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Original Article

Experimental study and safety evaluation of sensitized dendritic cell BxPC-3 tumor vaccine and immune response against pancreatic cancer

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Abstract: Objective: To evaluate the safety of sensitized DC-BxPC-3 by conducting tumorigenic experiments in BALB/c nude mice, and to explore the inhibitory effect of sensitized DC-BxPC-3 tumor vaccine-induced systemic immune effector cells (DCs derived monocytes cells, herein referred to as SIECs) on transplanted BxPC-3 tumors in BALB/c nude mice. Methods: After safety evaluation, the mice were randomly divided into 6 groups: DC-BxPC-3 tumor vaccine experimental group, sensitized DCs positive control group, BxPC-3 heat treatment group, BxPC-3 lysate positive control group, BxPC-3 positive control group, and culture medium negative control group. The mice in each group were subcutaneously injected (0.25 ml/mice), and the presence of tumor and tumor growth were observed every 5 days for 40 days. *In vivo* efficacy study, mice were randomly divided into 6 groups: sensitized DC-BxPC-3 tumor vaccine+SIECs group, DC-BxPC-3 tumor vaccine+SIEC group, sensitized DC+SIECs group, DC+SIECs group, SIECs group, and negative control group which were inoculated with the corresponding vaccine (1.0×10^8/nu, 0.25 ml/mice) on the same side as the tumor growth, while the negative control group was inoculated with cell culture medium. The tumor growth in each group was observed every 5 days for a total of 40 days. On the 40th day, the mice were sacrificed, and the implantation tumors were weighed. The tumor inhibition rate was calculated by the Kruskal-Wallis H test and the Mann-Whitney U test, and the pathological changes of transplanted tumors in the scapular region of observed by hematoxylin and eosin (H&E) staining. Results: *In vivo* safety evaluation: there were no implantation tumors in the inoculated areas except for the BxPC-3 positive control group. *In vivo* efficacy study: on the 15th day after implantation tumor inoculation in mice, implantation tumors appeared in the sensitized DC+SIEC group, DC+SIEC group, and the negative control group, and in the other groups on the 20th day. On the 40th day, a large number of the BxPC-3 cell necrosis and tissue liquefaction appeared in the implantation tumor tissue of the negative control group, the BxPC-3 cellular apoptosis was more obvious in the sensitized DC-BxPC-3 cell vaccine+SIECs group, DC-BxPC-3 cell vaccine+SIECs group and sensitized DC+SIECs group than in the DC+SIECs group and SIECs group. The sensitized DC-BxPC-3 cells vaccine+SIECs group ($P<0.05$) and DC-BxPC-3 cells vaccine+SIECs group ($P<0.05$) had significant tumor growth inhibition compared with the negative control group, and the sensitized DC-BxPC-3 cells vaccine+SIECs group showed the highest tumor inhibition rate (46.1%). Conclusion: Tumor cell immune vaccines such as sensitized DC-BxPC3 cell vaccines have good biosafety. SIECs induced by sensitized DC-BxPC-3 cell vaccine and DC-BxPC-3 cell vaccine have an obvious inhibitory effect on pancreatic transplanted tumors.

Keywords: Pancreatic cancer, systemic immune effector cells, dendritic cells, immunotherapy, DC-BxPC-3 cells vaccine

Introduction

Pancreatic cancer (PC) is a malignant tumor of the digestive system with high mortality [1]. Due to its hidden onset, the early manifestations are atypical symptoms such as epigastric discomfort, dyspepsia, or diarrhea, which are often confused with other digestive system diseases, and as a result patients have not been effectively examined and diagnosed. Risk fac-

