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Original Article

Pyrrolidinedithiocarbamic Acid Ammonium Salt Inhibits Apoptosis and Phenotypic Transformation of Co-Culture of Myeloma Cells and Renal Tubular Epithelial Cells by Reducing the Secretion of Light Chain Protein

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Abstract

Background: We investigate the effects of NFκB inhibitor pyrrolidinedithiocarbamic acid ammonium salt (PDTC) on the viability, apoptosis and cell phenotype of HK-2 cells in the co-culture system of myeloma cells in renal tubular epithelial cells.

Methods: This study was performed in Qiqihar Medical University, *Qiqihar, China* from Jun 2018 to Jan 2019. RPMI-8226 cells and HK-2 cells were inoculated in the co-culture chamber and cultured to establish the co-culture system. An immunoturbidimetric assay was performed to detect κ light chain and λ light chain in RPMI-8226 cells. The effect of PDTC on the secretion of κ light chain and λ light chain of RPMI-8226 cells was detected by immunoturbidimetry and the ratio was calculated.

Results: PDTC significantly increased the viability of HK-2 cells. PDTC reduced the apoptosis of renal tubular epithelial cells. After PDTC treatment, the expression of cell surface marker E-cadherin decreased, and the expression of α-SMA increased, which induced the renal interstitial fibrosis. The secretion of κ light chain and λ light chain of RPMI-8226 cells was significantly decreased after the addition of PDTC, but the ratio was not changed.

Conclusion: PDTC can inhibit the cell activity, promote apoptosis, and reduce the secretion of secretion of κ light chain and λ light chain through inhibiting the NF-κB pathway activation of myeloma cell RPMI-8226.

Keywords: Myeloma nephropathy; Renal tubular epithelial cells; Light chain protein

Introduction

Multiple myeloma (MM) is a hematological malignancy that often occurs in the elderly. About 20%-40% patients with MM had different degrees of kidney damage when they were first diagnosed (1). When MM occurs, however, syntheses of both light and heavy chains are accelerated, resulting in a large amount of light chain proteins remaining in the blood system (2). The proximal renal tubules' scavenging ability to remove unused light chain proteins is thus overwhelmed,

forcing extra light chain proteins to enter into the distal tubules and form a large number of casts. Cast formation in the renal tubules increases the tubular pressure, and reduces the glomerular filtration rate, making it even more difficult to scavenge the extra light chain proteins. While the concentration of light chain proteins keeps increasing, a vicious circle is formed, leading to and further aggravating kidney damage (3).

According to a study (4), the cell viability of HK-2 cells treated with different MM cell lines was significantly decreased, and the apoptosis was significantly increased. It was reported that the light chain obtained in patients with end stage renal disease could enter HK-2 cells to produce sufficient amount of hydrogen peroxide to stimulate the production of monocyte chemoattractant protein-1 (MCP-1), while MCP-1 is a key chemokine for proximal tubule activation (5).

In addition to symptomatic supportive therapy and renal replacement therapy, anti-myeloma medications such as thalidomide, lenalidomide and bortezomib are often used in the treatment of myeloma nephropathy (6, 7). These medications are less effective (8) and have many side effects and drug resistance (9), in some refractory MM because they have no targets with specificity, leading to MM chemotherapy failure and recurrence. Increased activity of NF-κB was observed in myeloma cell lines and fresh bone marrow mononuclear cells from patients with myeloma, compared to normal bone marrow mononuclear cells (10), which may be related to the mutations of NF-κB (11), genetic polymorphisms (12), intercellular contact (6), and receptors capable of activating NF-κB which only exist in malignant plasma cells (13). NF-κB can play an anti-apoptotic role by up-regulating interleukin-1, interleukin-2, macrophage colony-stimulating factor and other anti-apoptotic factors (14). It has been found that α-terpineol can inhibit the proliferation of MM cell line RPMI-8226 by inhibiting NFκB (15).

This study explored the effects of pyrrolidinedithiocarbamic acid ammonium salt (PDTC), a specific inhibitor of NFκB, on a series of biological functions of myeloma cells and renal tubular epithelial cells in a co-culture system in order to provide a reference to clinical treatment of MM nephropathy by targeting NF-κB.

