RESEARCH COMMUNICATION

Apoptin Induces Apoptosis in Human Bladder Cancer EJ and BIU-87 Cells

Hui Zhan, Jian-Song Wang*, Hai-Feng Wang, Yi-Gang Zuo, Chun-Hui Wang, Ming-Xia Ding

Abstract

Objective: To investigate whether apoptin is a apoptosis-inducing protein with a potential for bladder cancer therapy. Methods: We constructed a PCDNA3/Apoptin eukaryotic expression vector, and transfected this vector into bladder cancer cell lines BIU-87 and EJ, then observed the results by RT-PCR, transmission electron microscopy, MTT assay and the flow cytometry (TUNEL method). Results: PCDNA3/Apoptin successfully induced a high level apoptosis in both bladder cancer cell lines, compared with the controls ($p<0.05$). Conclusions: Apoptin can induce high level apoptosis in human bladder cancer EJ and BIU-87 cells, which suggests a potential for human bladder cancer therapy.

Keywords: Apoptin - urinary bladder neoplasms - apoptosis - therapy

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Introduction

Apoptin is a kind of viral protein from Chicken Anemia Virus, which can induce apoptosis in various tumor cells, but not in normal cells. This merit has attracted more and more attention from the researchers. Different studies have shown that Apoptin could kill almost all kinds of tumor cells, such as hepatoma carcinoma cells (Han et al., 2008; Han et al., 2010; Han et al., 2011) , oral cancer cells (Schoop, et al., 2008), ovarian cancer cells (Wang et al., 2011). However, the effect of this protein on bladder cancer cells still keep unknown. This research would try to find out the anti-tumor effect of Apoptin on human bladder cancer EJ and BIU-87 cells, and evaluate the prospect of this protein in treatment of human bladder cancer.

Materials and Methods

The prokaryotic expression vector of Apoptin pBV220/Apoptin was made by our institute (Yunnan Institute of Urology), the eukaryotic expression vector PCDNA3 and gene engineer bacteria E.coli DH-5α were provided by the Biological Institute of Chinese Medical Academy. The restriction endonuclease BamH I, Xhol, T4 DNA ligase, dNTP, pfuDNA Polymerase and DNA marker DL-2000 were bought from the TaKaRa company; the RPMI-1640 cell culture medium, the lipofectamineTM2000 Reagent and the RT-PCR kit were bought from Invitrogen company; the fetal bovine serum was came from Hyclone company; the plasmid extraction kit from OMEGA company; the Tunel Kit from Backman Coulter company, and the DNA gel extraction kit from Hua Shun company. The human bladder cancer EJ and BIU-87 cell lines were provided by Professor Xie Shusheng of Peking University.

Construction of the eukaryotic expression vector PCDNA3/Apoptin

We cultured the DH-5α with pBV220/Apoptin at 37°C for 12 hours, then collected the bacteria and extracted the plasmid. The primers of PCR were as follows: pl:5'-gt ggatccaccatgaacgctctccaagaagatact-3' and p2:5'- ggctcgagttacagtcttatacgccttcttgcg-3'. After the ctrophoresis, the product of PCR was extracted from the gel, and the double digestion of BamH I and Xhol was performed on the product of PCR and plasmid PCDNA3 respectively. Then we connected the product of PCR into the PCDNA3 with the help of T4 DNA ligase. Finally, the plasmid PCDNA3/Apoptin was identified with double digestion of BamH I and Xho I, and sequence.

