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1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) Induces Cell Apoptosis of Human Mesangial Cells

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ABSTRACT In China, primary glomerulonephritis is the leading cause of end-stage renal disease (ESRD), and patients who require kidney transplantation often with ESRD. Glomerulonephritis primary's most prevalent subtype is Mesangial proliferative glomerulonephritis (MsPGN). Although the cell apoptosis of human mesangial cells (HMCs) may be harmful in MsPGN, it plays a major role in the elimination of excessive HMCs and reparative glomerular remodelling after inflammation. A powerful mitogen for growing mesangial cells is epidermal growth factor (EGF). Therefore, it is possible to induce the proliferation of HMCs by incubating with EGF *in vitro*. 1,25$(OH)_2D_3$(VD3) may cause cell death in HMCs via activating AKT, caspase-3, caspase-9, JNK, and ERK, as well as elevating Bax and Bad levels.

INTRODUCTION

Primary glomerulonephritis is the main contributor to end-stage renal disease (ESRD) in China, and patients with ESRD frequently require kidney transplantation (Lin et al. 2014). The most common type of primary glomerulonephritis is mesangial proliferative glomerulonephritis (MsPGN). Although cell apoptosis of human mesangial cells (HMCs) may be harmful in MsPGN, it is critical in the elimination of excess HMCs and reparative glomerular remodelling following inflammation (Hughes et al. 2005). Epidermal growth factor (EGF) is a powerful mitogen for mesangial cell culture (Mishra et al. 2002).Therefore, it is possible to induce the proliferation of HMCs by incubating with EGF *in vitro*.

This investigation looked at how 1,25(OH)2D3 vitamin D3 affects the apoptosis of human mesangial cells (HMCs). Normal control (N), epidermal growth factor (EGF) (E), VD3 (V), EGF + VD3 (EV), and LY294002 were used to divide HMCs into four groups. Cell apoptosis was much lower in the E group than in the N group, but it was significantly higher in the V group ($p<0.05$). In contrast, the EV group had significantly more cell apoptosis than the E group ($p<0.05$). Caspase-3, caspase-9, JNK, and ERK were also activated in the V group. Furthermore, LY294002, a PI3K inhibitor, inhibited VD3-induced increases in AKT and Bad more effectively. 125MARRS and ERp57 were found in HMCs. EGF-induced mitogenesis is associated with PI3K/Akt and MAPK activation in a variety of cells, including mesangial cells (Mahimainathan et al. 2005).

VD3, the active metabolite of the secosteroid hormone vitamin D, is important in bone and mineral metabolism (Pazianas and Miller 2021). Furthermore, numerous studies show that chronic illnesses like diabetes and multiple sclerosis can be prevented by VD3 (Abe 2004). VD3 exerts its effects via both genomic and nongenomic mechanisms (Sutton and Macdonald 2003). VD3 mediates long-term, transcription-dependent events in the genome by binding to the intracellular vitamin D receptor (VDR), which combines with the retinoid X receptor to generate a heterodimer. The heterodimer subsequently recruits a plethora of nuclear transcriptional elements and coactivators to control gene transcription by binding to a vitamin D impact element in the promoter region of the target genes. Calcium and chloride channel activation, rapid intestinal calcium absorption, and acti-

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vation of intracellular massagers, concluding protein kinase A, protein kinase C, MAP kinases, and phosphoinositides are all examples of rapid nongenomic effects (Norman et al. 2004). However, there is no universal agreement on the mechanism of nongenomic action initiation. VD3 cannot regulate the activities of calcium and chloride ion channels in VDR knockout osteoblasts, indicating that the classic VDR is required for both genomic and nongenomic effects of VD3 (Zhang et al. 2021). Recently, the 1,25D3-MARRS protein, a 1,25D3 membrane binding protein, was discovered in chick intestinal epithelial cells (Janjusevic et al. 2022).It has been reported that the 1,25MARRS receptor is identical to ERp57, a classic member of the protein disulphid isomerase family (Khanal and Nemere 2007;Richard et al. 2010; Rohe et al. 2004).

The mechanism of VD3-induced apoptosis in HMCs, however, remains unknown. The researchers investigated the effect of VD3 on HMC apoptosis and the possible mechanism in this study.

Objective of the Study

This investigation looked at the effects of 1,25-dihydroxy vitamin D3 [VD3] on apoptosis in human mesangial cells (HMCs).

