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Inhibiting effect of immunoeffector cells induced by denderitic cells vaccine on growth of PC3 and BEL7402

Zao–Xi Sun[1]✉, Zheng–Ke Sun[1], Kai Li[1], Ping Long[1], Ya–Chen Xu[1], Qiu–Xi Yang[1], Hai–Rong Huan[2]✉, Cheng–Yi Sun[3]

[1]*Department of Hepatobiliary Surgery, First Affiliated Hospital of Hainan Medical University, Haikou, Hainan 570102, P.R. China*

[2]*Department of Epidemiology, School of Public Health of Hainan Medical University, Haikou, Hainan 571199, P.R. China*

[3]*Department of Surgery, Affiliated Hospital of Guiyang Medical University, Guiyang Guizhou, 550004, P.R. China*

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ABSTRACT

Objective: To compare effect of immunoprevention or immunotherapy based on denderitic cells (DCs), or supernatants on pancreatic carcinoma and hepatocelluar carcinoma *in vitro* and *in vivo*. **Methods**: DCs and monouclear cells (immunoeffecetor cells) were stimulated with hGM-CSF, hIL-4, hTNF-α, PC3 TA or BEL7402 TA and hIL-2, then DCs and immunoeffector cells were cocultured, and supernatants were harvested. *In vitro*, the immunoeffector cells were divided into A0 group (without DCs stimulated), A1 group (DCs stimulated, cultured with cytokines cocktail), A2 group (DCs stimulated, cultured with cytokines cocktail and tumor antigen, DCs vaccine). Cytoxicity assay was performed with lactate dehydogenase method. *In vivo*, the nude mice were allocated in 3 groups: prevention group, receiving immunoeffector cells activated by DCs vaccine 2 days before inoculation with PC3 or BEL7402; treatment group, receiving immunoeffector cells activated by DCs vaccine after development of implanted tumor in all nude mice; control group, receiving equivalents amount of RPMI1640 cultured liquid. On the 45th day, all the nude mice were sacrificed and the tumor was weighed. **Results**: The maximal inhibition rate of the A0, A1 andA2 were 3.5%, 68.1%, 81.0% in the BEL7402; 4.5%, 33.0%, 62.4% in the PC3. The differences in tumor weight among three groups were significant, but the differemce were not significant between the PC3 and BEL7402. **Conclusions**: DCs vaccine or supernatants may play an important role in treating and preventing against malignant tumor.

1. Introduction

Pancreatic carcinoma (PC) and hepatocellular carcinoma (HCC) are very common malignant tumors with the unsatisfied progonsis. It is first time to treat PC and HCC by standardization and comprehence ways in order to increase general curative effect[1-4]. Immunotherapy

✉Corresponding author: Prof. Zao-Xi Sun, No 31 Longhua Road, Haikou, Hainan Province, 570102 P.R. China.
Tel: 57-0898-66509465
E-mail: 13648665696@163.com
Prof. Hai-Rong Huan, No 3 Xueyuan Road, Haikou, Hainan Province, 571199P.R., China.
Tel: 57-0898-66960312
E-mail: 1822142889@qq.com

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in comprehensive treatment is to improve autologous immunity efficacy of antitumor[5]. Dendritic cells (DCs) are the best antigen presenting cells in nature, and play a pivotal role in the induction of specific T cell-responses, including the immune response against invading pathogens such as bacteria, viruses and malignant tumor[4,6]. Our study is to compare effect of immuneotherapy or immuneoprevention based on DCs vaccines or supernatants on PC and HCC. We found that peripheral blood mononucelear cells (PBMC) induced by tuomr antigen-pulsed-DCs vaccines could effectively inhibit the growth of tumor cell line PC3 cells and BEL7402 cells. These results may provide an immunotherapy or immunoprevention of autologous MoDCs vaccines for clinical application on PC and HCC patients.