tors include smoking, age, genetic and family history, and other risk factors of pancreatic cancer are screened for include [2-4] CA19-9, CA125, CEA, micro-RNA, and other markers including blood glucose laboratory examination [5-7]. Patients with middle and late stage symptoms such as anorexia and weight loss are often diagnosed by ① enhanced three-dimensional dynamic CT [8] scanning, MRI, magnetic resonance cholangiopancreatography (MRCP) [9], positron emission tomography computed tomography (PET-CT) [10], endoscopic ultrasonography (EUS) [11], and other physical examinations, and ② histopathology and/or cytology. At this time, most patients miss out on the most effective surgical treatment opportunities, radiotherapy, chemotherapy, and other traditional treatments, as well as traditional Chinese medicine. In recent years, tumor immunotherapy has played an important role in tumor therapy by improving self-immunity and removing residual tumor cells after surgery [12]. Chemoradiotherapy sensitizer and immune enhancer (Thymalfasin), as well as chimeric antigen receptor-modified T-cell (CAR-T) immunotherapy [13], Immune checkpoint inhibitors (ICIs), programmed cell death protein 1 (PD-1) [14] and monoclonal antibody pablizumab have improved the granulocyte-macrophage colony-stimulating factor modified tumor cell vaccine (GVAX) [15, 16], and cetuximab, dendritic cells (DCs)-based Algenpantucel-l vaccine [17-19] of programmed cell death ligand 1 (PD-L1) have shown some results in the immunotherapy of pancreatic cancer, but there are still many problems. For example, the occurrence of pancreatic cancer is affected by multiple factors as a result of polygenic mutations [2, 7, 20-23], the reduction and dysfunction of immune cells such as DCs are closely related to the tumor environment, and apoptosis is inhibited during the occurrence and development of pancreatic cancer [24, 25]. DCs are important professional antigen-presenting cells in the body. They induce the production of effector T cells by ingesting and processing antigens, and enhance the ability of effector T cells to recognize and kill tumor cells by overexpression of costimulatory molecules and adhesion molecules. By secreting TNF-α, IFN-γ, IL-12 further induces T cell differentiation and proliferation, promotes the production of cytotoxic T lymphocyte (CTL) and helper T cells (Th), and carries out immune response [26, 27]. This paper mainly discusses the biological safety of the sensitized DC-BxPC-3 cells vaccine and its anti-pancreatic cancer effect *in vivo*, so as to provide an experimental basis for the treatment of pancreatic cancer with the DC-BxPC-3 cell vaccine.

Material and methods

Materials

Animals and tumor strains

Six to eight week old male SPF grade BALB/c nude mice were purchased from Beijing Medical University Animal Center, and *in situ* human pancreatic cancer cells (BxPC-3) were aquired from Beijing Beina Chuanglian Institute of Biotechnology. All experimental procedures were carried out in accordance with the guidelines established by the National Institute of Health and approved by the Ethical Committee on the Use and Care of Animals at Hainan Medical University (No. HYLL-2022-268).

Reagents and instruments

Fresh peripheral venous blood from healthy people was anticoagulated with heparin sodium. Granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL-2, IL-4), RPMI 1640, and fetal bovine serum were purchased from the United States (GIBCO). Lymphocyte separation solution was purchased from Tianjin Haoyuan Huake Biotechnology Co., Ltd. Flow cytometry (BD Company, USA). For experimentation we used the following equipment: centrifuge (Ag company,Germany); cell counting Kit (muhisiencee company); multifunctional micropore detector (Synergy H1 microplate read, BioTek, USA); and a thermostatic water bath box (Grant company, USA).

Drug preparation [28]

Preparation of sensitive DCs tumor vaccine and DCs tumor vaccine: BxPC-3 cells in a logarithmic growth phase were taken and repeated freeze-thawing was performed, no cells were detected by microscopy, and no cell growth was observed in cell culture. Then BxPC-3 tumor-specific antigen (BxPC-3TSA) was obtained. Peripheral venous blood was diluted 1:1 with phosphate-buffered saline (1×, PBS), lymphocyte separation medium was added, and peripheral venous blood mononuclear cells (PBMCs) were obtained by centrifugation. The

PBMCs were re-suspended in RPMI 1640 medium and cultured in 6 wells. Plates were cultured at 37°C in a 5% CO_2 incubator for 3 h, and suspended cells (ie, SIECs) were collected. IL-4 (500 IU/mL), GM-CSF (1000 IU/mL), and IL-2 (250 IU/mL) were added to the culture medium, and after 48 h, some DCs were taken and BxPC-3TSA was added (ie, sensitive DCs). BxPC-3 cells in a logarithmic growth phase were put in a water bath at 40°C, mixed with sensitive DCs and DCs at a ratio of 1:1, add 500 mL of PEG400 containing 10% DMSO, put in a water bath at 40°C for 3 min, and then we stopped the reaction with PBS (1×) buffer. Then we centrifuged, and re-suspended the mixed cells in HAT complete medium to obtain sensitized DCs fusion tumor vaccine and DCs fusion tumor vaccine.

