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DNT cell inhibits the growth of pancreatic carcinoma via abnormal expressions of NKG2D and MICA in vivo

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ABSTRACT

This research aimed to investigate the effects of natural killer group 2 member D (NKG2D) and its ligands major histocompatibility complex class I chain-related molecules A(MICA) in DNT cell killing pancreatic carcinoma. Antibodies adsorption was used to separate DNT cell from human peripheral blood. Human pancreatic tumor models were established via implanting BXPC-3 cells into nude mice. Then randomly divided mice into blank group, gemcitabine group and DNT group. Mice weights and mice tumor volumes were measured every 5 days. 50 days later mice were euthanized at cervical dislocation method. Tumor weights were measured. Relative tumor volume and tumor inhibition rate were calculated. Western blot and qPCR were used to detect the expressions of NKG2D and MICA in the transplanted tumors of the three groups. DNT cell significantly increased over time. The blank group tumor volume and weight were significantly larger than the other groups ($p < 0.001$, $p < 0.001$), but there were no significantly difference between DNT group and gemcitabine group ($p > 0.05$). Gemcitabine and DNT cell tumor inhibition rate were 40.4% and 35.5%. Western blot and qPCR showed that MICA mRNA and protein levels in blank group were significantly higher than DNT group ($p = 0.001$, $p = 0.003$). NKG2D mRNA and protein levels in blank group were significantly lower than DNT cells group ($p < 0.001$, $p = 0.001$). In conclusion DNT cell can significantly inhibit the growth of pancreatic carcinoma in vivo, and the mechanism may be involved in abnormal expressions of MICA and NKG2D.

1. Introduction

Pancreatic carcinoma is one of the highest aggressive digestive system malignant tumors, the common features of pancreatic carcinoma is “cancer”, invasion and metastasis are very easy in early stage and closely related to the anatomical location of individual characteristics, namely easy to infringement of peripancreatic blood vessels and nerves, led to the removal of technical difficulties, naturally, this is the fact that does not dispute [1,2]. A latest statistics showed that pancreatic carcinoma is the ninth most common malignant tumor and the fourth leading cause of cancer-related deaths worldwide, with a 5-year survival rate<5% [3]. So far, the problem of pancreatic carcinoma treatment is still one of the most difficult problems to overcome in human medical history. In addition to the traditional surgery, radiotherapy and chemotherapy treatment, biological treatment gradually becomes a hot topic in recent years. Presently, the focus of the pancreatic carcinoma biological therapy is the adoptive cell immunotherapy. Adoptive cell immunotherapy (ACI) refers to the activation of autograft or allograft immune effector cells in vitro, function characterization and amplification at first, then inject to tumor patients, stimulate the body's immune response or directly damage tumor cells, thereby achieve the goal of treatment of tumor, especially in pancreatic cancer. ACI has the potential to be the development direction in the future [4]. Today, the tumor immunotherapy research field facing the biggest problems is how to identify and select the appropriate immune cells [5]. DNT cell for its special antitumor effect come into people's horizons. DNT cell (Double negative T cell) is a kind of T cell subset which not only can regulate and suppress the immune response but also kill tumor cells [6,7]. DNT cell can widely identify tumor antigen and specificity to kill a variety of tumor cells, which has a considerable advantage and great potential in tumor immunotherapy [8–10]. Although for DNT cell researchs have advanced,

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but the mechanism of DNT cells killing tumor is still not quite sure. Recently research shows that γδ T cells killing lung cancer cells might regulate via NKG2D [10], which interests us.

NKG2D can activate human immune system through identifying target cell surface b activation induced related ligand to transmit signals, thereby performing a target antitumor effect [11]. MICA as the main ligand of NKG2D also plays an important role in immune surveillance of tumor cells. There is increasing evidence that ligand expression can result in both immune activation and tumor evasion [12,13]. Research shows that MICA-NKG2D played a role in disease pathogenesis in the majority of patients with lymphocyte leukemia and further investigation into this signaling axis may provide potent therapeutic targets [14]. Whether NKG2D and MICA also play a role in DNT cell worthy our study. This experiment mainly through the establishment of nude mice pancreas transplantation tumor model, to observe the tumor inhibition rate of DNT cell. Using qPCR and Western Blot to detect the expressions of NKG2D and MICA in the tumor tissues to verify that DNT cell can inhibit pancreatic carcinoma through the MICA- NKG2D way.

2. Material and methods

2.1. Pancreatic cancer cell line culture

Human pancreatic cancer cells BXPC-3 was purchased from Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. Cells cultured in RPMI 1640 medium containing 10% FBS(Gibco, USA) in the constant temperature incubator (37 °C, 5% CO_2). BXPC-3 cells were cultured with trypsin enzyme-digesting technique and passaged in vitro every 2–3 days. When cells growth condition is good in a logarithmic growth period, rinse it twice with PBS, digesting for 30 s, cell count, and centrifugal (1200 r/min, at room temperature, 5mins) collecting cells, then join with medium, gently blowing, single cell suspension was made (1×10^7/ml).