Materials and Methods

Materials and reagents

The materials and reagents used in this study were purchased from commercial sources: human proximal tubular epithelial cell line HK-2 and human myeloma cell line RPMI-8226 were purchased from ATCC cell bank; fetal bovine serum (FBS), DMEM cell culture medium, and PRIM1640 cell culture medium were purchased from Gibco; NF-κB inhibitor PDTC was purchased from Selleck (catalog #: S3633); MTT cell proliferation and cytotoxicity assay kit (catalog #: C0009), caspase-3 activity assay kit (catalog #: C1115) and BCA kit (catalog #: P0009) were purchased from Beyotime Biotechnology Co., Ltd.; 6-well plate, 24-well plate and co-culture chamber (0.4 μm pore size) were purchased from BD; dimethyl sulfoxide (DMSO) was purchased from sigma; apoptosis assay kit was purchased from Sungene Biotech Co. Ltd. (catalog #: AO2001-02P-H); caspase-3 (catalog #: 9662), B cell lymphoma 2 (bcl-2, catalog #: 2872), B cell lymphoma 2 associated X protein (Bax, catalog #: 2774), E-cadherin (catalog #: 3195), α-smooth muscle actin (α-SMA, catalog #: 19245), and IκBα (catalog #: 4812) were purchased from CST; internal reference GAPDH (catalog #: 10494-1-AP) and horseradish peroxidase (HRP)-labeled secondary antibody (catalog #: SA00001-2) were purchased from Proteintech Group Inc. Immunoturbidimetric assay was performed on a Beckman Array 360 automatic protein analyzer (Beckman, USA). Consumable reagents such as diluents, standards, buffers, etc. were all purchased from Beckman. A 10 mM stock solution of DMSO was prepared.

Cell cultures

HK-2 and RPMI-8226 cells were cultured respectively in DMEM and PRIM1640 media supplemented with 10% FBS. The cell cultures were maintained at 37 °C in a humidified incubator

supplied with 5% CO_2. The media were changed every 2 days. Cells were passaged when reaching 80% confluency.

Establishment and grouping of co-culture system of myeloma cells and renal tubular epithelial cells

A co-culture system was established by seeding RPMI-8226 cells at $1x10^5$ cells per well in the upper chamber of a 6-well transwell and seeding HK-2 cells at 3×10^5 cells per well in the lower chamber. The cells were divided into HK-2 alone culture group, HK-2 alone + PDTC culture group, RPMI-8226/HK-2 co-culture group, and RPMI-8226/HK-2 + PDTC co-culture group after cultured for 24 h. In the HK-2 alone culture group and HK-2 alone + PDTC culture group, HK-2 cells were seeded in the lower chamber, whereas in the upper chamber were added RPIM-1640 medium only for the HK-2 alone culture group and RPIM-1640 medium containing 25 μM PDTC for the HK-2 alone + PDTC culture group. In the RPMI-8226/HK-2 co-culture group and RPMI-8226/HK-2 + PDTC co-culture group, HK-2 cells were seeded at 3×10^5 cells per well in the lower chamber, and in the upper chamber were seeded RPMI-8226 cells at $1x10^5$ cells per well for both groups. In addition, a supplement of 25 μM PDTC was added into the upper chamber for the RPMI-8226/HK-2 + PDTC co-culture group.

