Synthetic small peptides acting on B7H1 enhance apoptosis in pancreatic cancer cells

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Received January 19, 2012; Accepted April 11, 2012

DOI: 10.3892/mmr.2012.970

Abstract. The interaction of B7H1 in tumor cells with programmed death-1 (PD-1) in T cells plays an important role in the suppression of immune responses. However, the effects of the expression of B7H1 and the PD-1/programmed death 1 ligand 1 (PD-L1) complex on tumor cells themselves remain largely unknown. In order to clarify this, we induced apoptosis in a number of human pancreatic cancer cells with different expression levels of B7H1 and designed small peptides to interfere with the function of B7H1. In this study, we chose 2 human pancreatic cancer cell lines (BxPC-3 and Panc-1 cells). Cells with a high expression of B7H1 (BxPC-3 cells and Panc-1 cells treated with interferon-γ) presented much lower levels of drug-induced apoptosis after they were incubated with PD-1 immunoglobulin. Furthermore, the percentage of BxPC-3 apoptotic cells transfected with B7H1 siRNA was higher compared to that of cells transfected with control siRNA. Both of these results indicate that the PD-1/PD-L1 complex transfers a reverse signal to B7H1 pancreatic cancer cells upon drug-induced apoptosis in vitro. This effect may be a substantial factor for drug resistance during antitumor therapies. However, synthetic small peptides designed according to the amino acid residues of the PD-1/PD-L1 complex interrupt this effect successfully by their binding to cell membranes and acting like a blocking agent. This result may lead to a breakthrough in pancreatic cancer treatment.

Introduction

Pancreatic cancer is one of the malignancies with very poor prognosis and the 5-year survival rate is approximately 3% (1). It is regarded as the 8th most common cause of death from cancer worldwide (2), and the 4th leading cause of cancer-related death in the United States (3). The survival rate has not improved, as shown by studies on gemcitabine-based combination therapy (4). A breakthrough in the treatment of this disease may be required to improve the survival conditions.

B7H1 [CD274, programmed death 1 ligand 1 (PD-L1)] is a B7-related protein with an immunoglobulin (Ig)-like molecule first identified in 1999 (5). Ample evidence confirms that B7H1 is widely expressed in various human gastrointestinal cancers, including pancreatic (6), gastric (7), esophageal (8) and colon (5) cancers, and its expression is constitutive or inducible. B7H1 delivers an inhibitory signal to its receptor, programmed death-1 (PD-1), in T cells, causing the suppression of immune responses (9). The binding of B7H1 and PD-1 occurs through the formation of the PD-1/PD-L1 complex, which makes the 2 proteins interact through the conserved front and side of their Ig variable (IgV) domains containing some conserved residues (10). Blocking of the B7H1 and PD-1 interaction by neutralizing antibodies restores the cytotoxic T lymphocyte (CTL)-mediated lysis of tumor cells in vitro (11).

Most studies have focused on the mechanisms of the B7H1 suppressive effect on T cells mediated by PD-1/PD-L1. They have found that mechanisms of action are involved, such as the induction of apoptosis (12) and the exhaustion of T cells (13).

However, in a certain study, mixed B7H1⁺ and B7H1⁻ cells were cultured together with antigen-specific CD8⁺ CTL in vitro. The researchers did not detect the suppression of the cytolytic function of CD8⁺ CTL after short-term culture, while they found that B7H1⁻ cells presented preferential lysis (11). This conclusion suggests that B7H1 transfers a reverse signal to tumor cells themselves, apart from its role as a ligand to the PD-1 receptor, followed by certain mechanisms that induce the death of B7H1⁻ cells. To demonstrate this hypothesis, we induced apoptosis in a number of human pancreatic cancer cells with different expression levels of B7H1 and designed small peptides to interfere with the function of B7H1. Our results indicate that B7H1 expression in pancreatic cancer cells reduces drug-induced apoptosis and that synthetic small peptides interrupt this inhibition.
Materials and methods

Cell lines and cultures. The Panc-1 and BxPC-3 human pancreatic cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml of penicillin and 100 µg/ml of streptomycin at 37°C in a humidified incubator containing 5% CO₂. Treatments were performed after the cells adhered.

Peptide synthesis and treatment. Two small peptides with 6 hydrophilic amino acid residues were synthesized by solid phase synthesis (Hangzhou Angtai Biotech. Co., Ltd., Hangzhou, China). Peptide 1 (P1) was designed according to the amino acid sequence of the connecting site of the PD-1/PD-L1 complex; Peptide 2 (P2) was synthesized using the similar amino acid residues, but with an uncorrelated sequence as the control. Peptides were lyzed in phosphate-buffered saline (PBS) and incubated with the cells at the concentration of 50 µg/ml. Fluorescein isothiocyanate (FITC; 5 µl)-conjugated P1 and P2 (Angtai Biotechnology) at the concentration of 1 mg/ml were incubated with BxPC-3 cell samples for flow cytometry (FCM) assay.