2. Materials and methods

2.1. Materials

Phosphate buffered solution (Division of Biological Therapy, Belijing 302 Hospital of P.L.A.,China), raw blood (normal Chinese volunteers), lymphocyte separation solution (Pharmacial, USA), various cultured flasks (Sigma, USA), RPMI1640 supplemented with 1% *L*-glutamine and fetal bovine serum (Gibco, USA), human interleukin-2, human interleukin-4, human grnulocty-macrophage colony stimulating factor and tumor necrosis factor (hIL-2, hIL-4, hGM-CSF and hTNF-α, Peprotech, England), Cyto Tox 96~Non-Radioactoxic Assay kit (Promega,USA). Cells: HCC cell line BEL7402 (Division of Biological Therapy, Belijing 302 Hospital of P.L.A., China), PC cell line PC3 (Chinese Xieh Medical University). Aniamals: Balb/C nude mice (6-8 week old, Animal Center of Beijing Medical University, China).Tumor antigens were prepared from BEL7402 and PC3 cell line.

2.2. Methods

2.2.1. Produce tumor antigen of BEL7402 and PC3

PC cell line PC3 and HCC cell line BEL7402 were separately split by freezing and thawing at the -70 ℃(30 min) and 37 ℃(5-10 min) for three times. The antigen of the PC3 and BEL7402 were obtained, and there were no cells or no cells growth when materials splitted. They were cultured *in vitro* and observed under microscope, and then kept it at -20 ℃[7].

2.2.2. Separation of immunoeffector cells and DCs

Antiagglutinating raw blood in the same volume of phosphate buffered solution was diluted, and the diluted blood was added in the centrifugal tube with the same volume of lymphocyte separation solution (d=1.077 g/L), centrifugalized for 30 min (800×*g*, at the room temperature). PBMC were harvested, washed with phosphate buffered solution and centrifugalized for 20 min (400×*g*, 250×*g*, 250 ×*g*, at the room temperature) for three times. Washed PBMC (1×10^7/mL) were cultured for 3 h in 75 cm^2 cultured flasks in a humidified chamber at 37 ℃, 5% CO_2. The suspended PBMC was harvested separately and the DCs adhered to wall of the flask (digested by 0.20% pancreatic elastase supplemented with 0.20% EDTA).

2.2.3. Generation of immunoeffector cells

The immunoeffector cells (1×10^6/mL) was cultured with RPMI1640 supplemented with 1% *L*-glutamine, 10% fetal bovine serum for 6 d in the 75 cm^2 cultured flasks in a humidified chamber at 37 ℃, 5% CO_2. On first day, hIL-2 100 U/mL was added. 1/3 RPMI1640 culture solution and hIL-2 was exchanged every 2 d.

2.2.4. Preparation of DCs vaccine

DCs (5×10^5/mL) was cultured with RPMI1640 supplemented with 1% *L*-glutamine, 10% fetal bovine serum for 5 d in the 75 cm^2 cultured flasks in a humidified chamber at 37 ℃, 5% CO_2. On first day, hGM-CSF 500 U/mL and hIL-4 500 U/mL were added. On the second day, the DCs were divided into four groups, with BEL7402 or PC3 antigen pulsed (PC3 cells or BEL7402 cells:DCs=1:10) for two of them, on fourth days, hTNF-α 1 000 U/mL was added to all the four groups. To exchange 1/3 RPMI1640 culture solution, hIL-4 and hGM-CSF was exchanged every 2 d to every group, too.

2.2.5. Immunoeffector cells induced by DCs vaccine

On the sixth day, in every group DCs vaccine were mixed with immnuoeffector cells (DCs:immunoeffector cells=1:10) and were cultured for one day in 150 cm^2 cultured flasks in a humidified chamber at 37 ℃, 5% CO_2.

2.2.6. Harvesting and keeping of supernatant

The supernatant of DCs cultured with tumor antigen or DCs cultured with cytokines cocktail were collected and kept at -20 ℃.

