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Original Article

Establishment of stable multiple myeloma cell line with overexpressed PDCD5 and its proapoptosis mechanism

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Abstract: Objective: The transfected multiple myeloma cell line showing a stable doxycycline (DOX)-induced expression of PDCD5 was established. PDCD5 overexpression in the transfected cell line was analyzed for its effect on the dexamethasone (DXM)-induced apoptosis along with a discussion on the mechanism. Methods: (1) Lentiviral plasmid was used for the transfection of PDCD5 gene into the multiple myeloma cells. The screening was done by applying puromycin, and PDCD5 expression was induced by DOX. Real-time fluorescence quantitative PCR and Western Blot were performed to detect the expression levels of the target gene in the stable transfection group and the empty vector group; (2) The cell apoptosis rates of stable transfection group, blank group and empty vector group were measured by Annexin-APC/PI double staining flow cytometry; (3) Real-time fluorescence quantitative PCR and Western Blot were carried out to detect the expression levels of survivin, casepase-3 and Bcl-2 genes and proteins. Results: PDCD5 expression was significantly increased in the stably tranfected multiple myeloma cells compared with blank group and empty vector group. The cells in the transfection group were more sensitive to DXM, and the proportion of apoptotic cells was obviously higher than that of the blank group and the empty vector group ($P<0.05$). Survivin and Bcl-2 were considerably downregulated in U266/PDCD5 cells and combined DXM group than in the single agent group. However, caspase-3 was significantly upregulated. Conclusion: Multiple myeloma cell line transfected with endogenous PDCD5 gene was established. The endogenous PDCD5 overexpression accelerated the cell apoptosis under DXM induction. The proapoptotic action of PDCD5 gene had the effect of activating casepase-3 and downregulating survivin and Bcl-2, which further promoted the apoptosis of multiple myeloma cells.

Keywords: Programmed cell death 5 (PDCD5), lentivirus, dexamethasone (DXM), multiple myeloma, cell apoptosis

Introduction

Multiple myeloma (MM) is a clonal plasma cell proliferative malignancy with the second highest incidence of all hematological diseases. In the face of growing incidence every year, much progress has been made in the battle against MM, but MM still remains incurable. As indicated by many studies, apoptosis disorder is the major pathogenic mechanism of MM [1]. The onset, progression, and prognosis of MM are all associated with apoptosis disorder [2]. Programmed cell death 5 (PDCD5) is an important apoptosis-related protein discovered by Peking University Center for Human Disease Genomics. Found to be expressed abnormally in a variety of diseases such as tumors and rheumatism [3-6], PDCD5 can act in synergy with several agents to promote cell apoptosis [7-9]. We found through preliminary experiments that PDCD5 was downregulated in MM cells [10]. Adenovirus and liposomes were used for the transfection of exogenous PDCD5 gene into MM cells, but transfection efficiency tended to be low. The defects such as unstable target gene expression and deletion of PDCD5 gene in the passaged cells make it difficult to investigate the proapoptotic mechanism of PDCD5. In light of this, lentiviral plasmid was used to transfect the MM cells. The transfection of PDCD5 gene was proved stable, and the effects of PDCD5 on DXM-induced apoptosis of MM cells were observed to gain an understanding on the mechanism of proapoptotic activity of PDCD5.

Materials and methods

Materials

Myeloma cell line U266 was isolated from a patient with MM and donated by Molecular

