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ORIGINAL ARTICLE

Basic Study

Dendritic cells engineered to secrete anti-DcR3 antibody augment cytotoxic T lymphocyte response against pancreatic cancer *in vitro*

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Abstract

AIM

To investigate the enhanced cytotoxic T lymphocyte responses against pancreatic cancer (PC) *in vitro* induced by dendritic cells (DCs) engineered to secrete anti-DcR3 monoclonal antibody (mAb).

METHODS

DCs, T lymphocytes and primary PC cells were obtained from PC patients. DCs were transfected with a designed humanized anti-DcR3 monoclonal antibody heavy and light chain mRNA and/or total tumor RNA (DC-tumor-anti-DcR3 RNA or DC-total tumor RNA) by using electroporation technology. The identification, concentration and function of anti-DcR3 mAb secreted by DC-tumor-anti-DcR3 RNA were determined by western blotting and enzyme-linked immunosorbent assay. After co-culturing of autologous isolated PC cells with target DCs, the effects of secreting anti-DcR3 mAb on RNA-DCs' viability and apoptosis were assessed by MTT assay and flow cytometry. Analysis of enhanced antigen-specific immune response against PC induced by anti-DcR3 mAb secreting DCs was performed using a ^{51}Cr releasing test. T cell responses induced by RNA-loaded DCs were analyzed by measuring cytokine levels, including IFN-γ, IL-10, IL4, TNF-α and IL-12.

RESULTS

The anti-DcR3 mAb secreted by DCs reacted with

recombinant human DcR3 protein and generated a band with 35 kDa molecular weight. The secreting mAb was transient, peaking at 24 h and becoming undetectable after 72 h. After co-incubation with DC-tumor-anti-DcR3 RNA for designated times, the DcR3 level in the supernatant of autologous PC cells was significantly down-regulated ($P < 0.05$). DCs secreting anti-DcR3 mAb could improve cell viability and slow down the apoptosis of RNA-loaded DCs, compared with DC-total tumor RNA ($P < 0.01$). The anti-DcR3 mAb secreted by DC-tumor-anti-DcR3 RNA could enhance the induction of cytotoxic T lymphocytes (CTLs) activity toward RNA-transfected DCs, primary tumor cells, and PC cell lines, compared with CTLs stimulated by DC-total tumor RNA or control group ($P < 0.05$). Meanwhile, the antigen-specific CTL responses were MHC class I-restricted. The CD4+ T cells and CD8+ T cells incubated with anti-DcR3 mAb secreting DCs could produce extremely higher level IFN-γ and lower level IL4 than those incubated with DC-total tumor RNA or controls ($P < 0.01$).

CONCLUSION

DCs engineered to secrete anti-DcR3 antibody can augment CTL responses against PC *in vitro*, and the immune-enhancing effects may be partly due to their capability of down-regulating DC apoptosis and adjusting the Th1/Th2 cytokine network.

Key words: Dendritic cell; Antibody-encoding RNA; DcR3; Cytotoxic T lymphocyte response; Pancreatic cancer

Core tip: Dendritic cells co-transfection with tumor-associated antigens RNA and humanized anti-DcR3 monoclonal antibody mRNA may augment cytotoxic T lymphocyte responses against pancreatic cancer *in vitro*. This finding lays a good foundation for further investigation of tumor dendritic cells' vaccine targeting DcR3 protein against pancreatic cancer.

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INTRODUCTION

Pancreatic cancer (PC) is the fourth leading cause of cancer-related deaths in the US, with 40560 deaths in 2015 alone[1,2]. It has an extremely poor prognosis with a total 5-year survival rate of < 5%[3]. The poor prognosis and high mortality rate in PC patients may be attributed in part to lack of effective treatments[2]. Existing therapies for PC are limited to systemic chemotherapy and surgical resection. However, neither of these two strategies can cure PC completely[4]. Thus, more effective therapeutic methods are urgently needed.

Cellular immunotherapy is a promising alternative that is currently considered the fourth line of cancer treatment[5]. In this approach, different kinds of immune cells, such as cytokine-induced killer cells, lymphokine-activated killer cells, natural killer (NK) cells and dendritic cells (DCs) are adopted for immunotherapy. Of these, DC is the most commonly used immune effector cell because of its potent antigen-presenting function in the initiation of antitumor immune responses and its pivotal function in cancer immunosurveillance. Cytotoxic T lymphocytes (CTLs) are capable of eliminating cancer cells directly *in vivo*, but their activities are primarily managed by DCs. The use of DC-based tumor vaccines has therefore become a promising alternative treatment method for cancer[2,6].

In clinical practice, antigen choice is essential in the design of an effective vaccine. Because of lacking the expression of MHC class II molecules and co-stimulatory molecules, PCs, with low level of expression of tumor-associated antigens (TAA), display weak antigenicity and high heterogeneity[7]. Therefore, loading whole antigens from PC cells may be an alternative method that can both generate a broad T cell immune response to TAA and reduce the possibility of PC escape from immune recognition. We have previously reported that DCs transfected with total tumor RNA can effectively induce anti-PC tumor-specific CTL responses[2]. However, although this method has been demonstrated to generate a vaccine-induced rise in tumor-specific cells, the immune and tumor reactions stay modest, suggesting the need for novel strategies to improve antitumor immunity[8]. Evidently, one cause of this insufficiency is that tumor cells can produce certain immunosuppressive molecules to induce an immunosuppressive microenvironment and inhibit the function of tumor-associated cells, such as T lymphocytes and DCs[9]. Decoy receptor (DcR) 3 is possibly one of these cells in the tumor microenvironment (TME)[10].