MTT assay

Log phase RPMI-8226 cells were harvested by digestion with 0.25% trypsin. The cells were re-suspended in the medium and diluted to 3×10^4 cells per mL. To a 96-well plate was added the cell suspension at 100 μL per well. After the cells were attached, RPIM-1640 media containing a final concentration of 0, 10, 20, 30, 40, and 50 μM PDTC were added to sextuplicate wells. A blank well without cells was set for each concentration. The plate was incubated for 2 days, and cell viability was checked every 24 h. In the RPMI-8226/HK-2 co-culture group and RPMI-8226/HK-2 + PDTC co-culture group, RPMI-8226 cells were seeded at 5000 cells per well in the upper chamber of a 24-well plate. In the lower chamber of the 24-well plate, HK-2 cells were seeded at 5000 cells per well for the two co-culture groups as well as the two HK-2 alone culture groups. After the cells were attached, corresponding amount of the medium was added to the upper chamber for the HK-2 alone culture group and the RPMI-8226/HK-2 co-culture group. For the HK-2 alone + PDTC culture group and RPMI-8226/HK-2 + PDTC co-culture group, DMEM medium containing a final concentration of 25 μM PDTC was added to the upper chamber. Viability of the HK-2 cells in the lower chamber was measured after incubation for 24 h. The assay protocol was described below. MTT solution (5 mg/mL) was added to the plate, followed by incubation at 37 °C for 4 h. Then the culture solution was carefully aspirated, and 150 μL of DMSO was added. After shaking in the dark at room temperature for 10 min, absorbance of each well was measured using a microplate reader at 450 nm. Cell viability was calculated using formula: cell viability = (experimental well OD value – blank well OD value) / (control well OD value – blank well OD value) × 100%. IC_{50} was calculated with GraphaPad Prism software and was used as the drug concentration in the co-culture systems.

Immunoturbidimetric assay for light chain proteins

Concentrations of κ light chain and λ light chain in the RPMI-8226/HK-2 co-culture group and RPMI-8226/HK-2 + PDTC co-culture group were measured by scatter immunoturbidimeric assay using a Beckman Array 360 protein analyzer in accordance with the manufacturer's manual. The ratio of κ light chain to λ light chain κ/λ was calculated.

Effect of PDTC on apoptosis of renal tubular epithelial cells by flow cytometry

For all the 4 groups, after 24 h of incubation, supernatant in the lower chamber was aspirated, and cells were digested with trypsin. The cells were rinsed with PBS, followed by centrifugation at 1200 rpm to remove the supernatant. 1 mL of

1× binding buffer was added to the cells. The supernatant was aspirated after centrifugation at 1200 rpm. The cells were re-suspended in 1x binding buffer to get a concentration of $1x10^6$ cells/mL. To 100 μL of the cell suspension in each group was added 5 μL of Annexin V-FITC. After mixing, the mixture was incubated at room temperature for 10 min in the dark, followed by adding 5 μL of PI solution. After mixing, the mixture was incubated at room temperature for 5 min in the dark. PBS was added to get a total volume of 500 μL. After mixing, the mixture was subjected to flow cytometry. Apoptotic rate was sum of the early apoptotic rate (lower right quadrant) and the late apoptotic rate (upper right quadrant).

Total cell protein extraction and western blotting

Cells were harvested after centrifugation at 3000 rpm following tryptic digestion. Appropriate amounts of RIPA lysate and protease inhibitor were added, followed by ultrasound on ice for 5 min. After complete lysis, the mixture was centrifuged at 12000 g for 15 min in a low temperature centrifuge. The supernatant was collected, and 10 μL was used for total protein concentration measurement using BCA assay. To the remaining supernatant was added 5× loading buffer, followed by heating at 100 °C for 10 min. Equal amounts of total proteins for the 4 groups were loaded on gels containing 5% stacking gel and 10% separation gel for electrophoretic analysis. The gels were run at a constant voltage of 80 V until bromophenol blue entered the stacking gel with minimal distortion of the bands, when the voltage was changed to 120 V until the target bands were separated. The protein bands were transferred from gel to PVDF membrane by wet transfer method under a constant current of 275 mA for 80 min. After the membrane was blocked in TBST buffer supplemented with 5% milk at room temperature for 2 h, corresponding diluted primary antibody (dilution factor: 1:1000) was added. For cells in the upper chamber, levels of IκB protein were measured to assess the NF-κB pathway activation. For cells in the lower chamber, levels of caspase-3, bcl-2, Bax, E-cadherin, and α-SMA were measured. The membrane was incubated at 4 °C overnight within the corresponding primary antibody. After washing, the secondary antibody supplemented with 2% milk was added, followed by incubation at room temperature for 1 h. After development, the image was analyzed using Image J software for gray values of the bands. GAPDH was used as an internal reference. The ratio of the gray value of the target protein to the gray value of GAPDH was regarded as the expression level of that protein.