MATERIAL AND METHODS

Material

VD3 was dispersed in 100% ethanol and kept out of the light (Sigma Chemical Co., St. Louis, MO, USA). Before use, VD3 was diluted in cell culture medium. EGF was obtained from Peprotech in the United States. Sigma Chemical Co. provided the propidium iodide (PI) and LY294002 (a PI3K inhibitor) (St. Louis, MO,USA). Cell Signaling Technology provided fetal bovine serum (US Gibco Inc.), Dulbecco's modified Eagle's medium (DMEM), anti-caspase3, anti-Akt, anti-p-Akt, anti-Bcl-2, anti-Bax, anti-Bad, anti-P38, anti-p-P38, anti-ERK, anti-phospho-ERK, anti-JNK, anti-p-JNK, anti-VDR, and mouse anti- (MA, USA). Santa Cruz Biotechnology provided the anti-ERp57 antibody (Santa Cruz, CA).Thermo Fisher Scientific supplied anti-caspase8 and anti-caspase9 (MA, USA). Beijing Golden Bridge Biotechnology Co., Ltd. a secondary goat IgG antibody against a mouse, a secondary goat IgG antibody against a rabbit, donkey anti-goat IgG-FITC, and donkey anti-goat secondary antibody (Beijing, China).

Cell Culture

Xiangya Medical Center Laboratory at Central South University provided the HMCs (Changsha, China). The usual culture medium for the cells was DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 ug/ml streptomycin, and were kept in a humidified atmosphere with 95% O_2 and 5% CO_2. When the cells were about 80% confluent, they were adhered to the dishes and serially subcultured.The HMCs were incubated with EGF (10 μg/L), VD_3 (10^{-8} mol/L) or EGF (10^{-8} mol/L) + VD_3 (10^{-8} mol/L) for 48 h in a 6-well plate (10^5 cells/well), which were considered as E group, V group, or EV group. The untreated cells were designated as the normal control group (N group). In addition, HMCs were pretreated with LY294002 (2 mg/L) for 1 hour before being incubated with VD3 (10^{-8} mol/L) for 48 hours.

Annexin V/PI Staining

Following two PBS washes, the cells were trypsinized and centrifuged at 2000 rpm for five minutes. Each tube received 500μL of 1 buffer before being stained with 5 μL Annexin V and 10 μL PI according to the manufacturer's instructions (Bio Vision Inc, US). Each group's apoptosis was identified using flow cytometry (GER PARTEC). The experiment was repeated three times with the same sample to calculate the rate of apoptosis.

The Terminal dUTP Nick End-labeling (TUNEL) Staining

Using the in situ cell apoptosis detection kit, TUNEL labeling was performed as directed by the manufacturer (Roche Diagnostics). The cells were fixed with 4% (w/v) paraformaldehyde and permeabilized with 0.1 (v/v) Triton X-100. The TUNEL reaction mixture was then incubated on them for the following two hours at 37^0C and in the dark. POD was added after washing for 1 hour at 37^0C in the dark. TUNEL-positive cells were counted in five randomly chosen fields. Each slip had approximately 200 cells scored. The ratio of TUNEL-positive cells to normal cells served as the basis for calculating the apoptotic index (200).

qRT-PCR

To examine the mRNA expression levels of Caspase-3, Caspase-9, VDR, and 1,25D3-MARRS, total RNA was isolated using RNAeasy Mini-spin columns (Qiagen, Mississauga, Ontario, Canada) in accordance with the product's instructions. Complementary DNA (cDNA) was then synthesized using M-MLV reverse transcriptase (Invitrogen, USA). Power SYBR Green PCR Master Mix was used for amplification (Applied Biosystems, Foster City, CA). The target genes in this investigation were targeted using the following primers. â-actin: forward-5'-tagttgcgttacacccttttcttg-3'; reverse-5'-tcaccttcaccgttccagttt3'; Caspase-3: forward-5'-gaagcgaatcaatggactctg-3'; reverse-5'-ttgctgcatcgacatctgtac-3'; Caspase-9: forward-5'-agatgccaccccgttcc-3'; reverse-5'-cactgctcaaagatgtcgtcc-3'; 1,25D_3-MARRS: forward-5'-ctgagctagccgcaaaacc-3'; reverse-5'-cagagccttcccatcacg -3'; VDR: forward-5'-tgaccctggagactttgacc-3'; reverse-5'-gaatagtgccttccgcttca-3'. qRT-PCR was performed using a Light Cycler 480 Software Setup (Roche). The $2^{-\Delta\Delta ct}$ method was used to calculate relative mRNA expression levels with standardization against β-actin.