DcR3 is a decoy receptor for Fas ligand (FasL) and is a member of the tumor necrosis factor receptor (TNFR) superfamily[11]. DcR3 displays inducible expression, interacts with the herpes virus entry mediator (HVEM) and is presented by TNF-like molecule 1A (TL1A) and T lymphocytes (LIGHT)[12,13]. DcR3 lacks a transmembrane domain and works as a secreted protein instead of a membrane-bound one. DcR3 can bind to LIGHT and FasL, thereby blocking the interaction between LIGHT and LT-receptor (LTβR) or HVEM to inhibit the apoptosis induced by Fas-FasL interaction or LIGHT-mediated biological effects. DcR3 is frequently overexpressed in various

tumors, including lung cancers[14], gastrointestinal tract tumors[15], virus-associated lymphomas[16], and PCs[17]. It has been reported that overexpression of DcR3 is correlated with shortened total survival time of cancer patients[18]. DcR3 has been postulated to promote tumor growth by escaping FasL- and LIGHT-mediated immunosurveillance. Specifically, DcR3 is able to suppress the activation and differentiation of DCs and macrophages[19], enhance osteoclast differentiation and angiogenesis[20], and sensitize T lymphocytes to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in cancer patients[21]. In addition, DcR3 is also regarded as an important immunosuppressive factor in defects associated with immune effector cell function. Therefore, we sought to determine whether neutralizing DcR3 expression in the TME can augment the CTL responses against PC *in vitro* induced by DCs loaded with total tumor RNA.

In the current study, we evaluated the novel approach of co-transfecting DCs with total tumor RNA and mRNA encoding humanized heavy (H) and light (L) chains of an anti-human DcR3 mAb together to achieve anti-DcR3 protein stimulation. Through co-culturing of autologous isolated PC cells with DCs, we found that DCs transfected with these RNAs secrete operational immune modulating proteins that can reduce DcR3 expression in TME of cultured PC cells. Then we demonstrated that CTLs induced by DCs co-transfected with total tumor RNA and anti-DcR3 monoclonal antibody (mAb) mRNA show more effective cytotoxic activities against PC cells *in vitro* compared with DCs loaded only with total tumor RNA alone. Furthermore, the immune-enhancing effect of DCs engineered to secrete anti-DcR3 mAb is partly due to their capability of down-regulating apoptosis of DCs and adjusting the T helper (Th)1/Th2 cytokine network. These findings are crucial for the development of tumor DC vaccines targeting DcR3 protein against PC.

MATERIALS AND METHODS

Patient eligibility and tumor cells preparation

Fifteen HLA-A2+ PC patients (9 males and 6 females; median age of 53.5 years, ranging from 35 years to 72 years) were included in this study. According to the TNM classification of AJCC[22], there were 10 stage II patients and 5 stage Ⅲ patients. The location of tumor was divided into head (7 cases) and body/tail (8 cases). All patients underwent surgical resection and were pathologically diagnosed with invasive ducal adenocarcinoma.

Peripheral blood monocyte cells (PBMCs), isolated by Ficoll-Hypaque (Sigma, St Louis, MO, United States) density gradient separation, and was used as the nonmalignant control tissues. Pancreatic cancer specimens were obtained at the time of surgery and were stored in RNAlate (Ambion, Austin, TX, United States) at 4 ℃ until processing.

Autologous tumor cells were obtained as described by Wang *et al*[23]. Approximately 10 g of each tumor specimen was harvested in the operating room for primary cell culture. The tumor tissue was mechanically disrupted to generate approximately 1 mm^3 sections. The tissue was digested in 10 mL of RPMI-1640 medium supplemented with 0.05% collagenase (Hyclone, South Logan, UT, United States) with gentle agitation at room temperature for 4-6 h. After culturing for 7 d, the immunohistochemistry technique was used to detect the expression of DcR3 protein (anti-DcR3 mAb obtained from Sigma).

The human PC cell lines Capan-2 (HLA-A2+) and AsPC-1 (HLA-A2-), as well as the leukemia cell line K562, were obtained from the American Type Culture Collection (Manassas, VA, United States). The cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Hyclone), 50 U/mL penicillin, and 50 mg/mL streptomycin (Hyclone). All cells were cultured for 7 d and maintained in the logarithmic phase growth at 37 ℃ in a humidified atmosphere supplemented with 5% CO_2.

Preparation of RNA

Total cellular RNA was extracted from autologous PC cells and PBMCs by using TRIzol Reagent (Sigma) according to the manufacturer's instructions. Only RNA exhibiting a ratio of 28S:18S > 1 was subjected to further analysis.