Caspase-3 Activity Assay

Caspase3 activity detection kit was used. *p*NA standard curve was measured, and then 100 ul cell lysate was added to every 2×106 cells after treatment, and the supernatant was taken after ultrasonic lysis. Forty ul of buffer solution, 10 ul of Ac-DEVD-pNA (2 mM) and 50 ul of sample to be tested were added into the 96-well plate, and a blank control group was set to exclude the background absorption value. The mixture was incubated at 37 ℃ for 2 h, and OD value of the sample was detected with a microplate analyzer at the wavelength of 405 nm. The standard curve was used to calculate how much *p*NA was catalyzed in the sample, which was expressed as multiple of the control group.

Statistical Analysis

The SPSS 20 (Chicago, IL, USA) software was used for statistical analysis. Data were expressed as mean ± standard deviation. The paired *t* test was used for comparison between groups. A difference was statistically significant when $P<0.05$. Each test was repeated more than three times.

Results

Effect of NF-κB inhibitor PDTC on the activity of HK-2 in co-culture system

As shown in Fig. 1a, PDTC had a dose- and time-dependence on the inhibition of RPMI-8226 cell. The half-inhibition concentrations (IC_{50}s) of PDTC calculated by software were 25.59 μM and 3.03μM, respectively, for 24 h and

48 h. The concentration of 25 μM was thus chosen for subsequent experiments.

As shown in Fig. 1b, the activity of HK-2 cells in co-culture system was significantly decreased after the co-culture with RPMI-8226 (P<0.05), but activity of HK-2 cells was significantly increased after the addition of PDTC in co-culture system.

Effect of PDTC on apoptosis and cell phenotype transformation of HK-2 cells in co-culture system

Compared with the single culture group, the apoptosis rates of HK-2 cells and RPMI-8226 cells in co-culture group were significantly increased (P<0.05); compared with the co-culture group, the apoptosis rate of HK-2 cells in co-culture + PDTC group significantly decreased (P<0.05) (Fig. 2). Compared with single HK-2 cell culture group, the activity of caspase3 in HK-2 cells in co-culture system was significantly increased, and the ratio of bcl2 to bax was significantly decreased (P<0.05). Compared with the co-culture group, the activity of caspase3 in HK-2 cells decreased significantly after the addition of PDTC in the co-culture system, and the ratio of bcl2 to bax was significantly increased (P<0.05). The E-cadherin on the surface of HK-2 cells decreased significantly after the PDTC treatment in the co-culture group, and α-SMA increased significantly (P<0.05). PDTC could induce morphological changes of renal epithelial cells and produce renal interstitial fibrosis.