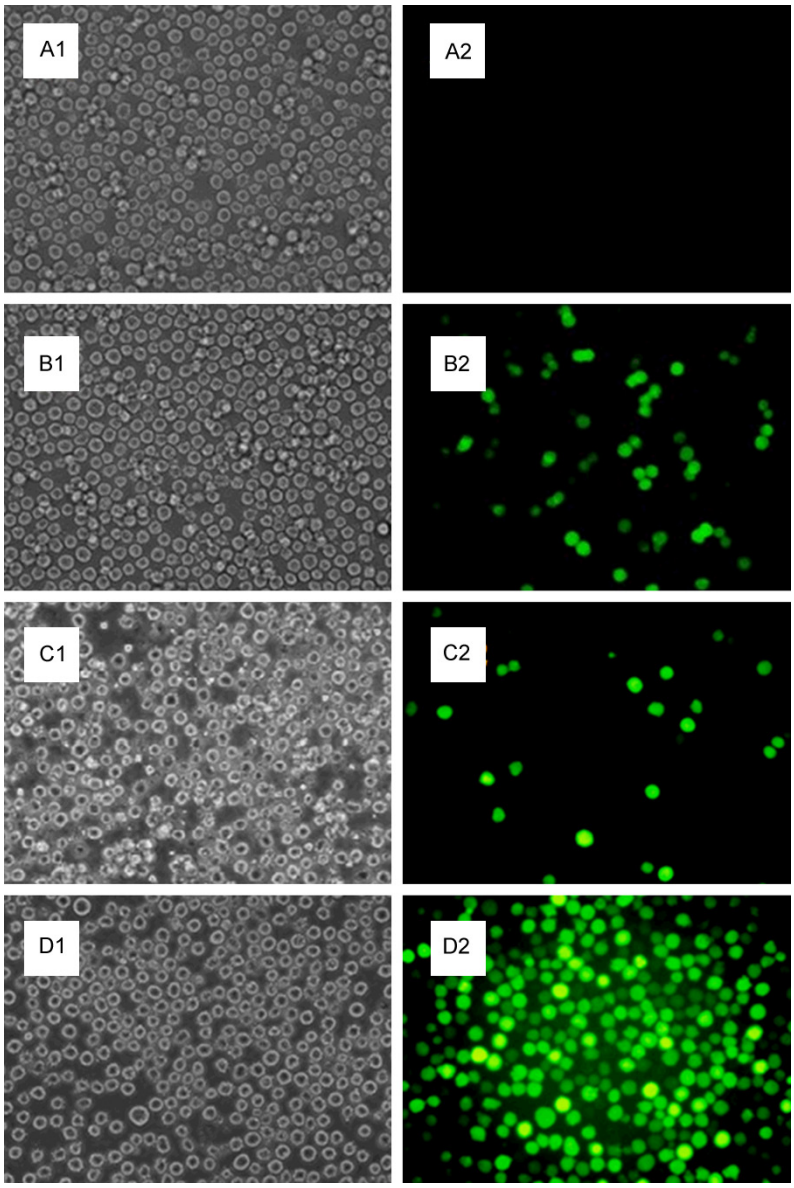

Figure 1. Microscopic observation before and after the screening of transfected cells with PDCD5 expression induced by DOX (20×). A. Non-transfected group; B. Empty vector group; C. Before the screening of transfected cells; D. After the screening of transfected cells. 1: Under the ordinary microscope; 2: Under the fluorescence.

Biology Laboratory of Central South University. Lentiviral plasmid was donated by Prof. Yang Hua from American University. Cell culture medium was purchased from Hyclone (USA); doxycline, polybrene and puromycin, Sigma (USA); Annexin V-APC/PI double staining flow cytometry assay kit, BD Company (USA); RNA extraction kit and reverse transcription kit, Fermentas Inc. (USA); anti-PDCD5, anti-Bcl-2, anti-caspase-2 and anti-β-actin antibodies, Cell Signaling Technology, Inc (USA).

Cell culture

U266 cells were cultured in 1640 medium containing 10% fetal bovine serum at 37°C using a 5% CO_2 incubator. The medium was replaced and the cells were passaged once every 2 days. The cells showing good growth were taken from the logarithmic phase, and corresponding treatments were administered after the cells grew to the specified number.

Detection of target gene expression by RT-qPCR

A total of 1×10^6 cells were collected for total RNA extraction using TRIzol reagent (Fermentas RNA Extraction Kit). The total extracted RNA was subjected to cDNA reverse transcription and then RT-qPCR. The conditions of RT-qPCR were as follows: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 seconds, annealing at 59°C for 50 seconds, extension at 59°C for 50 seconds, 40 cycles, then final extension at 60-95°C. The melting curves were plotted using β-actin as internal reference. Each sample had 3 replicates.