FCM assay. BxPC-3 and Panc-1 cells treated with or without interferon (IFN)-γ (500 U/ml) for 24 h in 6-well plates were harvested and washed twice with cold PBS. Each sample was incubated with 5 µl anti-human B7H1 phycoerythrin (PE), mouse IgG1 isotype control PE (eBioscience, San Diego, CA, USA), FITC-conjugated P1 or control P2 for 30 min at 4°C in the dark. After being washed twice with PBS again, the cells were resuspended in 500 µl PBS at the concentration of 1x10⁶/ml and analyzed by FCM (FACScan; BD Biosciences, Franklin Lakes, NJ, USA). In brief, for apoptosis analysis, cells grown in 6-well plates were pre-treated with P1, P2, recombinant human PD-1 Ig or human IgG (Sino Biological, Beijing, China) at the concentration of 50 µg/ml. After 24 h, the cells were treated with staurosporine (STS) at 0.5 µM for another 24 h at 37°C and were then collected. After being washed with PBS, the cells were incubated with Alexa Fluor 488 annexin V and propidium iodide (PI) (Invitrogen, Renfrew, UK) for 15 min at room temperature in the dark, and then the samples were measured by FCM.

siRNA interference. Transfection-related products were all purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. BxPC-3 cells were diluted in fresh medium without antibiotics and transferred to 6-well plates. Cells grown to a confluence of 50-60% were transfected with 8 µl of B7H1 siRNA or control siRNA per well according to the manufacturer's recommendations. After transfection for 24 h, the efficacy of the siRNA interference was determined by western blot analysis.

MTS assay. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays were performed according to the manufacturer's instructions (CellTiter 96 AQueous Non-Radioactive Cell Proliferation assay; Promega, Madison, WI, USA). Briefly, cells were seeded in 96-well culture plates at an optimal density (5x10⁴ cells/well) in triplicate wells. After 24 h, the medium was changed and the cells were treated with peptides (50 µg/ml) for another 24 h. Then, STS (0.5 µM) was added. After 24 or 48 h, 20 µl of MTS solution was added to each well (at the total volume of 120 µl/well) for 2 h at 37°C. The absorbance was measured at 570 nm using a microtitration plate spectrophotometer.

Western blot analysis. To evaluate the expression of the B7H1 protein and the active degrees of the apoptosis-related protein, poly (ADP-ribose) polymerase 1 (PARP-1), and caspase-3, cellular samples were analyzed by western blot analysis. Cell extracts were prepared with RIPA lysis buffer (Beyotime Biotechnology, Haimen, China). The total protein concentration was measured by the bicinchoninic acid (BCA; Beyotime Biotechnology) method using bovine serum albumin as the standard sample. After the samples were heat-denatured, a total of 40 µg of protein samples was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (6 or 12%, as required) and transferred to polyvinylidene fluoride membranes (Millipore, Bilherica, MA, USA). Membranes were then blocked with 5% non-fat milk in Tris-buffered solution with 0.5% Tween-20 (TBST) for 1 h at room temperature and incubated with primary antibodies specific to B7H1 (R&D Systems, Minneapolis, MN, USA), PARP-1 and caspase-3 (Epitomics, Burlingame, CA, USA) overnight at 4°C. After being washed 3 times, membranes were incubated with secondary antibodies (Zhongshan Goldenbridge Biotechnology Co., Beijing, China) or GAPDH (Shanghai Weike Biochemical Reagent Co., Shanghai, China) for 1 h at room temperature. Signals were detected by
enhanced chemiluminescence detection reagents (Millipore) using ImageQuant LAS-4000 (Fujifilm, Tokyo, Japan). The bands were analyzed using Multi-Gauge software (Fujifilm).

**Immunofluorescence.** Cells were cultured on microscope slides and washed 3 times with PBS for 15 min before being fixed with 4% paraformaldehyde at room temperature for 15 min. After being washed 3 times with PBS for 30 min, the cells were incubated for 2 h at room temperature with FITC-conjugated P1 or P2. After a further washing step, images were captured on a wide-field fluorescent microscopy (Zeiss, Jena, Germany).

**Statistical analysis.** All statistical analyses were carried out using the SPSS 19.0 statistical software package. All data were obtained from at least 3 individual experiments. Values are expressed as the means ± SD. Statistical analysis between groups was performed by one-way ANOVA. A value of p<0.05 was considered to indicate a statistically significant difference.