Western Blot Analysis

In cold RIPA buffer, the total protein of HMCs was homogenized (Beyotime Institute of Biotechnology, Shanghai, China). The total protein was centrifuged for 20 minutes at 4°C at 12000 rpm. Semidry blotting was used to transfer equal amounts of protein electrophoresed on SDS-PAGE (12 percent polyacrylamide gels) to nitrocellulose membranes (Sigma Chemical Co., St. Louis, MO, USA). 5% (w/v) nonfat milk powder was used to block membranes for two hours at room temperature. The primary antibodies were then applied to the membranes and left on them at 4^0C overnight. The membranes were then treated in secondary HRP-conjugated antibodies for an hour at room temperature. Enhanced chemiluminescence was used to see the protein bands, and X-ray film was used to capture the image. Utilizing the Versa Doc Gel Imaging System, densitometry measurement of bands was used to estimate the relative protein expression level (Bio-Rad, Hercules, CA).

Confocal Laser Scanning Microscopy

Before being permeabilized with 0.1 percent (v/v) Triton X-100, HMCs were seeded on coverslips and fixed with 4 percent (w/v) paraformaldehyde. After that, they spent the night at 4^0C being treated with an anti-ERp57 antibody. After washing, donkey anti-goat IgG-FITC antibody was added and stored at 37^0C away from light for 1 hour. Cells were only incubated with the secondary antibody as a negative control. The fluorescent mounting medium was used to mount the coverslips. Fluorescence was observed using a Zeiss Axiovert 135 inverted microscope and a Zeiss F-Fluar 40/1.3 oil immersion lens. A Zeiss Axiocam and Axiovision software were used to capture the images.

Statistical Analysis

The information is displayed as mean standard deviation (SD). Using the SPSS 24.0 program, ANOVA was used to compare numerous groups, while the LSD method was utilized to compare two groups (International Business Machines, corp., Armonk, NY, USA). Statistical significance was set at $p<0.05$.

RESULTS

1,25 $(OH)_2D_3$ Induced Cell Apoptosis in HMCs

The results showed that the rate of VD3-induced apoptosis was significantly higher in the V group than in the N group ($p<0.05$), whereas the rate of HMC cell apoptosis was lower in the E group than in the N group ($p<0.05$). Furthermore, the EV group had a higher rate of cell apoptosis than the E group ($p<0.05$). These findings suggest that VD3 can induce cell apoptosis and reverse EGF inhibition of HMC cell apoptosis *in vitro* (Fig. 1, Table 1).

1,25$(OH)_2D_3$ Induced the Activation of Caspases in HMCs

The kind of caspases present in VD3-induced apoptosis was identified by Western blot analysis. Caspase-3 and Caspase-9 were moderately activated in HMCs, whereas Caspase-8 was not activated (Fig. 2A). On the contrary, EGF treatment inhibited caspase-3 and caspase-9 protein expression levels. Furthermore, in comparison to the E

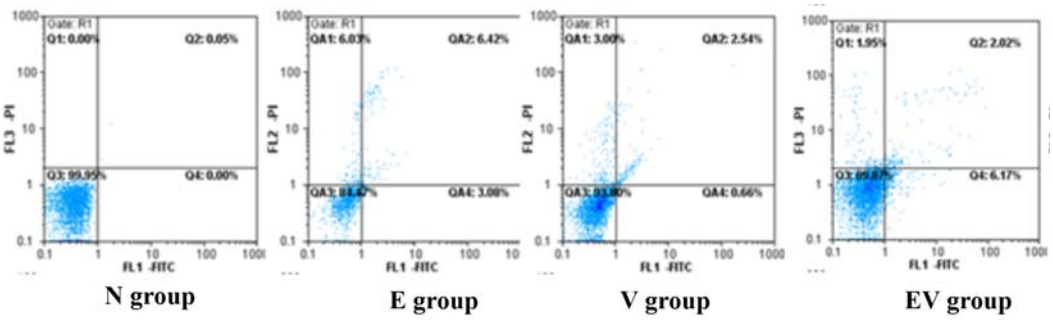

Fig. 1. The apoptosis rate of HMCs detected by flow cytometry
N group: normal control group;
E group: EGF group;
V group: 1,25(OH)$_2$D$_3$ group;
EV group: EGF + 1,25(OH)$_2$D$_3$ group

group, the EV group had higher levels of caspase-3 and caspase-9 protein expression ($p < 0.05$) . (Fig. 2A). These findings were confirmed by qRT-PCR, which revealed that VD3 activated caspase-3 and caspase-9 at the mRNA level ($p < 0.05$) . These results imply that VD3 induced cell apoptosis in HMCs via the caspase-9/3 pathway.