SIECs induction and culture: Sensitive DCs tumor vaccine, DCs tumor vaccine, sensitive DCs, DCs (5×10^6/mL), and SIECs were mixed at 1:20 and cultured at 37°C, 5% CO_2 for 24 h.

Methods

In vivo safety evaluation experimental method

Twenty-eight BALB/c nude mice were randomly divided into 7 groups according to body weight: sensitized DC-BxPC-3 tumor vaccine experimental group, DC-BxPC-3 tumor vaccine positive control group, sensitive DC positive control group, BxPC-3 heat treatment group, BxPC-3 lysate positive control group, BxPC-3 Positive control group and negative control group; 4 mice/group. Mice in the negative control group were subcutaneously injected with 0.25 mL of 10% RPMI 1640 medium, and the other groups were subcutaneously injected with 0.25 mL of immune effector cells or cancer cells (1.0×10^8 cells). The mice in each group were observed every 5 days whether a transplanted tumor appeared, and the size of the transplanted tumor was measured for a total of 40 days.

Experimental methods for in vivo efficacy study

Twenty-four BALB/c nude mice were randomly divided into 6 groups according to body weight: group A: sensitized DC-BxPC-3 tumor vaccine+SIECs group, group B: DC-BxPC-3 tumor vaccine+SIEC group, group C: sensitive DC+SIECs group, Group D: DC+SIECs group, Group E: SIECs group and Group F: negative control group; 4 mice/group. The mice in the negative control group were subcutaneously injected with 0.25 ml of 10% RPMI 1640 medium on the other side of the anterior shoulder after inoculation with BxPC-3 cells, and mice in the other groups were subcutaneously injected with 0.25 ml of corresponding immune cells (1.0×10^8 cells) on the other side of the anterior shoulder two days before inoculation with BxPC-3 cells. The mice in each group were observed every 5 days to observe whether a transplanted tumor appeared, and the size of the transplanted tumor was measured for a total of 40 days. On the 40^{th} day, the mice were sacrificed via cervical vertebral dislocation, and the implantation tumors were surgically dissected and weighed. The pathological sections of the implantation tumors were observed by H&E staining.

Statistical analysis and comparison of anti-tumor effect

SPSS 18.0 software was used to analyze the difference of implantation tumors mass between groups

In terms of *in vivo* biological efficacy, there were 4 BALB/c nude mice in each group. The weight data of transplanted tumors showed a non-normal distribution, which was expressed by median and interquartile range. The difference between groups was selected by the nonparametric Kruskal Wallis H test and pair comparisons were made using the Mann-Whitney U test. The weight of the tumors in each group and the difference between groups were described by Box chart. $P<0.05$ was considered statistically significant.

Descriptive methods were used to compare the anti-tumor effects of each group

Tumor inhibition rate = (average tumor mass in negative control group-average tumor mass in the treatment group)/average tumor mass in negative control group ×100%.

The anti-tumor effect of each group was compared by observing the apoptosis of BxPC-3 cells, tissue liquefaction and necrocytoses in the implantation tumor using pathological section method.

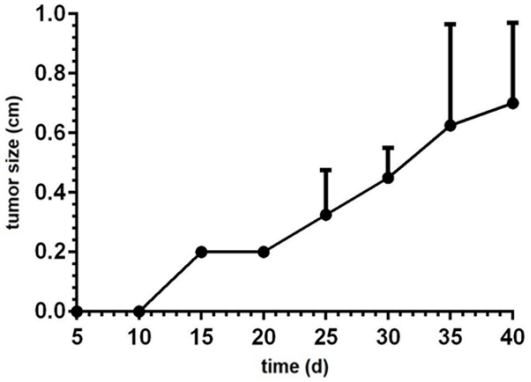

Figure 1. Growth of implantation tumor in mice of BxPC-3 positive control group.