Total RNA of anti-human DcR3 hybridoma clone 1B1 (a kindly gift from Dr. CF Wu of Jilin University, China)[24], was isolated with the RNeasy mini kit (Qiagen, Valencia, CA, United States). Five micrograms of RNA were used in a reverse transcription reaction with the RT primer 5′- ATT CTA GAG GCC GAG GCG GCC GAC ATG (T-30) VN-3′ and PowerScript RT (Clontech, Mountain View, CA, United States) and the primer 5′-AAG CAG TGG TAT CAA CGC AGA GTG GCC ATA TTG GCCr GrGrG 3AmMC7/-3′. The heavy (H) chain was amplified from 10% of the RT reaction by using Advantage 2 HF PCR mix (Clontech) and the primers 5′- AAA GAA TTC GGC CTT GTT GGC CTC ATT TAC CCA GAG ACC GGG AGA TG -3′ and 5′-GAA AAG CTT GGC CAT TGG GGC GGT ATC AAC GCA GAG TGG CCA TAT TG-3′. The L chain was amplified with the primers 5′-AAG AAT TCG GCC TTG TTG GCC TAA CAC TCA TTC CTG TTG AAG CTC TTG-3′ and 5′-GAA AAG CTT GGC CAT TGG GGC GGT ATC AAC GCA GAG TGG CCA TAT TG-3′. The resulting PCR fragments were digested with *Hind*Ⅲ and *Eco*RI and cloned into the *Hind*Ⅲ and *Eco*RI sites of the plasmid pSP73-Sph/A64, which possesses a T7 promoter and 64T nucleotides that allow for the production of *in vitro* transcribed RNA with a *polyA* tail of 64 residues. The gene encoding the full-length enhanced actin (as controls) was inserted into the pSP73-Sph/A64 plasmid, as well.

In vitro transcription of mRNA

All plasmids were digested with *Spe*I for use as a template for *in vitro* transcription reactions using the

mMESSAGE mMACHINE T7 kit (Ambion) according to the manufacturer's protocol. mRNA was purified with the RNeasy mini kit.

Generation and electroporation of DCs

DCs' generation was performed as previously described by Zhu *et al*[25]. A concentrated leukocyte fraction was isolated from PBMCs that processed 200 mL of blood during each collection. Leukapheresis products were separated by density-gradient centrifugation over polysucrose sodium diatrizoate (Sigma), and cells were resuspended in serum-free AIM-V medium (Gibco, Burlington, Canada). Cells were incubated in a humidified incubator for 2 h at 37 ℃ to allow plastic adherence. The non-adherent fraction was removed, and the adherent cells were cultured for 7 d in serum-free AIM-V medium supplemented with human rIL-4 (500 U/mL) and recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) (800 U/mL) (R&D Systems, Minneapolis, MN, United States) at 37 ℃ under 5% CO_2.

The DCs were transfected with RNA using a Gene Pulser II (Bio-Rad, Hercules, CA, United States). After 7 d, the immature DCs were transferred into a low-conductance medium (Cytofusion Medium Formula C; CytoPulse Sciences, Columbia, MO, United States) after centrifugation at 170 × *g* at 4 ℃ for three times, each time for 7 min. Viable cells were resuspended to a final concentration of (10-40) × 10^6 cells/mL in the low-conductance medium. Subsequently, 0.5 mL cell suspension was mixed with 3 μg per 10^6 DCs and electroporated in a 0.4 cm cuvette at an optimum condition[26]. The cells were recovered for 5 min, and then the same protocols were repeated with 10 μg of H chain antibody RNA and 5 μg of L chain antibody RNA per 10^6 DCs. Cells were recovered for 15 min, and then the transfected DCs were matured by adding 10 ng/mL of TNF-α (Roche Molecular Biochemicals, Mannheim, Germany) for 24 h. The electroporation of actin mRNA and PBMC RNA was used as controls. Inverted phase contrast microscopy (I × 70; Olympus, Tokyo, Japan) and electron microscopy (scanning electron microscope JSM-7300EX; Hitachi, Tokyo, Japan) were used for the morphological characterization of DCs transfected with different RNA. The DcR3 protein expression in RNA-DCs was low to negative (mature DC ⩽ 11%-14% and immature DC ⩽ 9%-11%, data not shown).

Western blot analysis

The recombinant human DcR3 protein (Sigma) was dissolved in 0.02 mol/L phosphate-buffered saline (PBS, pH 7.4). The concentration was determined by the BCA Protein Assay Reagent method (Pierce Chemical Company, Rockford, IL, United States). Then, the proteins were resolved on sodium dodecyl sulfate (SDS)-polyacrylamide denaturing gels, and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) overnight at 4 ℃. The membranes were blocked in Tris-buffered saline containing 2% non-fat dry milk (Bio-Rad) and 0.05% Tween 20 (Sigma) for 1 h. Using 1:5000 dilution of the supernatant from DCs co-transfected with autologous PC cell total RNA and anti-DcR3 mAb-encoding mRNA for 24 h, the supernatant from DCs was transfected with actin mRNA for 24 h (negative control) and the commercial anti-DcR3 mAb (positive control; Qiagen) as primary antibodies, followed by incubation for 1 h in a horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, United States).

Enzyme-linked immunosorbent assay

As described by our previous study[26], autologous tumor cells (1 × 10^6 cells) were co-incubated with DCs (1 × 10^6 cells) encoding anti-DcR3 mAb mRNA or actin mRNA (negative control) in 96-well plates in an overall volume of 200 μL at 37 ℃ for 0-72 h. A 1:5000 dilution of the commercial anti-DcR3 mAb was applied as positive control. Triplicate supernatant samples from these co-cultures were examined for specific DcR3 secretion using DcR3 enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, Waltham, MA, United States). Each well was measured at 450 nm, and the optical density values were used to calculate the concentration of the samples.