2.2.7. Cytotoxicity assay

Immunoeffector cells cytotoxicity was measured *in vitro* using lactate dehydogenase method (refer to directions of Cyto Tox 96~Non-Radioactoxic Assay kit). Immunoeffector cells were divided into A0 group (no DCs vaccine stimulated), A1 group (with DCs vaccine stimulated, cultured with cytokines cocktail), A2 group (DCs vaccine stimulated, cultured with cytokines cocktail and antigen of BEL7402 or PC3). Vigorous growth of BEL7402 cells or PC3 cells (2.0×10^4/100 μL/well, as target cells) were co-cultured separately with immunoeffector cells A0, A1 and A2 group at various ratios (effector:target=2.5:1, 5:1, 10:1, 20:1, 40:1, effector cell dilutions:100 μL/well) in 96-well round-bottomed plated for 8 h in a humidified chamber at 37 ℃, 5% CO_2. The cells of the target cell maximum lactate dehydogenase release control were completely lysed by lysis solution. After centrifugalizd for 4 min at 250 g, 50 μL aliquots were transferred from all wells to a fresh 96 well flat-bottom plate, 50 μL of reconstituted substrate mix was added to each well and was protected from light for 30 min at room temperature. Then 50 μL of stop solution was added to each well. Larg bubbles were popped, and the absorbance was recorded at 490 nm with enzyme-linked immunosorbet assays within 1 h at room temperature after the addition of stop solution. The percentage of cytotoxicity was calculated according to the following formula:

% cytotoxicity= (Experimental-Effector spontaneous-Target spontaneous)/(Target maximum-Target spontaneous)×100.

2.2.8. Animal models

A total of 36 Balb/C nude mice (6-8 wk old, Animal Center of Beijing Medical University, China, whole-body irradiation with 60 Co 200 Gy/mouse) were allocated into 3 groups: control group receiving equivalents amout of 10% RPMI1640 cultured medium in the front shoulder subcutaneous after developing implanted tumor of the PC3 or BEL7402; prevention group, 2 days before inoculation of tumor cells BEL7402 or PC3 in the front shoulder subcutaneous, received immunoeffecetor cells (1.0×10^8/nu-mouse/0.25 mL) induced by DCs vaccine (cultured with cytokines cocktail and the tumor antigen BEL7402 or PC3) in other side front shoulder subcutaneous; treatment grou, receiveing immunoeffecetor cells (1.0×10^8/nu- mouse/0.25 mL) induced by DCs vaccine (cultured with cytokines cocktail and the tumor antigen PC3 or BEL7402) in the same front shoulder subcutaneous tumor cells (PC3 or BEL7402) after developing implanted tumor (Diameter⩾2-4 mm). There were 6 nude mice per group for the BEL7402 or PC3, and were inoculated with 0.5×10^6 tumor cells of PC3 or BEL7402 /nu-mouse/0.25 mL. DCs cultured supernatant (0.3 mL/nu-mouse) was intermittently administrated 6 times in the same place of immunoeffector cells for the prevention group and treatment group.The response of nude mice on the first day was observed after inoculation of all kinds of cells at first time, and then observed every 3 days. All nude mice were sacrificed when implanted tumor emerged for 45 d tumor was weighed.

2.3. Statistics analysis

Results were expressed as mean ± SD, *Q* test was used for statistical analyis of the difference by SPSS12.0 statistical software. α =0.05 was regaredes as standard of test, and the difference was significant as P<0.05.

3. Results

3.1. Enchancement of immunoeffector cells cytotoxicity in vitro

In vitro study, killing rate of immunoeffective cells at various ratios (effector: target=2.5:1, 5:1, 10:1, 20:1, 40:1) were as Figure 1. The maximal inhibition rate of the A0, A1 andA2 were 3.5%, 68.1%, 81.0% in the BEL7402; 4.5%, 33.0%, 62.4% in the PC3. The rate of A2 was the highest at all ratios in HCC cells BEL7402 and PC cells PC3, followd by A1.