1,25(OH)$_2$D$_3$ Induced the Variation of Bax, Bad and Bcl-2 in HMCs

The protein expression levels of Bcl-2, Bax, and Bad were measured using Western blotting to investigate their roles in V-induced apoptosis. Among Bcl-2-associated X proteins, VD3 increased the

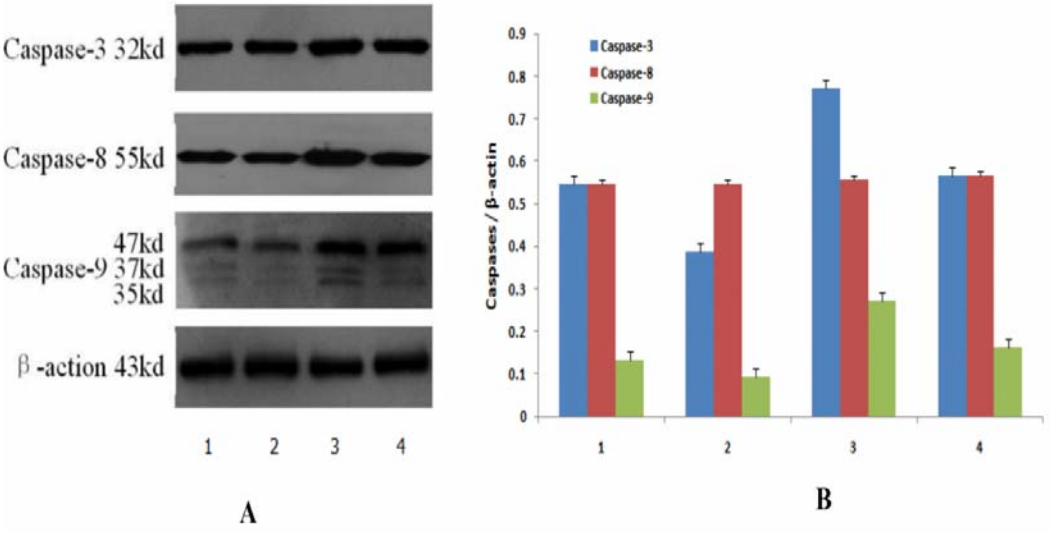

Fig. 2. The activation of Caspase-3, Caspase-8 and Caspase-9 in HMCs detected by Western blot and qRT-PCR
(A) the protein expression levels of Caspase-3, Caspase-8 and Caspase-9 in HMCs
(B) the mRNA expression levels of Caspase-3, Caspase-8 and Caspase-9 in HMCs
1: normal control group; 2: EGF group; 3: 1,25(OH)$_2$D$_3$ group; 4: EGF + 1,25(OH)$_2$D$_3$ group. Data are shown as means ± SD (n=3)
$^*p < 0.05$ compared with N group. $^\#p < 0.05$ compared with E group

protein expression levels of Bad and Bax ($p < 0.05$) (Fig. 3). Furthermore, VD3 decreased Bcl-2 protein expression ($p < 0.05$) (Fig. 3). These findings suggested that VD3 induced cell apoptosis in HMCs via a mitochondria-dependent pathway.

1,25(OH)$_2$D$_3$ Induced the Variation of Akt and p-Akt

The researchers then looked at how VD3 and EGF affected the protein expression levels of Akt and p-Akt as measured by Western blot. The V group had a considerably lower ratio of p-Akt/Akt than the N group ($p < 0.05$), but the E group had a greater ratio than the N group ($p < 0.05$) (Fig. 4A). According to these findings, VD3 reduced the p-Akt/Akt ratio in HMCs.

1,25(OH)$_2$D$_3$ Induced Akt Activation through a PI3K-dependent Pathway

To determine which pathway contributes to VD3-induced Akt activation, LY294002, a PI3K inhibitor, was used to inhibit PI3K expression. Pretreatment with LY294002 inhibited the Akt phosphorylation induced by VD3 in HMCs ($p < 0.05$) (Fig. 4B). These findings suggested that VD3 activated Akt via a PI3K-dependent pathway.

LY294002 Promoted 1,25(OH)$_2$D$_3$-induced Cell Apoptosis in HMCs

The protein expression levels of Bax, Bad, and Bcl-2 were examined by Western blot analysis to investigate the relationship between the PI3K-Akt pathway and active apoptosis proteins. The results showed that LY294002 had no effect on Bcl-2 and ax protein levels ($p > 0.05$) (Fig. 5). However, pretreatment with LY294002 decreased the expression of the Bad protein (Fig. 5). These results imply that by suppressing the expression of Bad, LY294002 may facilitate VD3-induced cell death via the PI3K-Akt pathway.