As previously described by Pruitt[8], an indirect ELISA was simultaneously used to measure the DcR3 mAb concentration in the supernatants of DCs co-transfected with total tumor RNA and anti-DcR3 mAb-encoding mRNA for 0-72 h. DCs transfected with actin mRNA were used as positive control.

Assay for DCs viability

As described by Chen[27], the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell proliferation assay was used to defined the cell viability. In brief, RNA loaded DCs were inoculated into 96-well tissue culture plates (BD PharMingen, San Diego, CA, United States) with a density of 3000 cells per well and co-incubation with or without 3000 autologous PC cells. The cells were handled according to the instructions of MTT at the time points of 0-96 h. The formazan crystals that we acquired were dissolved in dimethylsulfoxide (BD Biosciences, Franklin Lakes, NJ, United States). Absorbance was monitored at 490 nm and the percentage of exposed cells to controls was used to show the cell viability. Cells that did not receive RNA transfections were regarded as the control cultures.

Phenotypic analysis of DCs by flow cytometry

As described by our previous study[2], DCs were prepared using 2% paraformaldehyde after washing for three times with frigid PBS containing 0.5% of bovine serum albumin. Four fluorescein isothiocyanate

(FITC)-conjugated mAbs, including anti-HLA-DR, anti-CD80, anti-CD86 and anti-CD83, were used and which came from BD PharMingen. Flow cytometry was used to analyze those stained cells.

Flow cytometric analysis of DC apoptosis

Following the manner of Lin *et al*[28], apoptotic DCs were quantified by using annexin V-FITC and propidium iodide (PI) double staining. Briefly, autologous tumor cells were co-incubated with DC-total tumor RNA and DC-tumor-anti-DcR3 mAb mRNA for the specific times, and then 1×10^6 cells were resuspended in 100 μL binding buffer, after washing with frigid PBS. Two microliters of PI and FITC-annexin V were added into the cells that were resuspended and cultured for 15 min protecting from light. The cells were then added into 0.5 mL binding buffer and analyzed with flow cytometry.

Induction of antigen-specific CTL and in vitro cytotoxicity assay

Antigen-specific CTLs were produced utilizing a protocol described by Chen *et al*[2,26,27]. The PBMCs without adherence were cultured in serum-free medium containing 10 ng/mL IL-7 and 20 U/mL IL-2 (R&D Systems). The cells were encouraged weekly for a minimum of two times with RNA-DCs at a stimulator-to-effector ratio of 1:10. As determined by flow cytometric analysis, a minimum of 45% of purified effector cells were CD8+ T cells after 16 d of culture.

Target cells, including autologous DCs transfected with tumor antigen-encoding RNA and tumor cells, were resuspended in 1 mL RPMI-1640 medium at 37 ℃ in 5% CO_2 for 1 h, and which contained 100 μCi $NaCrO_4$ solution (Isotope Products, Beijing, China). The serial dilutions of effector CTLs at various E:T ratios and the 5×10^3 ^{51}Cr-labeled target cells were incubated in 200 μL RPMI-1640 in 96-well plates for 6 h. Fifty microliters of supernatant were then taken away, and ^{51}Cr secretion was measured by a gamma counter (Beckmann, Heidelberg, Germany). In all the tests, the spontaneous discharge was less than 15% of the total release of the detergent. Specific lysis percentage was computed as [(experimental cpm-spontaneous cpm)/(maximum cpm-spontaneous cpm)] × 100.

Analysis of cytokines released by T cells

As described by our previous study[2], after subjecting to different treatments, 5×10^3 DCs (DC-total tumor RNA and DC-tumor-anti-DcR3 RNA) were cultured in 96-well round bottom plates. T cells were isolated from proliferating peripheral blood lymphocytes (PBLCs) and 5×10^4 T cells were stimulated with RNA-DCs in a whole volume of 200 μL in 96-well plates for 24 h. The cytokines interleukin (IL)-12p70, interferon-γ (IFN-γ), IL-10, and TNF-α released by T cells were measured by ELISA kits (Endogen, Woburn, MA, United States). The results were obtained from triplicate wells and the examination of supernatant from cultured T cells alone for the four cytokines were used as control groups.

RNA-loaded DC-induced CD4+ and CD8+ T cell responses

The measurement of multi-antigen specific CD4+ and CD8+ T cell responses were conducted by cytokine release assay as described by Chen *et al*[2] and Miyazawa *et al*[29]. Through using the instrument of autoMACS™ (Miltenyi Biotec, Bergisch Gladbach, Germany), CD4+ and CD8+ T cells were separated from proliferating PBLCs and that were civilized after three cycles of re-stimulation *ex vivo*. The cells were then incubated with CD4 or CD8 microbeads (Miltenyi Biotec) for 15 min at 4 ℃ and washed before separation. Separation was executed adopting an autoMACS column (Miltenyi Biotec). The pillar was set in the magnetic field, and magnetically-labeled cells were preserved in the pillar and then flushed out as positively chosen cells while the magnetic field was turned off. The sorted populations' purity was determined by flow cytometry. The selected CD4+ and CD8+ T cells (5×10^4) were stimulated with RNA-DCs (DC-actin mRNA, DC-PBMC RNA, DC-total tumor RNA, and DC-tumor-anti-DcR3 mAb RNA, 5×10^3) in an overall volume of 200 μL of the entire medium in 96-well plates for 24 h. The supernatants were collected, and the IL-4 and IFN-γ levels were measured using IL-4 and human IFN-γ ELISA kits (Endogen). Each assay was carried out on duplicate samples.