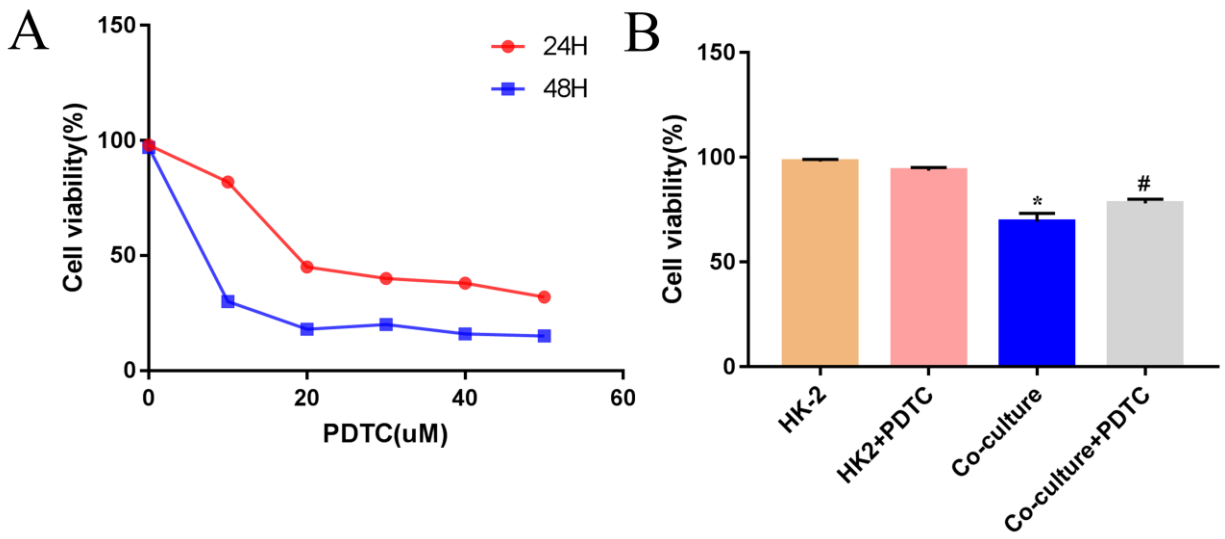

Fig. 1: Effect of PDTC on on the cell activity of RPMI-8226 and HK-2

A. Effects of different concentrations of PDTC on RPMI-8226 cell activity at different time. B. Effects of PDTC on the cell activity of HK-2 in single culture and co-cultured systems. *P<0.05, compared with the HK-2 alone culture group; #P<0.05, compared with the RPMI-8226/HK-2 co-culture group

Effect of PDTC on RPMI-8226NFκB pathway in myeloma cells

IκB protein levels were significantly increased after 24 h of 25 umol/L PDTC treatment, and the relative expression increased from 0.70±0.07 to 0.99 ± 0.02, with statistically significant difference (P<0.05). PDTC can increase the IκB protein of myeloma cell RPMI-8226 in the co-culture system, thereby inhibiting the activation of NFκB pathway (Fig. 3, 4).

Effect of PDTC on secretion of light chain proteins in myeloma cells

As shown in Table 1, levels of both κ light chain and λ light chain were reduced in the presence of 25 μM PDTC for 24 h. The differences were 8.30±1.39 and 0.91±0.35, respectively, and both were statistically significant (P<0.05).

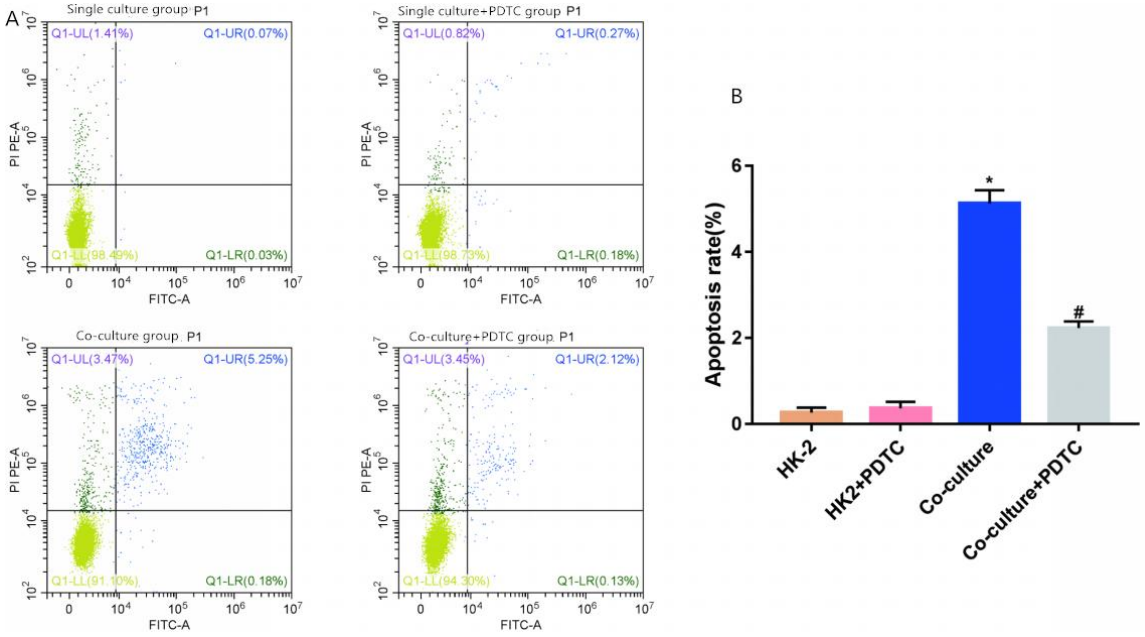

Fig. 2: Effect of PDTC on apoptosis of renal tubular epithelial cells HK-2 in each group