Detection of the expression of Apoptin protein in bladder cancer cell lines

Bladder cancer BIU-87 and EJ cell lines were cultured on the cell culture plate. The control group (only cell group, PCDNA3 + cell group and liposome + cell group) and experimental group (PCDNA3/Apoptin + cell group) were set for each cell line. 24 hours after transfection, we observed the expression of Apoptin mRNA by RT-PCR. While we used the immunohistochemistry and indirect immunofluorescence technique to detect the expression of Apoptin protein. The bladder cancer EJ cells in logarithmic growing phase were suspended in the medium

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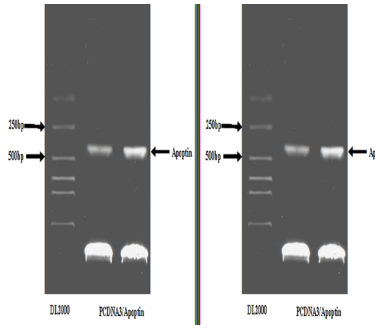

Figure 1. Identification of PCDNA3/Apoptin with Double Digestion of BamH I and Xhol

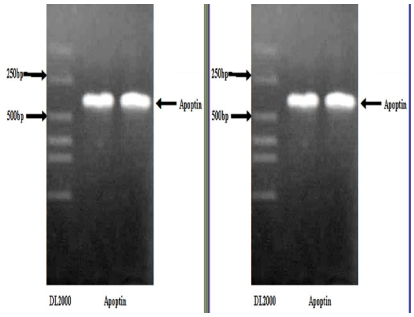

Figure 2. Checking the Expression of Apoptin mRNA by RT-PCR (EJ Cell).

at concentration of 1 × 10^4/ml and spread on sterile coverslips which were placed in six-well plate. After overnight culture, we removed media, washed twice with PBS. Then the cells were fixed in methanol at temperature of 4 C for 10min. The coverslips were washed with 0.25% Triton and 1% BSA. The first antibody and second antibody staining buffered glycerol fengpian. Staining was observed under fluorescence microscope (Nikon, Japan). We used PBS instead of first antibody as negative control.

Anti-tumor effects of Apoptin on bladder cancer cell lines

We examined the survival rate of the cells with MTT assay, the apoptosis rate with the flow cytometry (TUNAL method), and observed the changes of cell ultrastructure with transmission electron microscopy at 24, 48, and 72 hours after the transfection respectively.

Statistical Analysis

The data was tested by the statistic software SPSS 11.0, and the variance analysis was employed. The data was shown as mean number±standard deviation.

Results

Eukaryotic expression vector PCDNA3/Apoptin

The plasmid PCDNA3/Apoptin was identified with double digestion of BamH I and Xhol, a piece of DNA about 366bp was isolated from the vector (Figure 1), the molecular weight of which was similar to the gene of Apoptin. And the sequence also confirmed the gene of Apoptin had been cloned into the eukaryotic expression vector PCDNA3 successfully.

RT-PCR

24 hours after the transfection, expression of Apoptin could be checked by RT-PCR in both of the 2 cell lines (Figure 2).

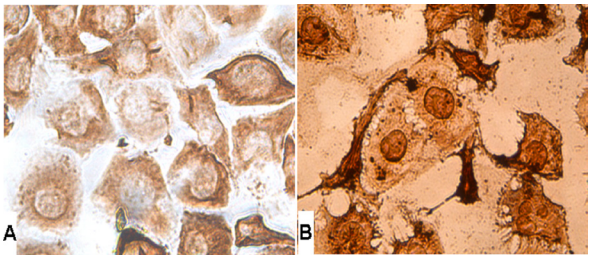

Figure 3. Checking the expression of Apoptin Protein by immunohistochemistry. A: Negative control group; B: Experimental group

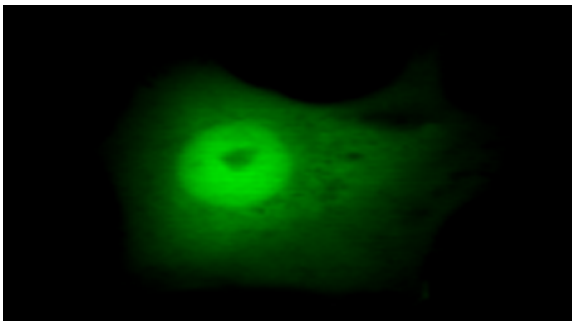

Figure 4. Checking the Expression of Apoptin Protein by Immunofluorescence. (PCDNA3-EGFP-Apoptin; EJ cell)