Statistical analysis

Quantitative data are presented as mean ± SD. The ANOVA and post hoc test (S-N-K method) analyses were performed using Excel software (Microsoft, Redmond, WA, United States). $P < 0.05$ was considered for statistical significance.

RESULTS

Generation of primary tumor cells and DCs

Primary tumor cells and DCs were cultured from HLA-A2+ PC patients. Most cultured primary tumor cells (> 90%) were found to be positive for DcR3 in the cytoplasm (Figure 1A). For DCs, cells cultured from PBMCs with stimulation of GM-CSF, TNF-α and IL-4 showed a series of typical morphologies of mature DCs. Most DCs without transfection with RNAs assembled non-cohesive colonies. Cells that were ablated from these colonies demonstrated typical villiform processes as shown by inverted phase contrast microscopy (Figure 1B). Both transfection with total tumor RNA (Figure 1C) and simultaneously loading with the total tumor RNA and anti-DcR3 mAb mRNA (Figure 1D) showed typical morphological characteristics of DCs.

DC-tumor-anti-DcR3 RNA secrete functional anti-DcR3 mAb

To determine if we could produce specific mAb by

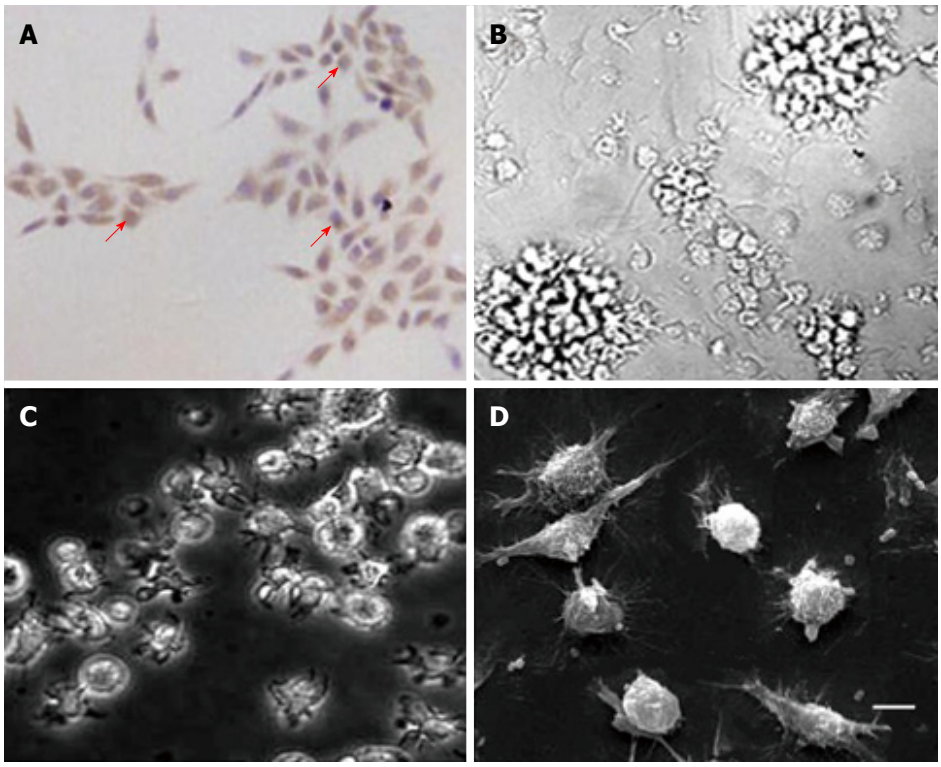

Figure 1 Cells obtained represented typical morphological characteristic of primary tumor cells and mature dendritic cells. Cells were derived from five pancreatic cancer (PC) patients and with similar shape of target cells. A: Most of the cultured primary tumor cells (over 90%) showed a DcR3-positive expression (magnification × 200); B: After cultured for 7 d, matured dendritic cells (DCs) were transfected without RNA and assembled into non-cohesive colonies, and the ablated cell showed a distinctive villiform process (magnification × 200). DCs transfected with total tumor RNA alone (C) or together with anti-DcR3 mAb mRNA (D) showed similar cell morphology to DCs transfected without RNA (C: magnification × 400; D: scanning electron microscope, bar represents 10 μm).

pulsing DCs with anti-DcR3 mAb-encoding RNA, we electroporated tumor-antigens-loaded DCs with IVT RNA encoding H and L mAb chains (anti-DcR3 H+L mRNA). The supernatants of DCs were obtained at the designated time points to identify the target mAb and measure their concentration. The supernatants of co-cultured autologous PC tumor cells and DC-tumor-anti-DcR3 RNA were harvested at specific time points to determine the effects of the mAb (Figure 2).

First, we used Western blotting to identify the anti-DcR3 mAb produced by DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA. As shown in Figure 2A, the supernatant of DC-tumor-anti-DcR3 RNA could specifically neutralize the recombinant human DcR3 protein and generate a band with slightly higher molecular weight than 30 kDa, which was in line with the theoretical molecular weight of DcR3 protein. A commercially available anti-DcR3 mAb was used as positive control, while DC-actin was used as a negative control.