**Results**

**B7H1 is constitutively expressed in Panc-1 cells and highly expressed in BxPC-3 cells.** We used FCM to determine the levels of B7H1 protein expression on the cell membrane in BxPC-3 and Panc-1 cells. There was a low-level constitutive expression of B7H1 in Panc-1 cells. This expression was upregulated 24 h after treatment with IFN-γ (500 U/ml), while B7H1 expression was high in BxPC-3 cells untreated with IFN-γ (Fig. 1A). We then extracted the total protein from cells treated as mentioned above to detect the B7H1 expression levels by western blot analysis. The results indicated a similar tendency. Panc-1 cells presented significantly higher levels of B7H1 expression after treatment with IFN-γ (Fig. 1B). The expression levels in BxPC-3 cells were effectively downregulated by siRNA silencing (Fig. 1C). In our study, following B7H1 siRNA transfection, Panc-1 and BxPC-3 cells had a low expression of B7H1, while Panc-1 cells treated with IFN-γ and BxPC-3 cells had a high B7H1 expression.

**Binding to PD-1 Ig protects tumor cells from STS-induced apoptosis.** Panc-1 cells and cells with a high expression of B7H1 were incubated with PD-1 Ig fusion protein (50 µg/ml) or control IgG (50 µg/ml) for 24 h after they adhered to 6-well plates. After extensive washing, the cells were treated with STS (0.5 µM) for 24-48 h. MTS assay was used to determine the cell proliferation in the different groups after treatment with STS (Fig. 2A). There was a higher percentage of cell viability in cells with a high expression of B7H1 pre-treated with PD-1 Ig compared to the controls (P<0.05), while the differences in Panc-1 cells with low B7H1 expression levels were not significant (P>0.05). There was an inhibitory action in the cell death caused by STS from the formation of the PD-1/PD-L1

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**Figure 2.** Binding to programmed death-1 (PD-1) immunoglobulin (Ig) protected tumor cells from staurosporine (STS)-induced apoptosis. Panc-1 cells treated with interferon (IFN-γ) (500 U/ml) for 24 h and BxPC-3 cells incubated with PD-1 Ig or control IgG for 24 h were treated with STS (0.5 µM). (A) MTS assays were used to measure the cell viabilities 24 and 48 h after STS treatment. Each point is the mean of triplicates with SD. (B) After 24 h of STS treatment, apoptosis was measured by flow cytometry assays double-stained with Alexa Fluor 488 annexin V and propidium iodide. The data are representative of 3 experiments.
To explore the mechanisms involved, we then examined cell apoptosis by FCM assay (Fig. 2B). After treatment with STS for 24 h, the percentages of early apoptotic cells in the cells with a high expression of B7H1 pre-treated with PD-1 Ig were significantly lower than those in the controls (P<0.05) and, as expected, there were no significant differences in the Panc-1 cells (P>0.05). These results suggest that the formation of the PD-1/PD-L1 complex suppresses STS-induced tumor cell apoptosis in cells with a high expression of B7H1.

**B7H1 knockdown increases apoptosis in BxPC-3 cells.** In order to examine whether the expression levels of B7H1 are related to cell apoptosis in BxPC-3 cells, FCM assay and western blot analysis were performed. The percentage of cells at the early stages of apoptosis in cells transfected with B7H1 siRNA was significantly increased compared to the control-transfected cells (P<0.05; Fig. 3A). Caspase-3 and PARP-1 play key roles during the process of cell apoptosis. We observed marked increases in the cleavage activities of caspase-3 and PARP-1 in the cells transfected with B7H1 siRNA, which indicated the enhancement of apoptosis. This suggests that high levels of B7H1 expression reduce STS-induced tumor cell apoptosis. On the other hand, a successful siRNA interference may increase apoptosis in these cells.

**Synthetic peptides designed according to PD-1/PD-L1 complex increase apoptosis in BxPC-3 cells.** Synthetic peptide P1 was a small peptide containing 6 amino acid residues. The amino acid sequence, Ser-Asn-Gln-Thr-Asp-Lys, was the same as human PD-1 residues, 73-78, which acted as the connecting site to PD-L1 during the formation of the PD-1/PD-L1 complex. Peptide P2, with the residues, Ala-Asp-Tyr-Lys-Arg-Ile, was synthesized to act as the control in our study. We used FITC-conjugated P1 and P2 to investigate the efficacy of these synthetic peptides binding to the cell membrane and whether this binding is relative to the expression levels of B7H1. BxPC-3 cell samples incubated with FITC-conjugated synthetic peptides for 30 min were then washed with PBS 3 times. Flow cytometry (FCM) assays were used to demonstrate the binding levels. (B) Cells fixed with paraformaldehyde were incubated with FITC-conjugated P1 and P2, and then observed by electron microscopy. (C) Apoptosis of cells pre-treated with P1 or control P2 was induced by staurosporine (STS) (0.5 µM) and was measured by FCM assays 24 h after STS treatment, using the method of double staining with Alexa Fluor 488 annexin V and propidium iodide. (D) Levels of poly (ADP-ribose) polymerase 1 (PARP-1), caspase-3 and their cleaved patterns in cells pre-treated with P1 and control P2 were analyzed by western blot analysis. GAPDH was used as the internal control. The data are representative of 3 experiments.