Detection of target protein expression by western blot

After the collection of 1×10^6 cells, protein lysis buffer was added to perform total protein extraction and protein concentration was measured. For electrophoresis analysis, 40 ug total protein was used. The products were transferred to membrane and incubated with primary antibodies and secondary antibodies (PDCD5 at a dilution of 1:200, β-actin 1:4000, Bcl-2, casepase-3, survivin 1:1000, secondary antibodies 1:3000), respectively. The membranes were washed before development, and the

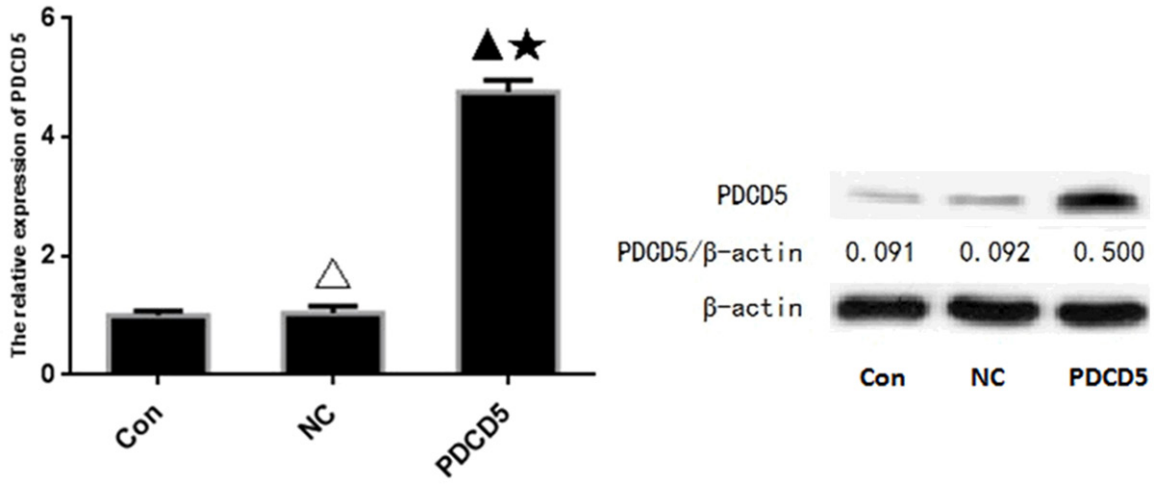

Figure 2. Detection of PDCD5 expression by qRT-PCR and Western blot. Left is the Mrna expression of PDCD5, and right the protein expression of PDCD5. ΔIndicates no significant differences between NC group and Con group, $P>0.05$. ▲Indicates significant differences between PDCD5 group and Con group, $P<0.05$. ★Indicates significant difference between PDCD5 group and NC group, $P<0.05$.

results were analyzed by using IPP6.0 software.

Cell transfection and screening

U266 cells were divided into control group (Con), negative control group (NC) and transfected group (PDCD5). Following inoculation to 6-well plate at the density of 5×10^5/ml, the cells were transfected with lentiviral plasmid containing or not containing the target gene. The medium containing 0.5 μg/ml puromycin was used for screening after co-culture for 48 h. The medium was replaced once 1-2 d to continue pressurized screening for a total of 10 d.

Induction of target gene expression

Into each group complete medium containing 2.0 μg/ml DOX was added. The medium was replaced after 48 hours of co-culture. Photos were taken under the fluorescence microscope and ordinary microscope for the same field of vision. Then the cells were collected and subjected to qT-PCR and Western blot to determine PDCD5 expression.

Detection of DXM-induced cell apoptosis by double staining flow cytometry

An equal volume of PBS was added into Con group, while for group Con+DXM, NC+DXM and PDCD5+DXM, complete medium containing 7.9 μg/ml DXM was added. The cells were cultured under the same conditions for 48 h, and 5×10^5 cells were collected from each group. For cells of each group, 500 μl of Bing Buffer, 5 μl of Annexin V-APC and 5 μl of PI were added successively. After cell cultures at room temperature for 15 min away from light, the cells were analyzed by a flow cytometer (BD Company, USA).