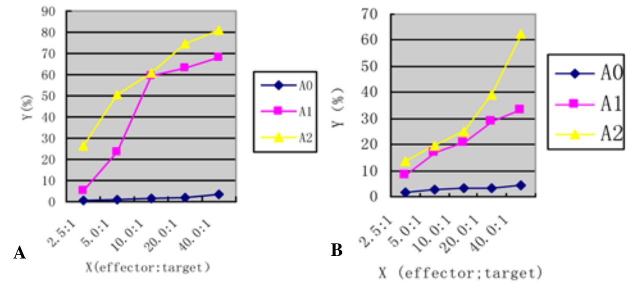

Figure 1.
Cytotoxicity assay of immunoeffector cells induced by DCs vaccine to BEL7402 (A) and PC3 (B).

3.2. Inhibiting effect of immunoeffector cells on tumor growth in vivo

Nude mice of the control group and treatment group in the PC3 and BEL7402 all developed implanted tumor on the 12th day. Among the 12 nude mice of the prevention group, on the 30th day and 45th day, one nu-mice challenged with BEL7402 developed implanted tumor, two challenged with PC3 developed implanted tumor (Table 1). The differences in weight of tumor among three groups were significant (P<0.01 and P<0.05 for BEL7402 and PC3 respectively), but the differemce were not significant between the PC3 and BEL7402 (P>0.05).There was no metastasis of implanted tumor in mice challenged with BEL7402 or PC3 of prevention group and treatment group, and no infiltrations of implanted tumor in mice of challenged with BEL7402 or PC3 of prevention group. But one mouse challenged with BEL7402 and another one challenged with PC3 showed infiltrations of implanted tumor in treatment group. Implanted tumor metastasis occurred in two mice challenged with BEL7402 and another two challenged with PC3 in the control group. All mice challenged with BEL7402 and PC3 in the control group developed infiltration.

Table 1
Inhibiting effect of immunoeffector cells on tumor growth *in vivo*.

Groups	*n*	Rate of implanted tumor		Weight of implanted tumor	
		PC3	BEL7402	PC3	BEL7402
Control	6	6/6	6/6	1.98±0.43	1.40±1.06
Prevention	6	2/6	1/6	0.14±0.21*	0.04±0.11△
Treatment	6	6/6	6/6	0.79±0.46△	0.72±0.54△

* compared with control group, P<0.05; △compared with prevention group, P<0.05.

4. Discussion

4.1. In vitro study

In vitro study, the killing rate of immunoeffector cells (no DCs vaccine stimulated, effector: target=40:1) were separately 3.5% and 4.5% against the BEL7402 and PC3. The results proved that the immunoeffector cells might activate each other and had very low anti-tumor capability. Some scholars have found that a coordinated cellular interplay is of crucial importance in both host defense against pathogens and malignantly transformed cells. The various interactions of DC, natural killer cells, and T helper cells can be influenced by a variety of pathogen-associated molecular patterns (PAMPs), and the Fas ligand-mediated cytotoxicity is initiated in natural killer cells through ligation of their activating receptors[8,9]. The killing rate of immunoeffector cells induced by DCs vaccine (DCs culture with cytokines cocktail, effector:target=40:1) were 68.1% and 33.0% against the BEL7402 and PC3, which indicated that the DCs pulsed by cytokines cocktail might enchance cytotoxicity of immunoeffector cells antitumor. Some experts have found that DC vaccines might induce long-term specific anticancer responses with immune memory cells, which could contribute to effective and lasting elimination of malignant cells. DC therapy, targeting activation and regulation of T cells, oncolytic virus vaccines and adoptive T cell therapies will all be considered, regarding the current situation and avenues for future exploration. Growth in either serum-containing or serum-free media supplemented with GM-CSF and IL-4 yielded a similarly heterogeneous population of cells. Tumor necrosis factor-alpha significantly augmented the number of these mature DC. The DC-CIK immunotherapy combined with TACE can improve the patients' progression-free survival time and the quality of life of the patients with advanced hepatocellular carcinoma and show good treatment safety[10-13]. The maximal killing rate of immunoeffector cells induced by DCs vaccine (DCs culture with cytokines cocktail and tumor antigen, effector:target=40:1) were 81.0% and 62.4% to the BEL7402 and PC3, which showed that the immunoeffector cells induced by pulsed DCs with cytokines cocktail and tumor antigen had best antitumor potential. The experts consider that the potent vaccines stimulate antigen presentation by DCs, hence driving the expansion of antigen-specific effector and memory T cells. Any mutation of tumor cells, they would always incite DC propagation and maturation, pulsing and antitumor immunity. Personalized DC-based anticancer vaccines in theory have the potential to present to the host immune system the entire repertoire of antigens harbored by autologous tumor cells[14-16]. The mechanism of differences in the efficacy of the killing rate of immunoeffector cells induced by DCs vaccine aginst the BEL7402 and PC3 needs to be studied furtherly.