2.2. DNT cell isolation and culture

DNT cell isolation and culture: adopt the international newest application “antibody adsorption” method [8,9] to isolate DNT cell from peripheral blood in vitro. Mix the Human CD4 Depletion Cocktail and Human CD8 Depletion Cocktail (Stemcell Technologies, Canada) with 10 ml peripheral blood from healthy people, incubating at room temperature for 20 min then joining with 10 ml PBS containing 2% FBS. Then slowly moved it to 15 ml Ficoll–Paque human blood lymphocyte separation medium (GE Healthcare, USA), the operation of pipette to centrifugal pipe wall to separate blood slowly drip into the lymph fluid, maintain stability of the interface. Centrifugal (1200 r/min, room temperature, 20 min), absorbs the gradient of the mist layer cells divide among serving OK3 antibodies (EBioscience, USA) after washing the 24 hole culture plate, the culture RPMI 1640 contains 2 ml recombinant cytokines IL-2, IL-4 (EBioscience, USA) and 10% FBS in the constant temperature incubator at 37 °C, 5% CO_2. Once every three days later in liquid, we collect the cells from the wells on day 12.

3. Establishment of animal model

In order to verify the DNT cells inhibition of pancreatic carcinoma. 30 SPF grade female Balb/c nude mice were used to establish a human pancreatic tumor model by subcutaneous injection of BXPC-3 cells (10^7 cells/mouse) into the right side of the armpit. The mice were aged 4–5 weeks and weighing 15–20 g from the Shanghai Slac Laboratory Animal Co. Ltd. The mice were feed in super clean laminar flow cabinet under the SPF condition in the animal experiment center of Anhui province hospital. Mice were used for all experiments and all procedures were permitted by the Animal Ethics Committee of Affiliated Provincial Hospital of Anhui Medical University. When the tumor volume reached about 50 mm^3, mice randomly average divided into three groups.

(1) blank group, (2) gemcitabine group: intraperitoneal injection of gemcitabine at a dose of 50 mg/kg, twice a week, total four times. (3) DNT group: DNT cell, (1×10^7, 0. 2 ml) through the mice caudal vein, twice a week, total four times. The mice body weights (BW) were measured every 5 days. Tumor diameters were measured with vernier caliper.

The tumor length (long diameter, A) and width (short diameter, B) were separately recorded every 5 days to calculate tumor volume (TV). And observing mice vitality and deaths. TV was calculated by the following formula: TV (mm^3) = (A, mm) × (B, mm)^2/2.

Relative tumor volume (RTV) was calculated on the basis of TV by the following formula:

$$\mathrm{RTV} = \mathrm{TV\ on\ measurement\ day}/\mathrm{TV\ on\ group\ assignment\ day}.$$

The animals were sacrificed by cervical dislocation, and the tumor was excised. After removal of adherent tissues, they were rinsed with saline, with removal of excess water using a paper towel, then tumor weight (TW) was measured. Tumor inhibition rate was also calculated by the following formula:

$$\text{tumor inhibition rate} = (\text{blank group} - \text{experimental group}/\text{blank group}) \times 100\%.$$

4. Quantitative real-time PCR analysis

qPCR was performed to investigate the MICA and NKG2D mRNA in mice pancreatic tissues. Total RNA was isolated from mice pancreatic tissue samples using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA) and cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (Invitrogen, USA). The PCR conditions were as follows: initial denaturation (95 °C, 5 min), followed by 40 cycles of denaturation (95 °C, 30 s), annealing (60 °C, 30 s) and extension (72 °C, 30 s) and a final extension step of 72 °C for 10 min, experiment was repeated three times. β-actin as standardization of RNA quality control. Relative gene expression data was analyzed by 2^{Ct} method [15]. All primers (Table 1) were purchased from Invitrogen.

5. Western blot analysis

For western blot, total proteins from mice tumor tissues were extracted, quantified, and subjected to 12% (w/v) SDS–PAGE. The gel was then transferred onto a PVDF membrane (Millipore, USA) in a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in Tris-buffered saline containing 0.2% Tween-20 (TBST) for 1 h with 5% non-fat dry milk and then membranes were incubated in primary antibody (rabbit anti-MICA antibody and goat anti-NKG2D antibody, Santa Cruz, USA) both diluted 1:500 at

Table 1
The primers of MICA, NKG2D and β-actin.

	Forward primer	Reverse primer
β-actin	5′-CCCATCTATGAGGGTTACGC-3′	5′-TTTAATGTCACGCACGATTTC-3′
MICA	5′-CAGGGCTTCTGGCTTCTATC-3′	5′-TGTCGTGGCTCAAAGATACC-3′
NKG2D	5′-TACTGTGGCCCATGTCCTAA-3	5′-CTTTCAGAAGGCTGGCATTT-3′

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