1,25(OH)$_2$D$_3$ Increased the Protein Expression Levels of p-JNK and p-ERK in HMCs

Western blot analysis was used to look at the levels of ERK, JNK, and P38 MAPK protein expression, to investigate the possible mechanism of antagonism. The results showed that JNK and ERK activation was higher in the VD3 group than in the normal control group (Fig. 6). However, there was no discernible difference in the levels of p-P38 MAPK protein expression between the VD3 group and the normal control group (Fig. 6).

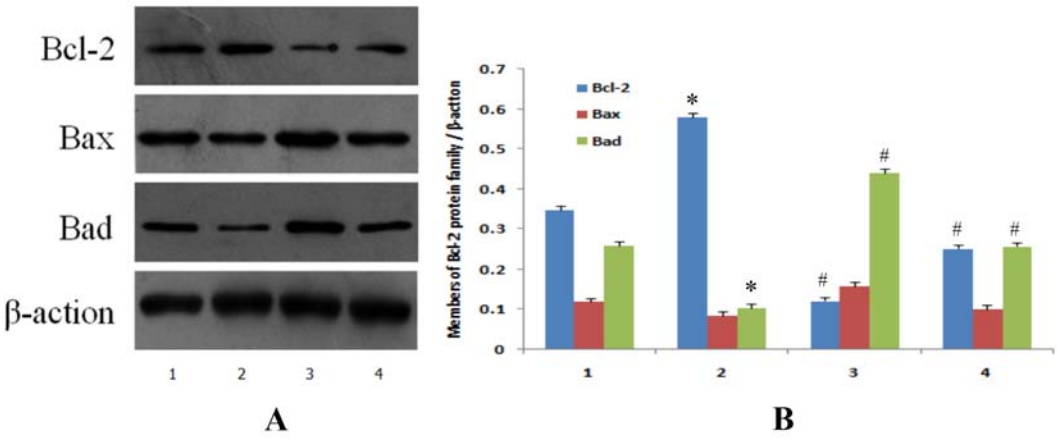

Fig. 3. The activation of members of Bcl-2 family in HMCs detected by Western blot
(A) the protein bands of Bax, Bad and Bcl-2 in HMCs
(B) the protein expression levels of Bax, Bad and Bcl-2 in HMCs
1: normal control group; 2: EGF group; 3: 1,25(OH)$_2$D$_3$ group; 4: EGF + 1,25(OH)$_2$D$_3$ group
Data are shown as means ± SD (n=3)
$^*p < 0.05$ compared with N group. $^\#p < 0.05$ compared with E group

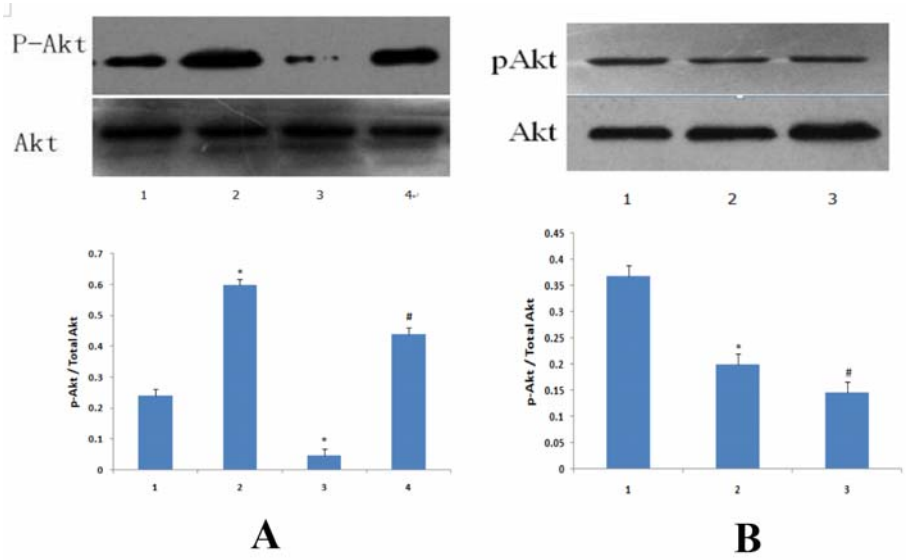

Fig. 4. The protein expression levels of Akt and p-Akt in HMCs detected by Western blot
(A) the p protein expression levels of Akt and p-Akt in HMCs induced by EGF and $1,25(OH)_2D_3$
(B) the p protein expression levels of Akt and p-Akt in HMCs induced by LY294002 and $1,25(OH)_2D_3$
A1: normal control group;
A2: EGF group;
A3: $1,25(OH)_2D_3$ group;
A4: EGF + $1,25(OH)_2D_3$ group
B1: normal control group;
B2: $1,25(OH)_2D_3$ group;
B3: LY294002 + $1,25(OH)_2D_3$ group
Data are shown as means ± SD (n=3)
$^*p < 0.05$ compared with N group. $^\#p < 0.05$ compared with E group in Fig. 4A or with V group in Fig. 4B