Lys-Arg-Ile, was synthesized to act as the control in our study. We used FITC-conjugated P1 and P2 to investigate the efficacy of these synthetic peptides binding to the cell membrane and whether this binding is relative to the expression levels of B7H1. BxPC-3 cell samples incubated with FITC-conjugated synthetic peptides for 30 min were washed extensively and analyzed by FCM. The level of P1 binding was significantly higher than the P2 control (Fig. 4A), which showed a similar tendency in B7H1 expression. To confirm our result, we performed immunofluorescence analysis to detect whether FITC-P1 binds to the cell membrane effectively. Cells with P1 treatment presented significantly higher fluorescence on the membrane than the control cells (Fig. 4B). With these results, we pre-treated BxPC-3 cells with synthetic peptides and then induced apoptosis by STS, as previously mentioned. In the FCM assays, the cell percentage in the early stages of apoptosis in P1 pre-treated cells was significantly higher than the P2 control cells (P<0.05; Fig. 4C). The detection of PARP-1 and caspase-3 showed markedly increasing levels of cleaved proteins in P1 pre-treated cells (Fig. 4D). This may be explained by the interference of the anti-apoptotic effect caused by the PD-1/PD-L1 complex formation, which occurred by the binding of P1.
Discussion

Previous studies have indicated that inhibitory signals are transferred to T cells by B7H1 in tumor cells, which lead to immune suppression (11,14,15). The blockage of B7H1 and its receptor, PD-1, in T cells by antibodies improves antitumor immunity (14). Disruption of the B7H1 gene upregulates T cell responses (16). These studies suggest that B7H1 and its receptor contribute to tumor cells escaping from immune destruction.

However, in this study, we attempted to explain the mechanism of the resistance to antitumor immunity in B7H1+ cancer cells from another point of view. The concept that programmed cell death by apoptosis serves as a natural barrier to cancer development has been established by compelling functional studies conducted over the past 2 decades. Yet, another research has revealed how apoptosis is attenuated in tumors that succeed in progressing to states of high-grade malignancy and resistance to therapy (17).

In the present study, we found that B7H1 expressed at a high level inhibits STS-induced cancer cell apoptosis and that the successful B7H1 knockdown increases apoptosis by disrupting this inhibition in the BxPC-3 human pancreatic cell line. A certain study previously demonstrated that B7H1 siRNA knockdown led to an increase in spontaneous apoptosis, as well as doxorubicin-induced apoptosis in breast cancer cells (18), and another study reported that B7H1 expression in cancer cells plays a role in the induction of the anti-apoptotic mechanism (19). All these results indicate that the expression levels of B7H1 are related to apoptosis in tumor cells. In due time, this may contribute to the inhibition of B7H1+ tumor cell lysis in immune responses. However, the underlying molecular mechanisms of the impact are unknown, and further research will help in developing tumor prognosis and therapy.

Furthermore, B7H1 is confirmed to bind its receptor, PD-1, and form the PD-1/PD-L1 complex. It is the structural basis that induces B7H1 to produce inhibitory effects on immune responses. This complex transfers reverse signals to B7H1+ tumor cells besides its forward direction to T cells. This was also detected in our study using PD-1 Ig to mimic the formation of the PD-1/PD-L1 complex. We found that drug-induced apoptosis in cells with a high expression of B7H1 increased significantly after treatment. Since the complex has several conserved domains described previously, small molecule drugs designed to interfere with its inhibitory signal may soon become a reality.

Small peptides less than 8-10 amino acid residues are easily absorbed by the gastrointestinal tract with little degradation. On the other hand, small peptides may cause less side-effects compared to other treatments, such as chemotherapy and radiotherapy. The amino acid residues binding to B7H1 may interrupt the integrity of the domains and cause some interference. In our study, we designed a small peptide containing 6 amino acid residues to act as a drug. After demonstrating its successful binding to cells with a high expression of B7H1, we discovered that its binding increased apoptosis in B7H1+ tumor cells.

In conclusion, our study demonstrates that the high expression of B7H1 and the formation of the PD-1/PD-L1 complex inhibit drug-induced apoptosis in pancreatic cancer cells in vitro. Synthetic small peptides enhance drug-induced apoptosis in pancreatic cancer cells with a high expression of B7H1. We are the first to demonstrate that synthetic small peptides can increase apoptosis in B7H1+ cancer cells. Our results may lead to a breakthrough in the treatment of pancreatic cancer.

Acknowledgements

This study was supported by the Key Social Development Project of Major Science and Technology (2011C13036-1). The study was performed at the Biomedical Research Center, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China.

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