Results

In vivo safety evaluation experimental results

The experiment revealed that on the 15th day after the subcutaneous injection of the mice, the mice in the BxPC-3 positive control group developed subcutaneous tumors, and with the passage of time. The volume of the subcutaneous tumors became larger and the skin in front of the shoulder of the mice in other groups was normal, as shown in **Figure 1**.

In vivo efficacy test results

The experiment showed that on the 15th day after subcutaneous tumor inoculation, tumors appeared in the anterior shoulder subcutaneously in the sensitized DC+SIECs group, DC+ SIECs group, and the negative control group. On the 20th day, implantation tumors appeared in all groups. On the 40th day, the pathological sections from mice in each group showed that a largeamount of cell necrosis occurred in the implantation tumor tissue of mice in the negative control group (as shown in **Figure 3**). The mass of implantation tumors in each group was compared by the Kruskal Wallis H test ($P>0.05$), and the experimental groups were compared with the negative control group by Mann Whitney U test analysis, they showed that the difference between the sensitized DC-BxPC3 tumor vaccine+SIEC group ($P<0.05$), the DC-BxPC3 tumor vaccine+SIEC group ($P< 0.05$) and the negative control group was statistically significant. The tumor inhibition rates of the experimental group and the positive control groups were 46.01%, 35.12%, 37.21%, 29.73% and 29.46% respectively (**Table 1** and **Figure 2**). The pathological section of the transplanted tumor showed that the sensitized DC-BxPC-3 cells vaccine+SIECs group, DC-BxPC-3 cells vaccine+SIECs group and sensitized DC+SIECs group had more BxPC-3 apoptosis, and the negative control group had more of tissue liquefaction and necrocytoses of BxPC-3 implantation tumor (**Figure 3**).

Discussion

DC cells play an important role in the body's immune response against endogenous or exogenous antigens. Tumor patients have low immunity with a lower number of DC cells in the body compared to normal people, and the functioning of DC cells is worse than that in normal people. At the same time, tumor cells have the ability of immune escape and immune resistance [29]. Therefore, improving the anti-tumor ability of tumor patients has become a major breakthrough in anti-tumor treatment. DC vaccine immunotherapy can improve the overall anti-tumor immune ability of the body by cultivating sensitized DC cells *in vitro* and inputting them into tumor patients, so as to achieve the effect of anti-tumor growth and even kill tumor cells [30]. How to improve the anti-tumor ability of DC vaccine *in vivo* and improve the survival rate of tumor patients is an important goal and development direction of tumor immunotherapy research.

At present, the main methods of sensitizing DCs are tumor antigen loaded DC culture and antigen carrying tumor gene infecting DCs. In some studies, dendritic cell vaccine loaded with tumor stem cell (CSCs) antigen combined with low-dose TP (paclitaxel + cisplatin) was used to treat mouse breast cancer, and it was found that the CSCs DC vaccine combined with low-dose TP was more effective than TP alone [31]. In some experiments, recombinant adenovirus carrying human papillomavirus (HPV)-16 E6/E7 gene was infected with DC to obtain DC vaccine, which cooperated with cytotoxic T lymphocyte anti cervical cancer experiments in nude mice, and the result was that the DC vaccine modified with HPV-16 E6/E7 gene could promote the proliferation of T lymphocytes in mice, and induce CTL to inhibit the growth of the transplanted tumor of cervical cancer in nude mice [32]. Some researchers have treat-