The amounts of anti-DcR3 mAb secreted by RNA-pulsed DCs were analyzed using an indirect ELISA assay. As shown in Figure 2B, mAb production by DC-tumor-anti-DcR3 RNA was transient and peaked at 24 h (containing 13.15 ± 1.9 ng of anti-DcR3 mAb per 1×10^6 cells) and then could not be detected after 72 h. However, no anti-DcR3 mAb was found in the supernatant of DC-actin RNA at any point.

The specific antigen-binding effect of anti-DcR3 mAb secreted by DCs co-transfected with total tumor RNA and humanized anti-DcR3 mAb mRNA was confirmed by ELISA. As shown in Figure 2C, the soluble DcR3 protein in the supernatant of autologous PC cells (1×10^6 cells) co-cultured with DC-tumor-anti-DcR3 RNA (1×10^6 cells) was significantly lower than that of tumor cells and the DC-actin RNA co-incubation group from 12 h to 72 h ($^aP < 0.05$).

Enhanced tumor-specific immune response induced by anti-DcR3 mAb secreting DCs

We next sought to determine whether anti-tumor responses could be enhanced by immunizing DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA. Using cells from HLA-A2+ PC patients, we evaluated the ability of DC-tumor-anti-DcR3 RNA to augment the induction of anti-PC CTLs in response to DCs. As shown in the left panel of Figure 3A, DC-tumor-anti-DcR3 RNA was used as not only stimulator cells but also target cells, while DCs transfected with total tumor RNA alone or other autologous RNA-DCs were used as targets. DC-tumor-anti-DcR3 RNA demonstrated further enhancement of antigen-specific CTL induction compared with DCs only loaded with total tumor RNA without any increase in CTL activity above non-specific background.

CTL activity against PC cells was also assessed.

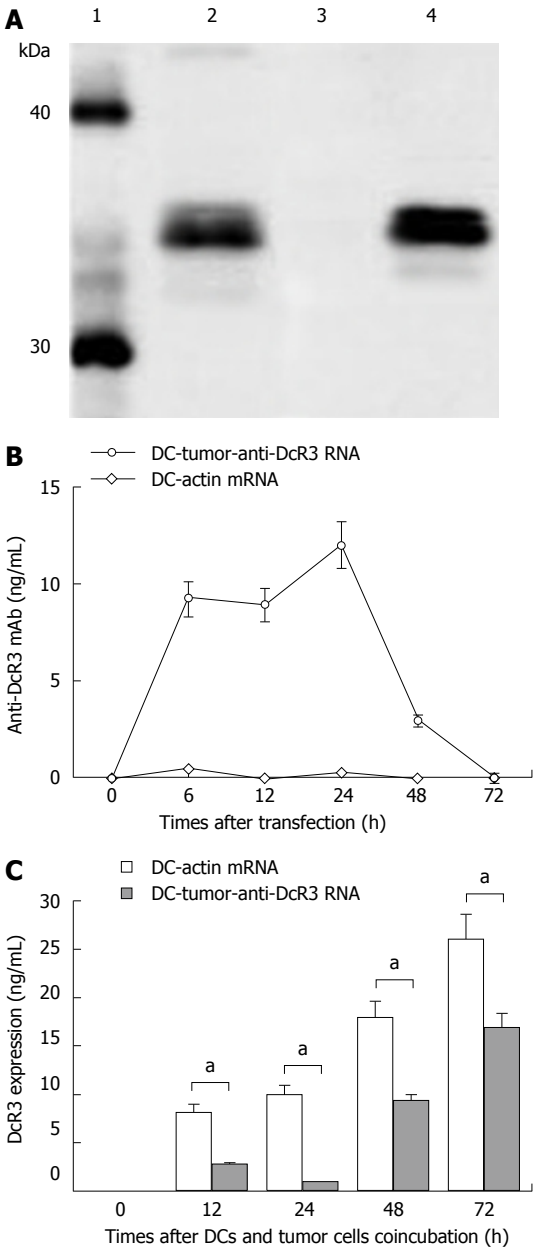

Figure 2 Identification, concentration and function of anti-DcR3 monoclonal antibody secreted by dendritic cells co-transfected with total tumor RNA and anti-DcR3 monoclonal antibody mRNA. A: Western blotting analysis showed that, similar to commercial anti-DcR3 mAb (lane 2), mAb secreted by dendritic cells (DCs) co-transfected with total tumor RNA and humanized anti-DcR3 H+L mRNA (lane 4) could also react with the recombinant human DcR3 protein (molecular weight of 35 kDa) and generate a band with molecular weight slightly greater than 30 kDa, whereas the supernatant harvested from DC-actin RNA could not bind the DcR3 protein (lane 3); B: The amounts of anti-DcR3 mAb produced by RNA transfected DCs were analyzed using indirect ELISA assay. The mAb secreted by DC-tumor-anti-DcR3 RNA was transient, peaked at 24 h, and then could not be detected after 72 h. However, no anti-DcR3 mAb was found in the supernatant of DC-actin RNA continuously; C: The specific antigen binding effect of anti-DcR3 mAb secreted by RNA transfected DCs was determined by measuring the levels of DcR3 protein in the supernatant of autologous tumor cells (1×10^6 cells) co-cultured with defined DCs (1×10^6 cells). After co-incubation with DC-tumor-anti-DcR3 RNA for 12-72 h, the soluble DcR3 protein level in the supernatant of autologous PC cells was significantly lower than those of tumor cells and the DC-actin RNA co-cultured group ($^aP < 0.05$). Except for those of western blotting and histogram, data represent the means of three experiments, and the histograms are representative of three experiments. Error bars represent SD. mAb: Monoclonal antibody.