A. Flow cytometry detects typical scatter plots of apoptosis in single culture group, single culture+PDTC group, co-culture group, co-culture+PDTC group. After the cells of all four groups were cultured for 24 h, Annexin V- FITC method showed that the apoptosis rate was the sum of early apoptosis (lower right quadrant) and late apoptosis (upper right quadrant). B. The histogram of the apoptosis rate of single culture group, single culture+PDTC group, co-culture group, co-culture+PDTC group

**P*<0.05 compared with the single culture group; #*P*<0.05 compared with the co-culture group

Table 1: Effect of PDTC on κ light chain level, λ light chain level and their ratio κ/λ in myeloma cells

Group	*κ light chain level*	*λ light chain level*	*κ/λ*
RPMI-8226/HK-2 co-culture group	16.45±1.62	1.70±0.23	9.85±2.02
RPMI-8226/HK-2 + PDTC co-culture group	7.92±1.04*	0.77±0.15*	10.67±2.72

* *P*<0.05, compared with RPMI-8226/HK-2 co-culture group

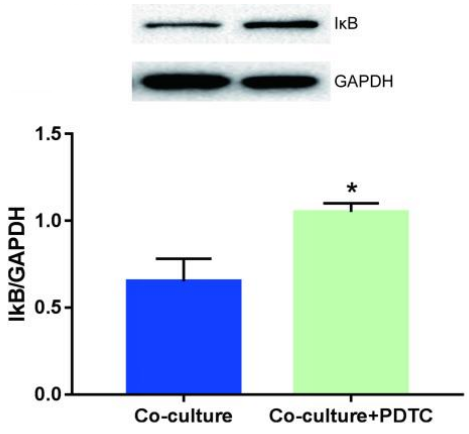

Fig. 3: Western blot analyses of related proteins

a, b apoptosis-associated proteins; c, d cell surface marker proteins

**P*<0.05 compared with the co-culture group

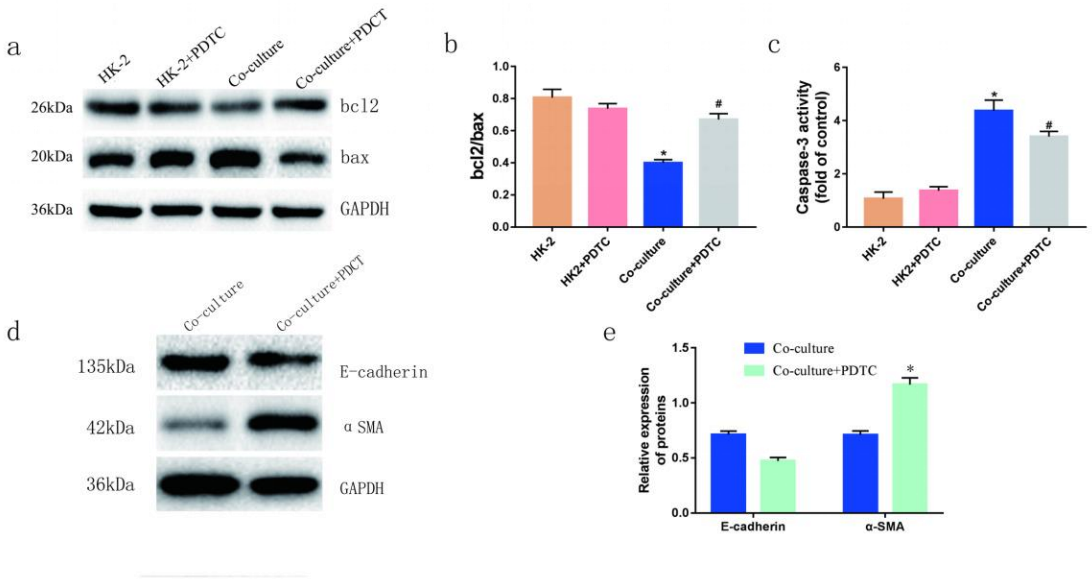

Fig. 4: Effect of PDTC on apoptosis protein and cell surface marker protein

A. Western blot was used to detect the effect of PDTC on the expression of apoptosis genes Bcl2 and Bax in single culture group, single culture+PDTC group, co-culture group, co-culture+PDTC group. GAPDH is the internal reference gene