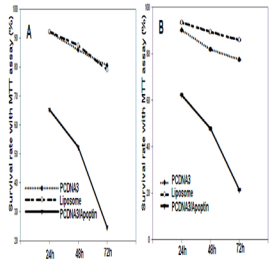

Figure 5. Examining the Survival Rate with MTT Assay: A: EJ Cell Line; B: BIU-87 Cell Line. *The survival rate of only cell group was set as 100%, so the survival rate of other groups = the OD value of each group/ the OD value of only cell group. *Compared the values of the group PCDNA3/ Apoptin +cell with those of other control groups, p<0.05, the difference was significant

Immunofluorescence

In experimental group, localization effects and morphological changes of cells were observed using immunohistochemistry method (Figure 3). In immunofluorescence assay, apoptin protein was stained light viridis in the EJ cells, indicating that it was expressed in the EJ cells (Figure 4).

Survival rate and apoptotic rate of the cells

The survival rate of each group with MTT assay at 24, 48, 72 hours was examined after the transfection. We found the growth of both bladder cancer cell lines transfected by Apoptin was inhibited obviously, and the survival rate declined step by step. 72 hours later, the survival rate was only about 20%. Compared with the control groups, the differences between them were significant (Figure 5).

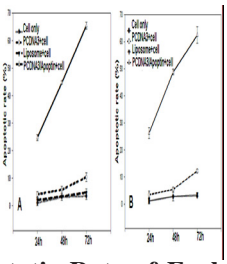

Figure 6. Apoptotic Rate of Each Group After the Transfection. A: EJ cell line; B: BIU-87 cell line. *Compared the values of the group PCDNA3/Apoptin + cell with those of other control groups, $p<0.05$, the difference was significant

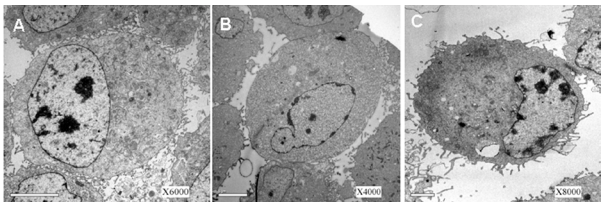

Figure 7. Ultrastructural Changes of Bladder Cancer Cells Attacked by Apoptin. A: Normal bladder cancer EJ cells; B: Early stage of apoptosis, chromatin margination could be seen in the cancer cells (BIU-87 cell); C: Late stage of apoptosis, fragmentation of cell nucleus and apoptotic bodies could be found (EJ cell)

The apoptotic rate of each group was observed with the help of Flow Cytometry (TUNAL method) at 24, 48, 72 hours after the transfection respectively. Both of the 2 cell lines transfected by Apoptin got a high level apoptotic rate, which increased with time. 72 hours after the transfection, the apoptotic rate was about 65%. Compared with the low apoptotic rate of the control groups, the difference between them were significant (Figure 6).

Transmission electron microscopy assay

The ultrastructural changes of the cancer cells were observed with transmission electron microscopy at 24, 48, 72 hours after the transfection respectively. Typical ultrastructural changes of apoptosis could be found in both of the two bladder cancer cell lines, which also aggravated with the passage of time. 48 hours after the transfection, such changes as karyopyknosis, karyorrhexis, chromatin margination and apoptotic bodies could be seen in most of the cancer cells (Figure 7).

Discussion

Bladder cancer is one category of common genitourinary cancers in china, and about 70 percent of which is superficial cancer (Gu et al., 2003; Pruthi et al., 2008). Nowadays, the major therapy of bladder cancer is transurethral electroresection of bladder tumors (TURBT) or partial cystectomy. And after operation, long term intravesical instillation with drugs of chemotherapy often be performed to inhibit the recurrence. However, because of the natural or accepted drug resistance of tumor cells, instillation of single drug often be failed to control the recurrence. Although the combined chemotherapy can get a better result than that of single drug instillation, the rate of recurrence still stay at a high leve (Schulze et al., 2007; Pruthi et al., 2008). So it is necessary to find some new drugs with new anti-tumor mechanism.