Both CTLs induced by DC-tumor-anti-DcR3 RNA and DC-total tumor RNA were able to lyse their own cancer cells effectively, while CTLs induced by DC-PBMC RNA or DC-actin RNA were not, as shown in Figure 3A (right panel). Furthermore, DC-tumor-anti-DcR3 RNA showed greater effectiveness and superior ability to recognize and lyse HLA-A2+ autologous PC cells ($P < 0.05$). Meanwhile, with increase in the E:T ratio (from 10:1 to 40:1), the killing intensity increased concomitantly ($P < 0.05$).

No lysis of normal PBMCs or NK-sensitive K562 cells was observed. However, evident lysis against the cultured PC cell line occurred (Figure 3B). Effector T cells (HLA-A2+) stimulated by DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA could lyse the Capan-2 cells, which expressed the HLA-A2+ antigen endogenously. On the other hand, HLA-A2- AsPC-1 cells were not identified and lysed. DC-tumor-anti-DcR3 RNA was more potent at inducing cytotoxicity in CTLs against Capan-2 cells with HLA-A2 adaptation in comparison with the CTLs induced by DC-total tumor RNA alone ($P < 0.05$). The expression of HLA alleles other than A2 was not evaluated in these experiments.

Improvement in cell viability by anti-DcR3 mAb secreting DCs

The effect of anti-DcR3 mAb secreting DCs on viability of RNA-loaded DCs was determined by MTT assay. As shown in Figure 4A, when RNA-DCs were co-incubated without autologous tumor cells, cell viability did not vary significantly at designed times, with around 85% survival throughout ($P > 0.05$). By contrast, the viability of DC-total tumor RNA cultured with tumor cells was evidently lower than that of DC-tumor-anti-DcR3 RNA ($P < 0.01$). The results of DC-PBMC RNA and DC-actin RNA were similar to those of DC-total tumor RNA. Meanwhile, both the DC-tumor-anti-DcR3 RNA and DC-tumor RNA demonstrated positive expression of CD80, CD83, CD86 (co-stimulatory molecules) and HLA-DR (MHC II molecules) after co-incubation with PC cells for 24 h, but there was no significant difference between the two methodologies (data not shown).

Annexin V and PI double staining was performed to demonstrate the inhibition of apoptosis of DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA. After incubation with autologous PC cells for 0-96 h, annexin V-positive apoptotic cells were found to increase sharply in a time-dependent manner in DC-total tumor RNA, whereas apoptosis of DC-tumor-anti-DcR3 RNA were mitigated by anti-DcR3 mAb (Figure 4B).

T cell cytokine production pulsed by RNA-DCs

There were no significant differences in the amount of IL-10, TNF-α, IL-12p70 and IFN-γ cytokines released in the culture supernatants of T cells when measured by ELISA (data not shown). However, high levels of cytokines could be secreted by T cells after pulsing