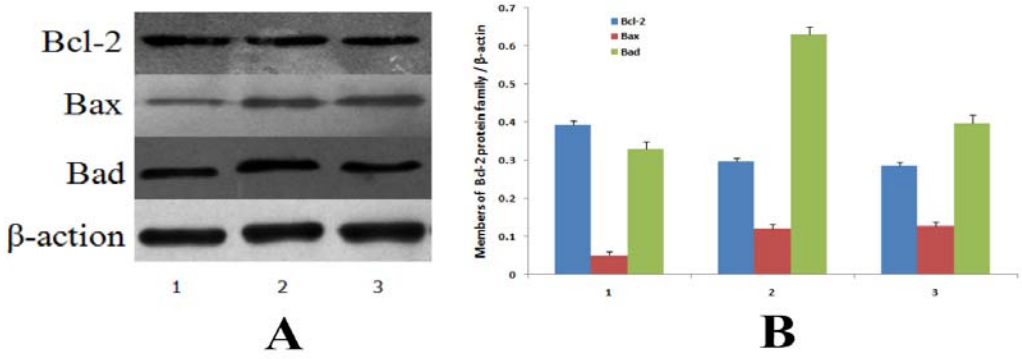

Fig. 5. The protein expression levels of Bcl-2 protein family induced by LY294002
(A) the protein bands of Bax, Bad and Bcl-2 in HMCs
(B) the protein expression levels of Bax, Bad and Bcl-2 in HMC.
1: normal control group;
2: $1,25(OH)_2D_3$ group;
3: LY294002 + $1,25(OH)_2D_3$ group
Data are shown as means ± SD (n=3).
$^*p < 0.05$ compared with N group. $^\#p < 0.05$ compared with V group

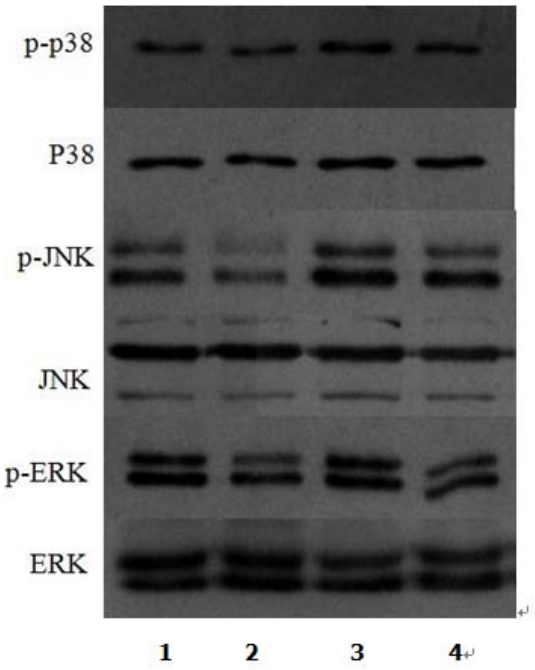

Fig. 6. The activation of MAPKs in HMCs detected by Western blot
1: normal control group;
2: EGF group;
3: $1,25(OH)_2D_3$ group;
4: EGF + $1,25(OH)_2D_3$ group

The Expression Levels of VDR and 1,25D3-MARRS in HMCs

The researchers first used qRT-PCR, Confocal laser scanning microscopy, and examining the levels of VDR expression using a western blot and 1,25MARRS in HMCs. At the mRNA level, 1,25MARRS was expressed in HMCs, and ERp57 was expressed in HMCs (Fig. 7A). At the protein level, 1,25MARRS was expressed in HMCs with a 57 kDa band (Fig. 7B). Confocal laser scanning microscopy revealed the presence of 1,25MARRS receptors in the membrane and nucleus of HMCs (Fig. 7C). However, neither mRNA nor protein levels of the well-known VDR were detected in HMCs (data not shown).