Detection of apoptosis-related genes

Expressions of survivin, casepase-3 and Bcl-2 genes were detected by qT-PCR and Western Blot.

Statistical analyses

Each experiment was repeated for at least three times to ensure the repeatability of the experiment results. All measurement data were expressed as X ± S, and data analyses were performed using SPSS 19.0 software. The significance level was set as 0.05. LSD-T test was used for intergroup comparison.

Results

Screening of U266 cells stably transfected with PDCD5 gene

For PDCD5 group, the medium was replaced and 2 μg/ml puromycin was applied for screening for 10 d after 48 hours of transfection. On day 3 of screening, all cells in Con group died and 90% of the cells in PDCD5 group died. The

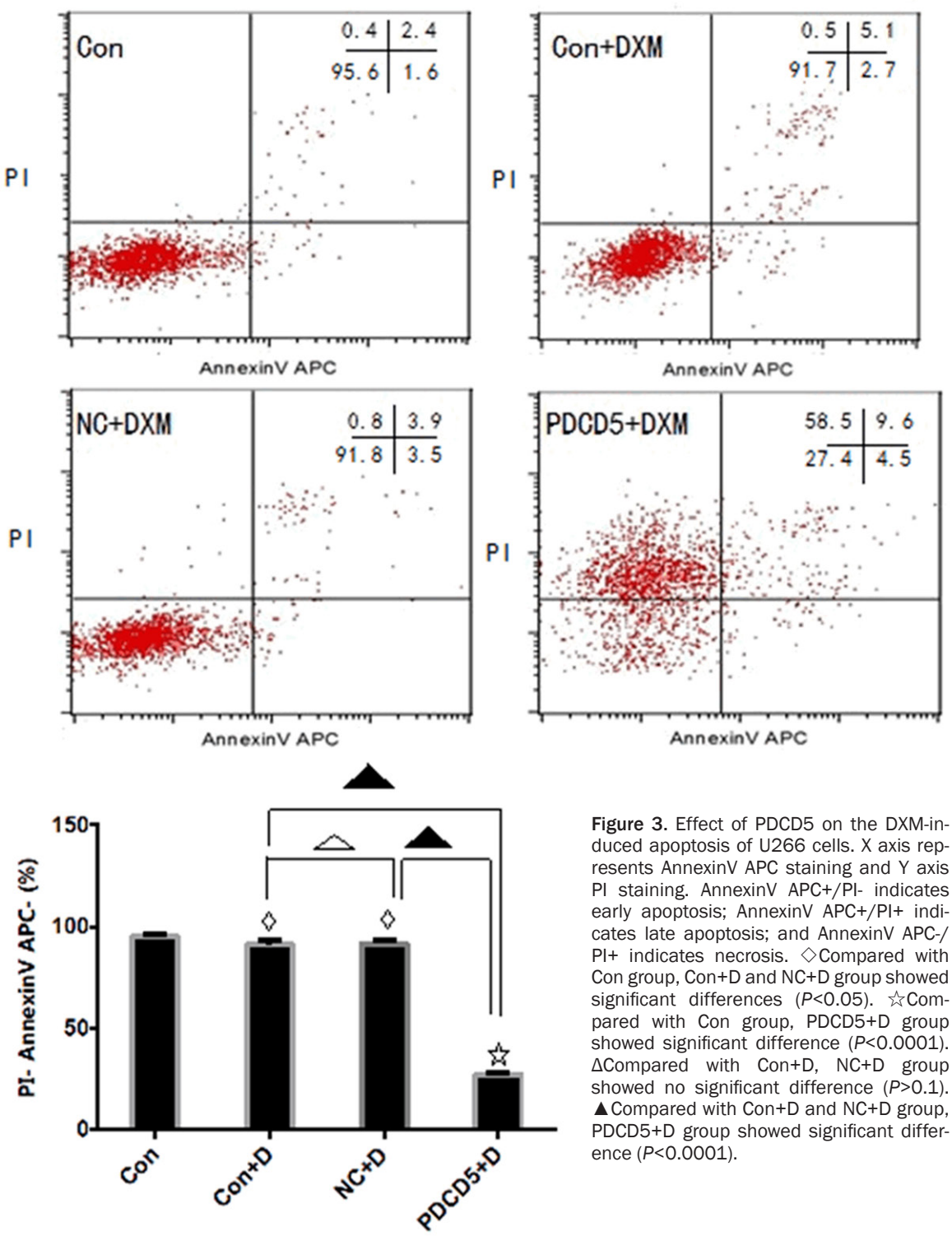

Figure 3. Effect of PDCD5 on the DXM-induced apoptosis of U266 cells. X axis represents AnnexinV APC staining and Y axis PI staining. AnnexinV APC+/PI- indicates early apoptosis; AnnexinV APC+/PI+ indicates late apoptosis; and AnnexinV APC-/PI+ indicates necrosis. ◇Compared with Con group, Con+D and NC+D group showed significant differences (P<0.05). ☆Compared with Con group, PDCD5+D group showed significant difference (P<0.0001). ΔCompared with Con+D, NC+D group showed no significant difference (P>0.1). ▲Compared with Con+D and NC+D group, PDCD5+D group showed significant difference (P<0.0001).

surviving cells were resistant to puromycin, and these cells were successfully transfected with PDCD5 gene. On day 5-7, the survival cells in PDCD5 group began to proliferate. On day 9, the cells in PDCD5 group grew to 90% confluence and showed good growth. When using 2 μg/ml DOX to induce PDCD5 expression, GFP fluorescence could be clearly seen under the inverted fluorescence microscope (**Figure 1**), which indicated stable transfection.

