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Effect of 1,25(OH)$_2$D$_3$ on the proliferation of human mesangial cells and their expression of Ki67

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ABSTRACT. Previous studies have found that 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$ or VD$_3$] exerts many biological effects, including the inhibition of cell proliferation and induction of apoptosis, but its mechanism of action remains unclear. The goal of our investigation was to explore the effects of 1,25(OH)$_2$D$_3$ on the proliferation of cultured human mesangial cells and their expression of Ki67 *in vitro*, and to establish its mechanism of action. Cultured human mesangial cells were randomly divided into the following four groups: normal control (N group; administered Dulbecco's modified Eagle's medium containing 5% fetal bovine serum), proliferation [epidermal growth factor (EGF) group; administered 10 μg/L EGF], VD$_3$ intervention [administered 10^{-8} M 1,25(OH)$_2$D$_3$], and proliferation and intervention [EGF+VD$_3$ group; administered 10 μg/L EGF and 10^{-8} M 1,25(OH)$_2$D$_3$]. Cells were incubated for 48 h with the corresponding treatment, and fluorescence immunocytochemistry and reverse transcription-

quantitative polymerase chain reaction were used to detect expression of Ki67 protein and mRNA, respectively. Compared to the N group, Ki67 levels were found to be higher in the EGF group but significantly lower in the VD$_3$ intervention group. Moreover, expression of Ki67 by cells in the EGF+VD$_3$ group was significantly lower than that of those in the EGF group. All of these differences were statistically significant (P < 0.05). In conclusion, 1,25(OH)$_2$D$_3$ inhibited Ki67 expression and the proliferation of human mesangial cells; therefore, Ki67 may be regarded as a potent therapeutic target in mesangial proliferative glomerulonephritis.

INTRODUCTION

Mesangial proliferative glomerulonephritis (MsPGN) is a common form of primary glomerulonephritis. MsPGN is characterized by the proliferation of mesangial cells (MCs) and the accumulation of mesangial matrix, leading ultimately to end-stage renal disease. Thus, there exists an urgent need to clarify the pathogenesis of this disease and develop an appropriate therapeutic strategy (Liu et al., 2011). Previous studies have reported expression of the proliferation markers Ki67, proliferating cell nuclear antigen, and topoisomerase IIα in both proliferative and non-proliferative cells in glomeruli (Yang et al., 2004). Ki67 protein indicates cellular proliferation, and has been used for the assessment of several cell types, including neural stem cells, tumor cells, and MCs (Nam et al., 2014; Takagi et al., 2014). Recent studies have suggested that 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$ or VD$_3$] inhibits proliferation of MCs and induces their apoptosis (Zhang et al., 2016). Moreover, it has been reported that 1,25(OH)$_2$D$_3$ can alleviate glomerulosclerosis and promote restoration of renal functions in the Thy-1.1 rat nephritis model (Panichi et al., 2001), but its mechanism of action is unclear. Here, we describe the effects of 1,25(OH)$_2$D$_3$ on the proliferation of human MCs and their expression of Ki67, providing a theoretical basis for the development of clinical treatments for proliferative glomerular diseases.

MATERIAL AND METHODS

Materials

Cell line

Human renal MCs (catalog No. 4200) were purchased from the Central Laboratory of Xiang Ya School of Medicine (Changsha, China).

Reagents

Dulbecco's modified Eagle's medium (DMEM; low glucose) and trypsin were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained

from Sijiqing Biotech (Hangzhou, China). Penicillin-streptomycin solution and DMEO were purchased from Solarbio (Beijing, China). Epidermal growth factor (EGF) and 1,25(OH)$_2$D$_3$ were supplied by Sigma (St. Louis, MO, USA). Mouse monoclonal anti-human β-actin and rabbit monoclonal anti-human Ki67 antibodies were produced by Cell Signaling Technology (Beverly, MA, USA). Goat anti-rabbit immunoglobulin (Ig)G and goat anti-mouse IgG secondary antibodies were purchased from ZSGB-Bio (Beijing, China). SYBR Green PCR Master Mix was manufactured by Applied Biosystems (Foster City, CA, USA).

Apparatus

An LSM 510 laser scanning microscope confocal microscope was purchased from Zeiss (Oberkochen, Germany). A quantitative polymerase chain reaction (qPCR) instrument and LightCycler 480 software were supplied by Roche (Basel, Switzerland).

