was detected in tumor cells in complete media and those treated with solvent. The intensity and pattern of DNA laddering were different in tumor and normal cells treated with 80µM of calcitriol. The ladder was clearly detected in tumor cells. The intensity of DNA ladder was stronger and clearer in tumor cells while normal epithelial cells were slightly affected (Figure 2).

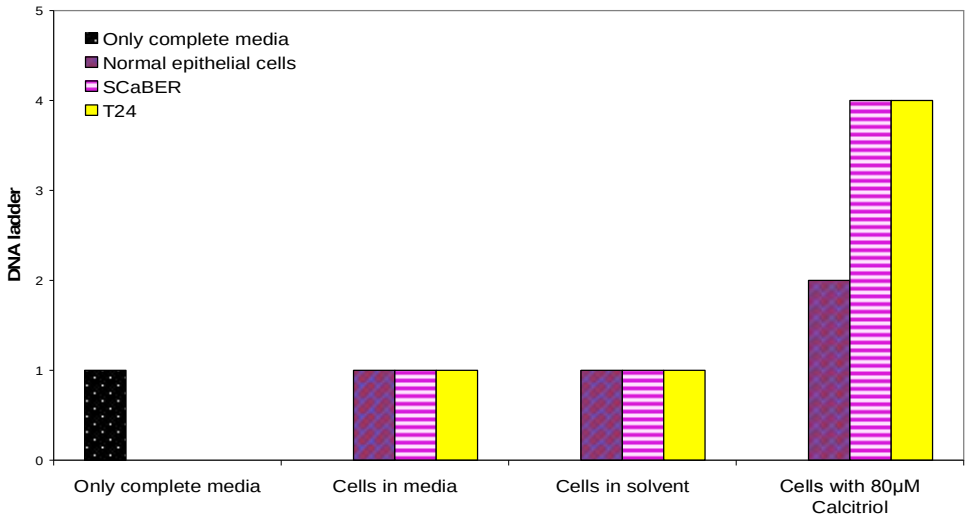

Figure 2: Effect of calcitriol (80µM) in inducing apoptosis of normal and cancer cells after 24 hours of incubation that determined by DNA laddering assy.

DISCUSSION

We explored in our study the cell growth inhibition of human squamous and transitional bladder carcinoma cell lines that treated with different concentrations of calcitriol. Previous studies demonstrated that cell culture, in vivo, and genetic evidence suggesting that greater exposure to vitamin D could have a role in protecting against bladder cancer [4,17]. Higher serum 25-hydroxyvitamin D may be associated with greater urinary excretion of free and conjugated vitamin D metabolites [18]. Increased exposure of the bladder mucosa to these metabolites could promote transitional cell differentiation and apoptosis and thus, reduce epithelial proliferation and neoplasia [12].

In addition, individuals with low levels of plasma 25-hydroxyvitamin D3 my be at high risk of more aggressive forms of urothelial bladder cancer [19]. Vitamin D receptors have been demonstrated in bladder cancer tissues. This receptor expression increased in tumors of higher stage and grade [20]. Furthermore, Konety with his colleagues observed that treatment of 253J bladder cancer cell line with calcitriol resulted in significantly greater proportion of apoptotic cells compared to treatment with vehicle ethanol alone [4].

In accordance with the last considerations, our study indicates that calcitriol has a definite antiproliferative effect on bladder cancer cells. Treatment of SCaBER and T24 cancer cells with calcitriol resulted in a significantly inhibition in the proliferation and growth of these cells as compared with the calcitriol treated normal epithelial cells after 24 and 48 hours of incubation. Our results were interpreted by the findings of Rao et al [21] who found that calcitriol treatment of prostate cancer cell line caused G0/G1 cell cycle arrest and apoptosis by inducing the expression of the cyclin-dependent protein kinase (CDK) inhibitor p21. Others showed that calcitriol treatment of canine transitional cell carcinoma cell line resulted in the accumulation of cells in the G0/G1-phase and the reduction of cells in the S-G2/M-phase at 24 and 48 hours after treatment [11]. As well as, Blutt with his coworkers [22] have demonstrated that the growth inhibitory effects of calcitriol are mediated by induction of apoptosis.

In consistence with these findings, our results showed that the SCaBER and T24 carcinoma cell lines treated with calcitriol exhibit greater and significant proportion of apoptotic cells compared to that treatment with ethanol alone. Apoptosis of tumor cells upon calcitriol treatment appeared after 24 hours and increasing up to 48 hours of exposure. Treatment of normal epithelial cells with calcitriol resulted in very mild effect, and the viability of normal epithelial cells treated with media only or with calcitriol was not comparable due to the convergence of the proportion of cells. The fact that vitamin D receptor expression increases with stage and grade of bladder tumors could enhance calcitriol inhibition of advanced bladder tumor cells and explain the mild effect on normal epithelial cells.

In conclusion calcitriol induced growth inhibition and gave rise to apoptosis in the two different types of bladder cancer cell lines. Calcitriol showed promise as a chemopreventive agent for preventing and treating bladder cancer.

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