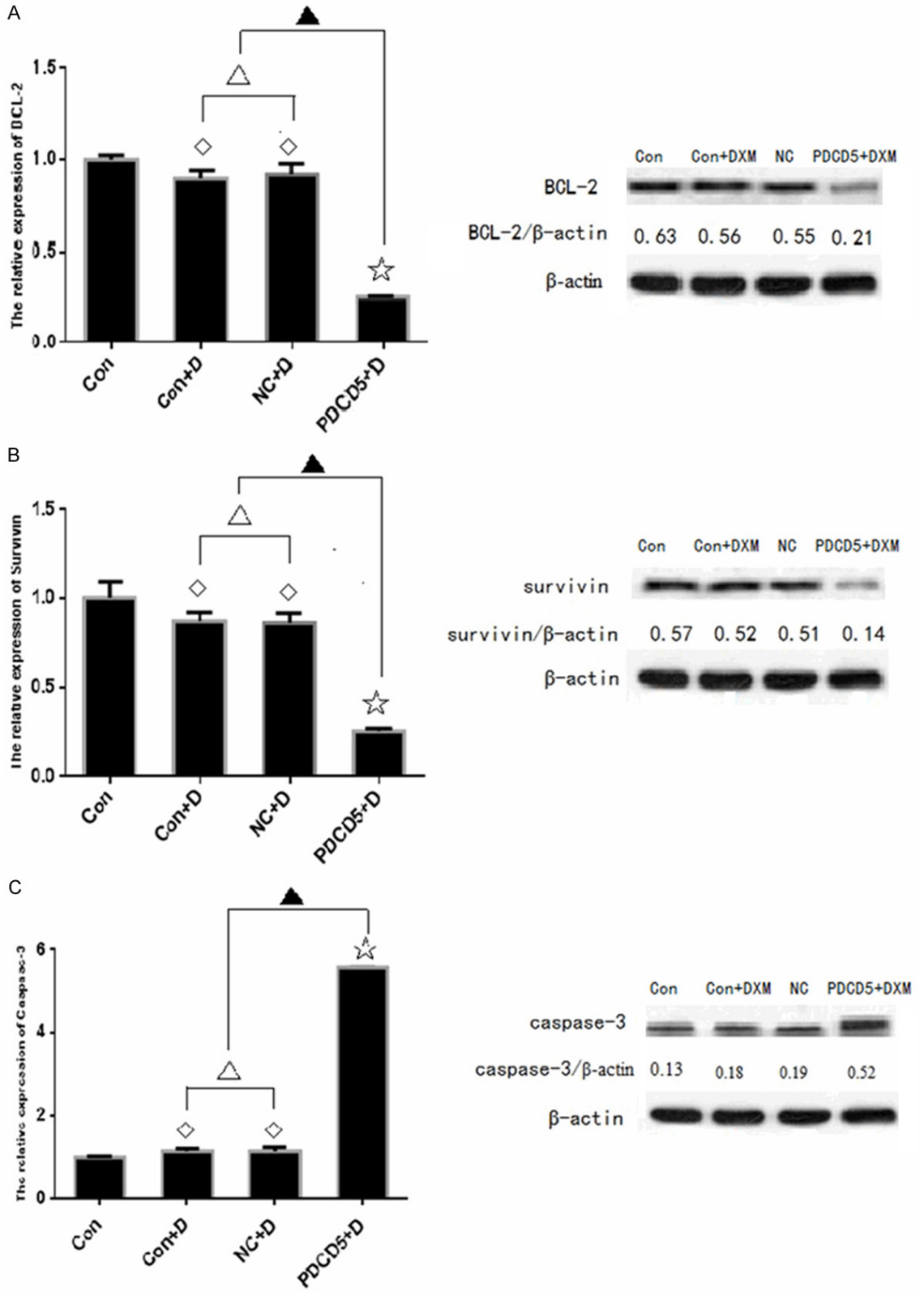

Figure 4. Detection of target gene expression in each group by qRT-PCR and Western blot. A-C are the mRNA and protein expressions of Bcl-2, survivin and caspase-3 in each group, respectively. Left is mRNA expression, and right protein expression. ◇Compared with Con group, the mRNA expressions of Con+D and NC+D group showed significant differences ($P<0.05$). ☆Compared with Con group, PDCD5+D group showed significant difference ($P<0.0001$). ΔCompared with NC+D group, the mRNA expression of Con+D group showed significant difference ($P>0.1$). ▲Compared with Con+D and NC+D group, PDCD5 group showed significant difference ($P<0.0001$).

Detection of PDCD5 expression

The mRNA and protein expressions of PDCD5 were detected in each group using qRT-PCR and Western Blot. As shown in **Figure 2**, the two detection methods produced consistent results. There were no significant differences between NC group and Con group, whereas the mRNA and protein expressions of PDCD5 group were greatly increased compared with Con group ($P<0.05$).

Effect of PDCD5 on DXM-induced apoptosis of U266 cells