DISCUSSION

The researchers found that PI3K/Akt, as well as caspases and the Bcl-2 protein family, play a role in VD3-induced cell apoptosis in HMCs. The researchers discovered that VD3 activates the PI3K-dependent mitochondrial pathway in HMCs, causing cell apoptosis. Furthermore, the researchers demonstrate that JNK and ER activation occurs during an antagonistic effect between EGF and VD3 in HMCs.

VD3 has been shown to have anticancer and antifibrosis effects in numerous epidemiologic and experimental studies. These effects are essentially predicated on the reduction of cell proliferation and the activation of differentiation and death (Ma et al. 2006). An energy-dependent cascade of chemical processes is a key component of the extremely complex mechanism known as apoptosis. It is primarily mediated by three pathways. (1) perforin pathway, (2) death receptor pathway, and (3) mitochondrial pathway (Palai and Mishra 2015). Unknown processes underlie VD3-induced cell death. In prostate cancer LNCaP and ALVA-31 cells, VD3

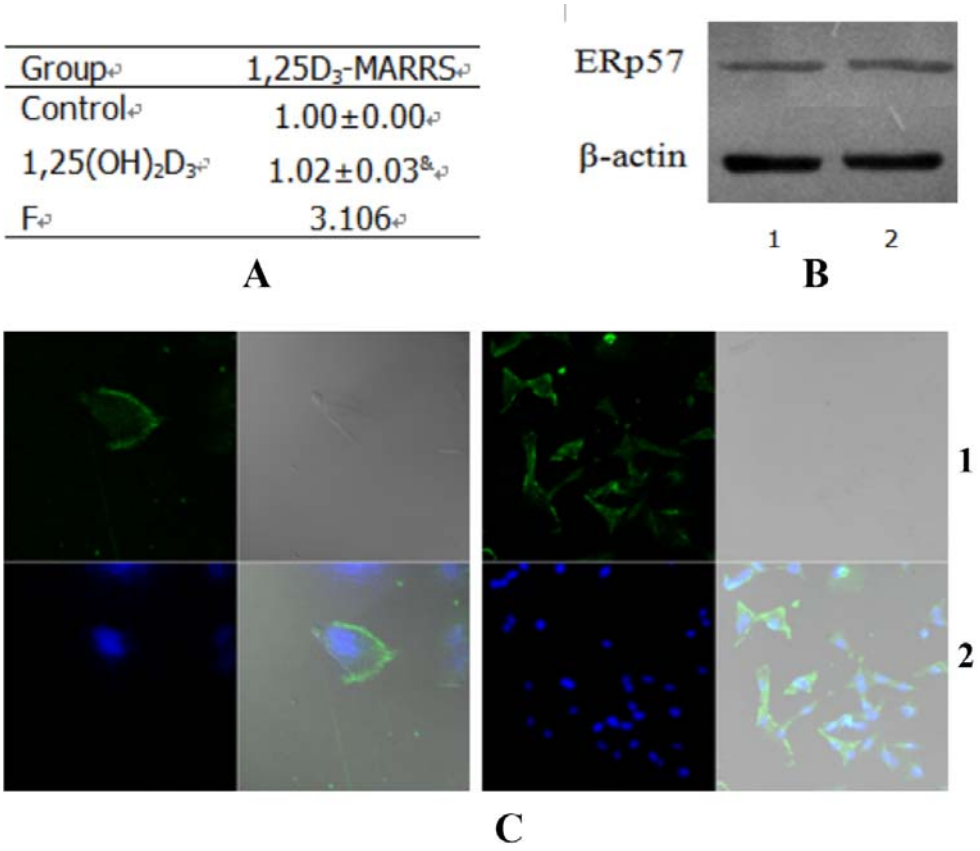

Fig. 7. The expression levels of 1,25MARRS in HMCs
(A) qRT-PCR;
(B) Western blot;
(C) Immunofluorescence
1: Normal control group; 2: 1,25(OH)$_2$D$_3$ group.
Data are shown as means ± SD(n=3)
$^{\&}p$ > 0.05 compared with normal control group

prevents the activation of anti-apoptotic Bcl-2 family member proteins and IAP proteins. It also causes caspase-3 and caspase-9 activation (Guzey et al. 2002). Through nongenomic activation of the PI3K/Akt and MAPK pathways, VD3 induces cell apoptosis in squamous cell carcinoma (SCC) cells. Furthermore, Akt activation inhibits VD3-induced cell apoptosis and promotes an earlier onset of apoptosis in SCC cells, whereas ERK is not involved in this apoptosis (Ma et al. 2006). However, by preventing caspase-8 activation and raising the Bcl-2/Bax ratio, VD3 prevents osteoblast apoptosis (Duque et al. 2004).