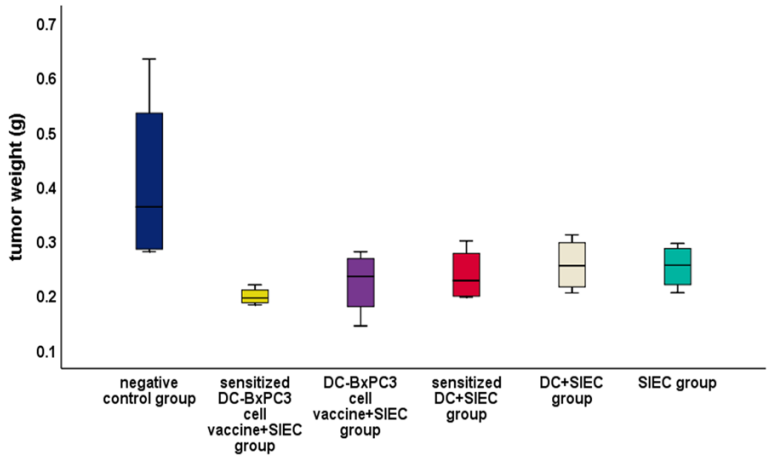

Figure 2. Inhibition of BxPC-3 cells growth in each treatment group *in vivo*. Note: The weight of the tumor in each group was analyzed by the Kruskal Wallis H test (P>0.05), and compared with the negative control group by Mann Whitney U test (*P<0.05).

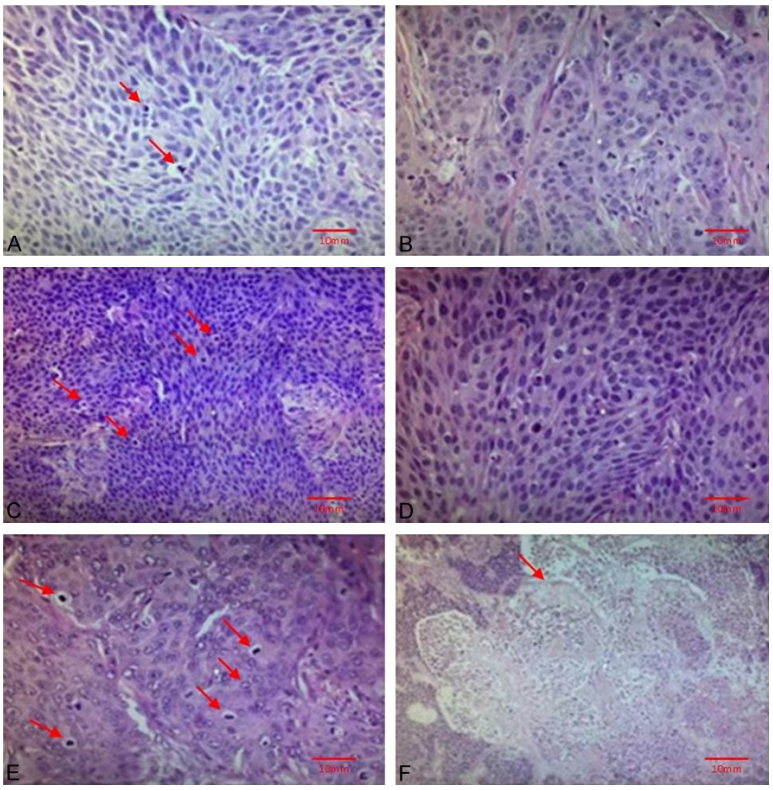

Figure 3. Pathological findings: The BxPC-3 cells apoptosis in the (A-C) are more obvious than (D) and (E) (H&E ×100, Scale bar. 10 mm). There is the tissue liquefaction and necrocytoses of BxPC-3 implantation tumor in the (F) (H&E ×100, Scale bar. 10 mm).

ed patients with pancreatic ductal carcinoma with DC immunotherapy containing nephroblastoma derived gene peptide (dc/wt1-i), which can result in a longer survival time [33]. However, in view of the 100-year history of tumor immunotherapy, the results of its clinical trials are unsatisfactory. The possible reasons for this may be that the immune cells such as DCs in tumor immunotherapy are derived from ill patients, the number of DCs in the patient's peripheral blood is less than that in healthy young people, and the DC in the patient's peripheral blood are dysfunctional. After *in vitro* treatment, their function and number still cannot reach the normal state, or due to the immune resistance of tumors and tumor-microenvironment, multifactor and multi-gene mutations, result in drug resistance [34-37]. Therefore, in order to improve the effect of DC immunotherapy on pancreatic cancer and other tumors, many factors should be considered. A systematic immunotherapy perspective is required, for instance, the source of DCs and immune effector cells, and the type and number of antigens presented by DCs should be considered, so as to maximize the autoimmune and antitumor ability of patients with pancreatic cancer and other tumors.