The effect of PDCD5 on DXM-induced apoptosis of U266 cells was characterized through cell apoptosis rate using Annexin-APC/PI double staining flow cytometry after DXM treatment (7.9 μg/ml) for 48 h. As shown in **Figure 3**, the living cell rate of Con group, Con+DXM group, NC+DXM group and PDCD5+DXM group was 96%±0.5%, 91.7%±2.0%, 91.8%±1.7% and 27%±0.67%, respectively. Compared with non-transfected group, all DXM treatments promoted the apoptosis of U266 cells ($P<0.05$), and PDCD5+DXM group had the highest apoptosis level ($P<0.0001$).

Mechanism of the effect of PDCD5 on the DXM-induced apoptosis of U266 cells

The mechanism of the proapoptotic action of PDCD5 in U266 cells under the induction by DXM was further investigated. After 48 hours of DXM treatment, the expressions of survivin, casepase-3 and Bcl-2 genes and expressions were detected by RT-PCR and Western Blot, respectively. The results are shown in **Figure 4**. Compared with other groups, survivin and Bcl-2 were obviously downregulated in PDCD5+DXM group ($P<0.05$), while casepase-3 was significantly downregulated ($P<0.05$). Pairwise comparison did not reveal significant differences between Con group, Con+DXM group and NC+DXM ($P>0.1$).