4.2. In vivo study

In vivo study showed that immunoeffector cells induced by DCs vaccine (DCs culture with cytokines cocktail and tumor antigen) could effectively inhibit growth, infiltration and metastasis of implanted tumor. In the prevention group, we found there were one mouse challenged with BEL7402 developed implanted tumor, two nu-mice challenged with PC3 developed implanted tumor, on the 30th day and 45th day. A total of 24 nude mice in the control group and treatment group developed implanted tumor on the 12th day. The differences in the weight of implanted tumor among three group were significant (P<0.01 and P<0.05 for BEL7402 and PC3 respectively). The mice challenged with BEL7402 or PC3 of prevention group and treatment group showe no metastasis. Some scholars have discussed the perspectives of DC-based anti-tumor immunotherapy and optimizing strategies of DC vaccination in humans in light of results obtained in mouse models. Multi-cytokines or tumor allogenic antigen stimulated normal DC to induce immunoeffector cells may play an important role in the prevention and treatment of malignant tumor in human hepatocellular carcinoma cell line BEL-7402 and human pancreatic carcinoma cell line PC-3[17-19], but two cases challenged with BEL7402 and another two cases challenged with PC3 in control group had implanted tumor metastasis. Every nude mice in the control group developed implanted tumor infiltration. One nude mouse challenged with BEL7402 and another one challenged with PC3 in the treatment group has infiltration, and no infiltration occurred in the prevention group. These results indicted that the DCs and immunoeffector cells induced by DCs vaccine could secrete cytokines cocktail of anti-tumor, and they had coordinative anti-tumor potential *in vitro* and *in vivo*. The experts have found that the DC-based vaccination can stimulate an antitumoral T cell response in patients with advanced or recurrent pancreaticcarcinoma receiving concomitant gemcitabine treatment. HCC/DRibbles-pulsed DCs immunotherapy might be useful for suppressing the growth of residual tumors after primary therapy of human HCC. Treatment with the improved DC vaccine which was tumor cell lysate pulsed with M2 and OK (HMO-D), compared with H-D and HM-D, significantly increased cell surface markers (MHC-Ⅰ and Ⅱ, CD40, CD80, CD86 and CD11c) expression on DCs, enhanced Th1-type cytokines (IL-12, TNF-α and IFN-γ) production. Immunization with HMO-D effectively reduced tumorprogression and enhanced the survival of mice with H22 tumors. β-elemene combined DC/Dribble vaccine could induce specific immune cells to secrete secretory cells. The immunological effects might be associated with enhancing the DC antigen presenting function[20-23]. But, the differences of implanted tumor weight between the BEL7402 and PC3 was no significant (P>0.05), the reason might be that DCs vaccine and immunoeffector cells from normal person were unspecific.

In summary, immunoeffector cells induced by DCs vaccine could effectively prevent and inhibit growth, metastasis. Infiltration of tumor; the immunoeffector cells and DCs vaccine from normal person have general cellullar immune effect. The coadminstration with supernatant of DCs cultured and immunoeffector cells induced by DCs vaccine is a potential cocktail way to inhibit malignant tumor.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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