B. Histogram of the relative expression of Bcl2 protein and Bax protein. The ratio of the co-culture group was significantly lower than that of the single culture group. The ratio of co-culture + PDTC group was higher than that of the co-culture group. Compared with the single culture group, * P<0.05; compared with the co-culture group, #*P*<0.05

C. Histogram of Caspase-3 enzyme activity of cells in single culture group, single culture+PDTC group, co-culture group, co-culture+PDTC group. Caspase-3 enzyme activity was detected by spectrophotometry. A microplate reader was used to detect the absorbance of the sample at 405 nm. The Caspase-3 enzyme activity in the co-culture group was higher than that in the control group, and the activity in the co-culture + PDTC group was lower than that in the co-culture group. Compared with the single culture group, **P*<0.05; compared with the co-culture group, #*P*<0.05

D. Western blot was used to detect the expression of cell surface markers E-cadherin and α-SMA in the single culture group, single culture + PDTC group, co-culture group, co-culture + PDTC group

E. Histogram of relative expression levels of E-cadherin and α-SMA protein in co-culture group and co-culture + PDTC group. Compared with the co-culture group, the expression of E-cadherin in the co-culture + PDTC group decreased and the expression of α-SMA increased. Compared with the co-culture group, **P*<0.05

Discussion

In the United States, MM has become the second most common blood cancer (16). It is characterized by Bence Jones protein (BJP). It can freely pass through the glomerular filtration membrane due to its small molecular weight. It was believed that kidney damage due to BJP was associated with BJP's catalytic activities (17-19). Therefore, it was postulated that inhibition of BJP's catalytic activities would attenuate BJP-mediated apoptosis (20). K light chain protein can enter the nucleus and activate lysosomes, and eventually leading to apoptosis (21). In this study, HK-2 were co-cultured with RPMI-8662. It was found that the light chain proteins produced by myeloma cells can mediate apoptosis of HK-2. The underlying mechanism may be related to the catalytic activity of light chain proteins.

Light chain proteins in urine can cause damage to renal tubular epithelial cells, thereby aggravating renal interstitial lesions (22). Damaged tubular epithelial cells secrete large amounts of pro-fibrotic factors, which promote fibrosis formation (23). In this process, damaged tubular epithelial cells promote expression of key genes such as α-SMA and snail (24, 25). In this study, the E-cadherin was down regulated, while the interstitial cell surface marker α-SMA was up-regulated when HK-2 cells were co-cultured with RPMI-8226 cells. This finding indicated that light chain proteins could mediate cell phenotype transformation.

Activation of NF-κB pathway was observed in myeloma cell lines and myelomonocytes of patients with myeloma (10, 15). When cells are stimulated by external stimuli, IκB phosphorylation happens, releasing the NF-κB dimers from their inhibitory proteins and activating NF-κB. The NF-κB complexes then enter into the nucleus to exert their biological effects. In this study, the IκB level in RPMI-8226 cells was significantly increased after the cells were treated with PDTC. The addition of PDTC to the upper chamber in the HK-2 alone culture group did not cause any changes of apoptosis-associated proteins in the HK-2 cells. However, when PDTC was added to the upper chamber in the RPMI-8226/HK-2 co-culture group, the apoptotic rate of HK-2 cells was reduced, and levels of E-cadherin and α-SMA were reversed to some extent. Secretion of light chains was significantly reduced. These findings suggested that the NF-κB pathway in RPMI-8226 cells was inhibited by PDTC, leading to increased apoptosis, decreased secretion of light chain proteins, and less effect on HK-2 cell apoptosis and cell phenotype transformation. A study (4), also found that when HK-2 cells were treated with RPMI-8226 cell culture medium, E-cadherin of HK-2 cells increased, while Vimentin decreased, which was not consistent with the results of this study. We speculate that it may be caused by some signal communication between the two kinds of cells in the co-culture system used in this study.

In the culture medium of RPMI-8226 cells, there was only one factor secreted by cells, lacking the signal molecule produced by the communication between the two cells.

Conclusion

PDTC inhibited activation of the NF-κB pathway in RPMI-8226 cells, leading to increased apoptosis, reduced secretion of light chain proteins, and decreased effect on apoptosis and cell phenotype transformation of HK-2 renal tubular epithelial cells.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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