Discussion

Apoptosis disorder is one important pathogenic mechanism of MM [1]. PDCD5 gene is a novel apoptosis-related gene which shows a high expression in the early apoptosis of cells and promotes cell apoptosis. Our earlier findings that PDCD5 gene was lowly expressed in MM cells [10] prompted the use of lentiviral plasmid for the transfection of PDCD5 gene into MM cells. We attempted to reveal the mechanism of the proapoptotic activity of PDCD5 through the detection of apoptosis-related genes.

Lentiviruses are the tool for high-efficiency and high-stability transfection that is currently in use [11]. Compared with ordinary plasmids, liposomes and adenoviruses, lentiviruses can effectively infect non-periodic and post-mitotic cells. By integrating itself into the host genome, lentiviruses achieve a continuous, stable expression of the exogenous genes [12] and cause no immunoreactivity and damage to host cells [13, 14]. The lentivirus inducible expression vector was used to transfer the exogenous gene for its advantages in transfection stability and the specificity of gene silencing. The use of DOX and other analogs for the regulation of PDCD5 gene expression overcame the defect of uncontrollability of target gene carried in the vector. We first performed transfection of PDCD5 gene into U266 cells using lentivirus and then the stably transfected cells were selected by using puromycin. For these cells, EGFP expression was induced by DOX, and the fluorescence emitted by EGFP was observed under the inverted fluorescence microscope. The transfected cells showed a significant upregulation of PDCD5, and the deletion of PDCD5 gene did not happen after cell passage. We successfully established the transfected MM cells with a stable, high expression of PDCD5.

As an important apoptosis-related gene [15], PDCD5 was shown to be downregulated considerably in a variety of tumors [16, 17]. Fan, GL et al. suggested the role of PDCD5 as an effective apoptosis promoter rather than as an apoptosis inducer. Although PDCD5 alone did not produce a significant impact on cells, its synergy with proapoptotic factors could effectively promote cell apoptosis [18-20]. We detected cell viability through flow cytometry in Con, NC and PDCD5 group after DXM treatment. It was found that the proportion of apoptotic cells in PDCD5 group was obviously higher compared with that in Con and NC group. We can reasonably infer that PDCD5 possesses a remarkable proapoptotic effect. Our results agreed well with the experimental findings in other solid tumors, confirming the proapoptotic effect of PDCD5 in the presence of other apoptosis-inducing factors.

However, the mechanism of the proapoptotic action of PDCD5 remains unclear. Xu S et al. found that the PDCD5 expression in apoptotic cells was evidently higher than that in normal cells, and they confirmed that the proapoptotic action of PDCD5 depends on the mitochondrial pathway [21, 22]. Nuclear translocation of PDCD5 in early apoptosis can evoke mitochondrial permeability transition and the changes of membrane potential. This will lead to alteration of mitochondrial functions and amplified apoptotic signaling, which facilitates cell apoptosis [23]. In the mitochondrial pathway of apoptosis, caspase-3 is the terminal effector caspase [24], whereas Bcl-2 and survivin are the important apoptosis-related genes [25-27]. We discussed the mechanism of proapoptotic action of PDCD5 in MM cells by detecting the expressions of genes related to mitochondrial apoptosis in MM cells with PDCD5 overexpression induced by DXM. Results showed that the MM cells with PDCD5 overepxression also had an obviously increased apoptosis and the downregulation of survivin and Bcl-2. However, caspase-3 was significantly upregulated, indicating the possible proapoptotic effect of PDCD5 through the inhibition of Bcl-2 and suvivin and the activation of caspase-3.