The safety of DC tumor vaccine is a key factor in the antitumor treatment of using a DC tumor vaccine. We used non-immunized mice as carriers to observe whether sensitized DC-BxPC-3 tumor vaccine, related reagents and tumor cells or tumor cell lysates can cause tumors, for safety evaluation. The results showed that sensitized DC-BxPC-3 tumor vaccine, related reagents and tumor cells or tumor cell lysates did not

Table 1. Inhibitory effects of immune effector cells on the growth of pancreatic cancer *in vivo*

Group	*N* (pair)	Median and interquartile range of tumor weight (g)	Tumor inhibition rate (%)
Negative control group	4	0.3636 (0.1458)	-
Sensitized DC-BxPC3 tumor vaccine+SIEC group	4	0.1963 (0.0096)*	46.01
DC-BxPC3 tumor vaccine+SIEC group	4	0.2359 (0.0402)*	35.12
Sensitized DC+SIEC group	4	0.2283 (0.0542)	37.21
DC+SIEC group	4	0.2555 (0.0556)	29.73
SIEC group	4	0.2565 (0.0418)	29.46

Note: The weight of the tumor in each group was analyzed by the Kruskal Wallis H test (P>0.05), and compared with the negative control group by Mann Whitney U test (*P<0.05).

form tumors in non-immune mice. Therefore, sensitized DC-BxPC-3 tumor vaccine immunotherapy is safe.

Another key to the anti-tumor therapy of the DC tumor vaccine is the efficient induction of immune response *in vivo* by DC tumor vaccine. Similarly, we used non-immunized mice as carriers to observe the anti-tumor effect of the sensitized DC-BxPC-3 tumor vaccine and its related DC tumor vaccine, or related inducers, to induce immune effector cells SIECs. The results showed that the inhibition rate of SIECs induced by sensitized DC-BxPC-3 tumor vaccine on BxPC-3 transplanted tumor was the highest (46.01%), which was significant compared with the negative control group. The pathological section showed that sensitized DC-BxPC-3 vaccine induced SIECs had a significant tumor apoptosis effect on BxPC-3 transplanted tumor cells, while the BxPC-3 transplanted tumor in the negative control group showed necrosis and liquefaction due to excessive growth. Therefore, the sequential DC tumor vaccine, namely sensitized DC-BxPC-3 tumor vaccine, has a more efficient anti-tumor effect on inducing immune effector cells [28, 38-40].

The biological efficacy experiment showed that SIECs had a certain inhibitory effect on transplanted tumors in non-immune mice, and the inhibitory effect was basically consistent with that of SIECs induced by antigen free DCs. The reason may be that DCs without antigen have no induction effect on SIECs. Systemic immune effector cells mutually activate and kill tumor cells.

The biological efficacy experiment also found that the tumor inhibition rate of DC-BxPC-3 tumor vaccine-induced SIECs on BxPC-3 xenografts was lower than that of sensitized DCs-induced SIECs on BxPC-3 xenografts. The reason may be that when DCs are fused with BxPC-3, the cells are damaged, resulting in the low initial function of the newly fused cells-BxPC-3 [28].

In this study, no difference in tumor inhibition rate between groups was found, which may be related to the low number of experimental animals (n=4). In addition, only one tumor cell line (BxPC3) was used in this study, which has certain limitations, and further experiments with multiple pancreatic cancer cells are needed. The source of DCs in this experiment needs to be further explored, including the difference between DC cell lines and primary DCs in this experiment, as well as the comparison of DCs derived from peripheral venous blood, bone marrow and umbilical cord blood.

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Disclosure of conflict of interest

None.

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