Methods

Cell culture

Human MCs of passage 3-8 cultured in DMEM containing 10% FBS were used for experiments and maintained in a humidified atmosphere of 95% O$_2$ and 5% CO$_2$. The cell passage number was selected according to the stability of cellular physiological functions and protein expression (in preliminary tests, human MCs of passage 3-8 were sufficiently stable to be used in experiments). The cells were randomly divided into the following four groups: normal control (N group; administered DMEM containing 5% FBS), proliferation (EGF group; administered 10 μg/L EGF), VD$_3$ intervention [administered 10^{-8} M 1,25(OH)$_2$D$_3$], and proliferation and intervention [EGF+VD$_3$ group; administered 10 μg/L EGF and 10^{-8} M 1,25(OH)$_2$D$_3$]. Cells were incubated for 48 h with the corresponding treatment. All experiments were repeated thrice.

Ki67 immunofluorescence analysis

Cells were seeded on the tops of coverslips in six-well plates and cultured as described above. The culture medium was aspirated from each well and discarded when cells were approximately 80% confluent (2 x 10^5 cells). Cells on coverslips were then fixed with 4% paraformaldehyde and permeabilized with 3‰ Triton X-100 for 20 min. Following blocking, incubation with the primary antibody (diluted 1:500) was carried out at 4°C overnight. The fluorescein isothiocyanate-conjugated goat anti-rabbit IgG secondary antibody (diluted 1:200) was then incubated with the cells for 1 h at room temperature in the dark. With the exception of blocking, each step was followed by three 5-min washes with 0.01 M phosphate buffered saline. All samples were mounted using glycerol and imaged on a Zeiss LSM 510 META laser scanning confocal microscope for analysis of fluorescence intensity.

Reverse transcriptase-qPCR (RT-qPCR) analysis of Ki67 expression

To investigate expression levels of the gene encoding Ki67 in each group, total RNA was extracted using RNeasy Mini spin columns (QIAGEN, Toronto, Canada) following the

manufacturer's protocol, and reverse transcribed into complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized with the sense primer H-Ki67-Fx (5'-CCCCATTCACCCACCTTG-3') and the antisense primer H-Ki67-Rx (5'-TGATGTCACCTGGCTCCC-3'), yielding a product of 141 bp. The reaction program was as follows: 95°C for 3 min, then 40 cycles of 95°C for 15 s and 60°C for 40 s. qPCR was carried out using 96- or 384-well plates with β-actin as an internal control, and the products were analyzed with LightCycler 480 software. Semiquantitative analysis was performed to assess the expression level of Ki67/β-actin using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All data were processed by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) and measurement data are reported as means ± standard deviation. ANOVA was used to compare groups, while the least significant difference test was employed for multiple comparisons between groups. P values less than 0.05 were considered to represent statistical significance.

RESULTS

Morphology and number of human MCs in each group

Expression of Ki67 in MCs was measured using AIM software, based on laser scanning confocal microscopy of fluorescence intensity. Cells in the N group were transparent and fusiform, irregularly stellate, or dendritic in shape, with clear cytoplasm and nuclei. Cells in the EGF group exhibited similar morphological characteristics, but a greater number of cells were observed. Those in the VD_3 group demonstrated apoptosis, karyotheca shrinkage, and varied morphology. In addition, the number of cells was significantly lower compared to those in the N group. Cells of the EGF+VD_3 group were similar to those in the N group. This suggests that EGF can promote MC Ki67 expression and proliferation. In contrast, VD_3 is able to inhibit the expression of Ki67 and reverse the effects of EGF (Figure 1).

Normal control group   EGF (10ng/ml) group   1,25(OH)$_2$D$_3$(10^{-8}mol/l)group   EGF+1,25(OH)$_2$D$_3$ group
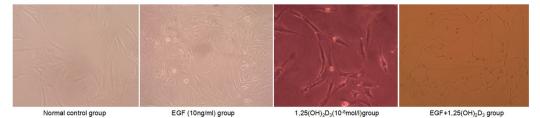
Figure 1. Morphology of human mesangial cells in different groups incubated for 48h (200X).

Ki67 immunofluorescence

The intensity of fluorescence associated with Ki67 in the N, EGF, VD_3, and EGF+VD_3 groups was 35.958 ± 2.563, 49.872 ± 2.745, 22.415 ± 2.277, and 36.567 ± 2.270, respectively (F = 123.429, P < 0.05). Cells in the EGF and VD_3 groups showed stronger and weaker fluorescence, respectively, than those of the N group. No significant difference was established

between the EGF+VD$_3$ and N groups in terms of fluorescence intensity. EGF+VD$_3$ cells demonstrated weaker fluorescence than that by EGF cells; however, the VD$_3$ and EGF groups did not significantly differ in this respect (Figure 2).
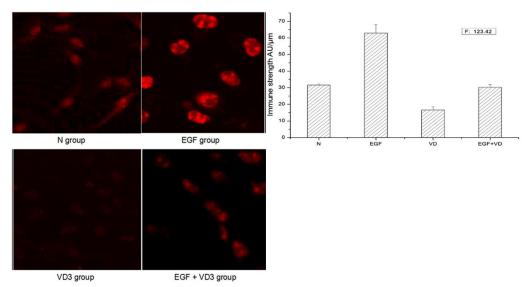

Figure 2. The immunofluorescence of Ki67 of cells in different groups (the red fluoresce indicated positive products located in cell nuclei, 400X).