Bcl-2 is known for its apoptosis inhibitory effect [28]. Li H et al. [29] showed that PDCD5 promoted the cisplatin-induced apoptosis of glioma cells, which occurred alongside an inhibition of Bcl-2 expression. Then it was inferred that PDCD5 protein reduced the inhibitory effect of Bcl-2 on proapoptotic BAX protein through the inhibition of Bcl-2 expression. As the charge balance of the mitochondrial outer membrane is altered, the opening of mitochondrial permeability transition pores is facilitated. The release of cytochrome c into the cytoplasm will activate the terminal effector caspase, thereby inducing cell apoptosis. The Bcl-2 expression was measured by RT-PCR and Western Blot in DXM-induced apoptosis of MM cells. Compared Con and NC group, the Bcl-2 expression in PDCD5 group was significantly inhibited, and the two showed a negative correlation. The two detection methods produced consistent results, which corresponded to the findings in solid tumors [30, 31]. We infer that PDCD5 promoted tumor cell apoptosis by directly or indirectly inhibiting Bcl-2 expression through a particular signaling pathway.

Survivin is the most powerful apoptosis-inhibiting gene discovered so far. With regulatory effects on cell apoptosis and cell cycle, survivin is closely related to tumor cell differentiation and proliferation [32, 33]. In solid tumors, PDCD5 expression is negatively correlated with that of survivin [34]. Both regulate the caspase expression through mitochondrial pathway, though in an opposite direction. Hence it can be reasonably inferred that PDCD5 gene inhibits the binding of survivin with cell cycle protein CDK4 by either directly or indirectly inhibiting the activity of survivin. In this way the inhibitory effects of survivin on caspase-3 and caspase 7 are weakened, thus promoting cell apoptosis. Furthermore, PDCD5 can inhibit the expression of survivin gene and thus promote the release of mitochondrial cytochrome c, leading to the activation of downstream caspase. We studied the proapoptotic effect of PDCD5 gene under the induction by DXM. Survivin expression in Con group (DXM treatment only) was significantly reduced. It was indicated that PDCD5 may degrade survivin mRNA or inhibit survivin expression, thus reducing the inhibition on caspase-3. The mechanism of proapoptotic action of PDCD5 may be possibly related to survivin gene.

Caspase-3 gene is the terminal effector caspase in the apoptosis pathways of most cells. However, all apoptosis pathways involve the caspase-mediated cascade reaction. Relevant studies [35] have shown that PDCD5 overexpression ensures the functioning of mitochondrial apoptosis by sustaining the activity of caspase-3. We found that blank control group (DXM treatment alone) and empty vector group both had a mild upregulation of caspase-3 under the induction by DXM. In PDCD5 group, caspase-3 was upregulated more significantly, indicating the possible connection between proapoptotic action of PDCD5 gene and caspase-3 activation in MM cells.

Caspase-3 normally exists as inactive procaspase, the activation of which is the early event in apoptosis. Bcl-2 and survivin as apoptosis inhibitors function through the blocked activation of caspase-3. Study [36] showed that PDCD5 gene could bind to caspase-3 to form a complex, which prolonged the half-life of caspase-3 and induced cell apoptosis. Chen LN et al. [37] achieved a low expression of endogenous PDCD5 through siRNA intervention. As a result, caspase-3 activation was blocked and

the release of cytochrome c was inhibited. It was proved from another perspective that PDCD5 regulated the caspase-3 activity through the cellular apoptosis pathway. These findings point to the possibility that the pro-apoptotic action of PDCD5 is related to the inhibited caspase-3 activity: (1) PDCD5 may bind to caspase-3 to form a complex, thus affecting the half-life of caspase 3; (2) PDCD5 may block the signaling pathways of Bcl-2 and survivin, thereby reducing the inactivation of caspase-3 by the two proteins. The three proteins act in synergy to promote the apoptosis of MM cells.

Stably transfected MM cell line showing PDCD5 overexpression was constructed, and the pro-apoptotic action of PDCD5 gene was analyzed. We confirmed that the proapoptotic action of PDCD5 gene was associated with the inhibition of survivin and Bcl-2 and caspase-3 activation. It was inferred that the proapoptotic effect may depend on the mitochondrial pathway, but the molecular mechanism of signaling needs further investigation.

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Disclosure of conflict of interest

None.

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