Both genomic and nongenomic mechanisms are used by VD3 to operate. The classic genomic action involves VD3 bidding to the VDR, which regulates target gene transcription. Recently, the 1,25D3-MARRS protein, a novel VD3 receptor, was discovered and described. However, its role in VD3-induced cell inhibition is still in its early stages. VDR was not found in HMCs, according to the data. The researchers looked at whether the nongenomic mechanisms that VD3 can act through are also involved in the cell apoptosis that compound causes in HMCs. Cell apoptosis depends

on the PI3K/Akt-mediated signal pathway. As a result, the researchers looked into the function of PI3K/Akt activation in the apoptosis phenomenon, specifically in HMCs. The researchers discovered that VD3 activated Akt via a PI3K-dependent pathway during HMC cell apoptosis. The researchers also discovered that LY294002 promoted VD3-induced cell apoptosis in HMCs by down-regulating Bad expression.

Akt may induce cell apoptosis via a variety of mechanisms that appear to be cell type and stimuli dependent. In mammals, Akt may modulate IAP family members to suppress cell death during endoplasmic reticulum stress (Hu et al. 2004). Akt, on the other hand, has been shown to phosphorylate and regulate post-mitochondrial proteins such as GSK-3, Bad, and Caspase-9. Furthermore, during cisplatin-induced apoptosis, it has been demonstrated that XIAP is phosphorylated by Akt at serine 87, which prevents further ubiquitination and auto-ubiquitination (Dan et al. 2004). In this study, VD3 induced cell apoptosis by promoting proteolytic processing of pro-caspase-9, decreasing Bcl-2 expression, and influencing Bax and Bad activation. According to the findings, VD3 activates a PI3K-dependent mitochondrial pathway for cell apoptosis in HMCs.

The researchers also show that EGF and VD3 have an antagonistic effect on cell apoptosis in HMCs. The mechanisms of the antagonistic effect appear to be cell type dependent and are not fully understood. VD3 inhibits the stimulatory effect of EGF on cell mitosis in Caco-2 cells by reducing VDR-mediated control of EGF receptor levels (Kallay et al. 1998). The researchers discovered that VDR was not present in HMCs in this study. Because EGF and VD3 can act through a variety of signal pathways, the researchers investigated whether these pathways could also play a role in the antagonistic effect in HMCs. According to the findings of this study, EGF can inhibit cell apoptosis in HMCs via a PI3K-dependent mechanism. However, VD3 effectively reverses this EGF inhibition. Furthermore, EGF appears to be cell type and stimulus dependent in its ability to regulate apoptosis via MAPK activation. EGF inhibits ceramide-induced cell apoptosis in renal proximal tubular cells by activating ERK and JNK (Iwayama and Ueda 2013). VD3 protects human HaCaT keratinocytes by inhibiting stress-induced activation of p38 MAPK and JNK rather than enhancing cellular survival pathways (Diker-Cohen et al. 2006). JNK and ERK activation was affected during an antagonistic effect between EGF and VD3, but not p38MAPK, in this study.

CONCLUSION

The researchers found that PI3K/Akt, caspase activation, and the Bcl-2 protein family all play roles in VD3-induced cell apoptosis in HMCs. VD3 induces cell apoptosis via a PI3K-dependent mitochondrial pathway in HMCs. Furthermore, the researchers demonstrate that JNK and ERK are activated during the antagonistic effect of EGF and VD3. To begin, the researchers must confirm the presence of 1,25MARRS in HMCs. More research is needed to see if 1,25MARRS influences HMCs' VD3-induced cell death.

RECOMMENDATIONS

To begin, the researchers must confirm the presence of 1,25MARRS in HMCs. More research is needed to see if 1,25MARRS influences VD3-induced cell apoptosis in HMCs.

ABBREVIATIONS LIST

1,25-(OH)2D3: 1,25-dihydroxyvitamin D3
ESRD: end-stage renal disease
MsPGN: Mesangial proliferative glomerulonephritis
HMCs: human mesangial cells
EGF: Epidermal growth factor
PI: Propidium iodide
PI3K: phosphoinositide3-kinase
JNK: c-Jun N-terminal kinases
IgG: immunoglobulin G
TUNEL: Terminal-deoxynucleoitidyl
RNA: RibonucleicAcid
DNA: deoxyribonucleic acid
RT- PCRÿReverse Transcription-Polymerase Chain Reaction
SD: standard deviation
ANOVA: Analysis of Variance
HMCs: human mesangial cell apoptosis
VDR: vitamin D receptor

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

There are no potential conflicts of interest to disclose.

FUNDING

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Not applicable.

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