RT-qPCR detection of Ki67

Ki67 mRNA was detected using RT-qPCR. Compared with that in the N group, Ki67 expression was significantly higher in the EGF group, but significantly lower in the VD$_3$ group ($P < 0.05$). Ki67 mRNA levels were similar in the EGF+VD$_3$ and N groups ($P > 0.05$), but significantly lower in the former than in the EGF group ($P < 0.05$). No significant difference was detected between the VD$_3$ and EGF groups in Ki67 mRNA expression (Table 1).

Table 1. Reverse transcriptase quantitative polymerase chain reaction analysis of Ki67 mRNA expression.

Group	Ct value	$2^{-\Delta\Delta Ct}$
N	34.263 ± 0.578	1.000
EGF	31.054 ± 2.252[a]	2.479[a]
VD$_3$	32.726 ± 0.182[a]	0.584[a]
EGF+VD$_3$	32.935 ± 0.266[b]	1.176[b]
P		<0.05

[a]$P < 0.05$ compared with the N group; [b]$P < 0.05$ compared with the EGF group. N = normal control; EGF = epidermal growth factor; VD$_3$ = 1,25(OH)$_2$D$_3$.

DISCUSSION

As the most biologically active metabolite of vitamin D$_3$, the steroid hormone 1,25(OH)$_2$D$_3$ is associated with multiple cellular functions, inducing cell cycle arrest, inhibition of cell division, and DNA breakage, thus preventing cell proliferation (Ramos-Martínez et al.,

2013; Liaudat et al., 2014). This molecule has been reported to be an effective immunomodulator that acts on MCs to inhibit their proliferation, decreases albuminuria and inflammatory cell infiltration, and accelerates repair of glomerular injury. For these reasons, $1,25(OH)_2D_3$ is used for the treatment of human proliferative glomerulopathy (Panichi et al., 2001).

Ki67 protein is a widely used cellular marker of proliferation present during all active phases of the cell cycle, but absent in resting cells (G0). Thus, Ki67 expression indicates entry into the cell cycle. Changes in Ki67 expression have been shown to signal excessive proliferation of cells during neoadjuvant endocrine therapy (Sundara Rajan et al., 2014) and the inhibition of proliferation by $1,25(OH)_2D_3$ (Urata et al., 2014). In the present study, the effects of $1,25(OH)_2D_3$ on Ki67 expression in human MCs were investigated. Immunofluorescence experiments suggested that human MCs treated with $1,25(OH)_2D_3$ had lower nuclear Ki67 levels than those of normal control cells. Similarly, RT-qPCR analysis demonstrated that Ki67 mRNA expression of MCs administered $1,25(OH)_2D_3$ was lower than that in the N group. Our results indicate that $1,25(OH)_2D_3$ restricted not only Ki67 protein production, but also Ki67 mRNA expression in inhibiting human MC proliferation. Thus, Ki67 can be regarded as a potent therapeutic target in MsPGN.

EGF is a multi-functional growth factor that stimulates cell growth and proliferation both *in vitro* and *in vivo*. Kumar et al. (2010) reported that EGF stimulates proliferation of MCs. Given the abnormal proliferation of these cells in MsPGN, using EGF, we constructed a microenvironment to promote the proliferation of human MC cultures in order to study the effects of $1,25(OH)_2D_3$ on this process. Our experiments showed that expression of Ki67 at both the protein and mRNA level was increased in the EGF group than the N group, suggesting that EGF can promote the expression of Ki67 in MCs. Furthermore, we established that Ki67 mRNA and protein levels were decreased in the EGF+VD$_3$ group, implying that $1,25(OH)_2D_3$ inhibited Ki67 expression in human MCs treated with EGF. It was also shown that $1,25(OH)_2D_3$ restricted the proliferation of EGF-treated human MCs by reducing Ki67 expression.

In conclusion, by limiting Ki67 expression, $1,25(OH)_2D_3$ inhibits the proliferation of human MCs, and can diminish the promotion of this process by EGF. However, the mechanism by which $1,25(OH)_2D_3$ acts on human MCs remains unclear and needs further investigation, including an examination of its possible influence on signaling pathways affecting proliferation of these cells. Future studies should focus on the effect of $1,25(OH)_2D_3$ on Ki67 expression *in vivo*.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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