Apoptin is a kind of viral protein from Chicken Anemia Virus (CAV), which can induce apoptosis of tumor cells. This protein, molecular weight of which is 13600, is composed by 121 amino acids, and includes 2 functional regions (Backendorf et al., 2008). In other researchers' reports, following merits of Apoptin in anti-tumor therapy were mentioned: Apoptin can induce apoptosis in almost all kinds of the malignant tumor cells (Backendorf et al., 2008) ; Apoptin can kill tumor cells but not the normal cells (Schoop et al., 2008); Apoptin can induce high level apoptosis in tumor cells (Rohn et al., 2004); The anti-tumor effect of Apoptin do not depend on the expression of p53 (Zhuang et al., 1995); Apoptin can induce high level apoptosis in tumor cells with the expression of anti-apoptosis protein Bcl-2 and bcl-xL (Danen-van et al., 1999; Noteborn et al., 2004; Schoop et al., 2004). This viral protein has been looked as a candidate of new anti-tumor drugs.

The anti-tumor effect of Apoptin on many kinds of tumors has been observed by different researchers. However, the anti-bladder cancer effect of this protein is still unknown. So we performed this study to observe the anti-tumor effect of Apoptin on different bladder cancer cell lines. In our research, we found Apoptin can induce high level apoptosis in bladder cancer EJ and BIU-87 cell lines. 24 hours after the transfection of PCDNA3/Apoptin, the apoptotic rate of bladder cancer cells was about 25%, 72 hours later, the apoptotic rate was about 65%, at the same time, the survival rate measured by MTT was less than 25%, and the difference between the testing group and other control groups was significant, which meant both of the bladder cancer cell lines were sensitive to the anti-tumor effect of Apoptin. Because we expressed Apoptin in cells, the drug concentration was limited by the expressing ability of the cells, and some researchers have reported the anti-tumor effect of Apoptin was concentration dependent(Wadia et al., 2004). Consequently, if we increased the concentration of Apoptin with appropriate method, as a result, the apoptotic rate of the bladder cancer cells induced by Apoptin would rise further. Besides, the transfection rate also affected our results.

At present, whole mechanism of the anti-tumor effect of Apoptin still keep unknown, according to other researchers' reports, following mechanisms play an important role in this process: Apoptin can concentrate in the nuclear of tumor cells, but distribute in the cytoplasm of normal cells, which was looked as the major mechanism of the selective anti-tumor effect of this protein, and the trigger of the apoptotic process (Danen-van et al., 2003). The DNA connecting effect: when Apoptin concentrate in the nuclear of tumor cells, this protein can connect with the DNA, and may cut off the DNA to induce the apoptosis(Leliveld, et al., 2004). Phosphorylation of Apoptin: Rohn (Rohn et al., 2002) and Poon (Poon, et al., 2005) reported respectively that phosphorylation of Apoptin took place in many tumor cells but not in normal cells, and the phosphorylated region was located on the C terminal of this protein. If this region was mutated, Apoptin could induce apoptosis in normal cells too, which

meant this effect had something to do with the selectively anti-tumor effect of Apoptin. The interaction between Apoptin and other small molecular protein: researchers have found several kinds of molecular chaperones could cooperate with Apoptin in the selective anti-tumor process, for example: the Nmi DEDAF and APC1 et al. (Danen-van et al., 2004; Teodoro et al., 2004; Noteborn et al., 2005).

As we mentioned before, Apoptin can kill the tumor cells through a new mechanism different to traditional chemical drugs. Consequently, this protein will provide a new choice to therapy of multi-drug resistant bladder cancer, and this protein may provide a new hope for human bladder cancer therapy.

Acknowledgements

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