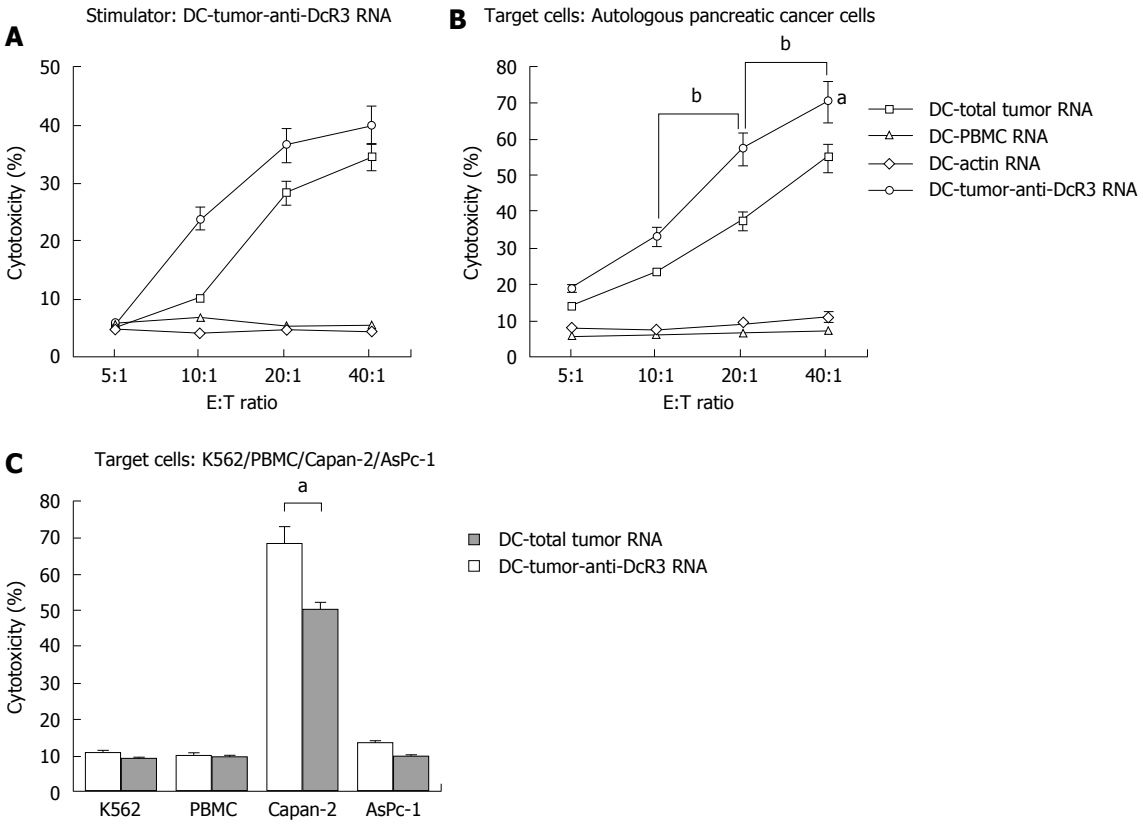

Figure 3 Antigen-specific immune response against pancreatic cancer is enhanced by anti-DcR3 monoclonal antibody secreting dendritic cell. Dendritic cells (DCs)-total tumor RNA, DC-PBMC RNA, DC-actin RNA and DC-tumor-anti-DcR3 RNA were used to stimulate autologous T cells weekly for two times followed by a cytotoxic T lymphocyte (CTL) assay. Induction of tumor antigen-specific CTLs was measured by using RNA-transfected DCs and tumor targets (primary tumor cells, K562, Capan-2 and AsPC-1 cell line cells). A: Left panel: DC-tumor-anti-DcR3 RNA was used as not only stimulator cells but also target cells, and CTLs stimulated by DC-tumor-anti-DcR3 RNA could recognize and lyse tumor antigen-specific cancer targets (DC-total tumor RNA and DC-tumor-anti-DcR3 RNA). No cross-reactivity was apparent against DCs loaded with normal tissue surrounding PC or actin (DC-PBMC RNA and DC-actin RNA). Compared with CTLs stimulated by DC-total tumor RNA, DC-tumor-anti-DcR3 RNA further enhanced the induction of CTL activity. Right panel: Both DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA and DCs transfected with total tumor RNA alone showed an effective and superior ability in recognizing and lysing HLA-A2+ autologous PC cells, whereas T cells activated by DC-PBMC RNA or DC-actin RNA could not ($^{a}P < 0.05$). Moreover, CTLs induced by DC-tumor-anti-DcR3 RNA could produce a more powerful killing activity toward the tumor cells compared with the DC-total tumor RNA with the E:T ratio increasing from 10:1 to 40:1 ($^{b}P < 0.05$); B: At the E:T ratio of 40:1, the effector T cells (HLA-A2+) stimulated by DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA could lyse the Capan-2 cell line cells, which endogenously expressed the HLA-A2 antigen effectively. By contrast, the cells of the AsPC-1 line (HLA-A2-) were not recognized and lysed. DC-tumor-anti-DcR3 RNA showed a more powerful capability in inducing the cytotoxicity of CTLs against HLA-A2-matched tumor cell line (Capan-2) compared with that induced by DC-total tumor RNA alone ($^{a}P < 0.05$). The experiment was repeated thrice representatively, and the data are shown as mean ± SD. mAb: Monoclonal antibody; DC: Dendritic cell; CTLs: Cytotoxic T lymphocytes.

with RNA-DC (Figure 5A). The IFN-γ and IL-12p70 produced by DC-tumor-anti-DcR3 RNA pulsed T cells were higher than those secreted by T cell pulsed by DC-total tumor RNA ($P < 0.01$). At the same time, compared with the DC-total tumor RNA group, the TNF-α levels detected in the DC-tumor-anti-DcR3 RNA group was not changed significantly ($P > 0.05$). In addition, the IL-10 level was lower when it was detected in DCs co-transfected with both total tumor RNA and anti-DcR3 mAb RNA ($P < 0.05$).

CD4+ and CD8+ T cell responses induced by anti-DcR3 mAb secreting DCs

As shown in Figure 5B, the CD4+ and CD8+ T cells incubated with DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA produced significantly higher level of IFN-γ than those incubated with control DC (DC-actin RNA) or DCs exposed to normal tissues (DC-PBMC RNA) ($P < 0.01$). In addition, the CD4+ and CD8+ T cells that were cultured with DCs co-transfected with both total tumor RNA and anti-DcR3 mAb RNA produced a higher level of IFN-γ than those cultured with DC-total tumor RNA ($P < 0.01$). Meanwhile, CD4+ and CD8+ T cells incubated with DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA were able to produce IL-4, but negative or weak secretion of IL-4 was observed in control groups (DC-actin RNA and DC-PBMC RNA) ($P < 0.01$). Furthermore, decreased levels of IL-4 production were detected in CD4+ T cells stimulated by DC-tumor-anti-DcR3 RNA compared with DC-total tumor RNA ($P < 0.01$). No significant difference in IL-4 secretion was detected between CD8+ T cells stimulated by DC-tumor RNA and those stimulated by DC-tumor-anti-DcR3 RNA.

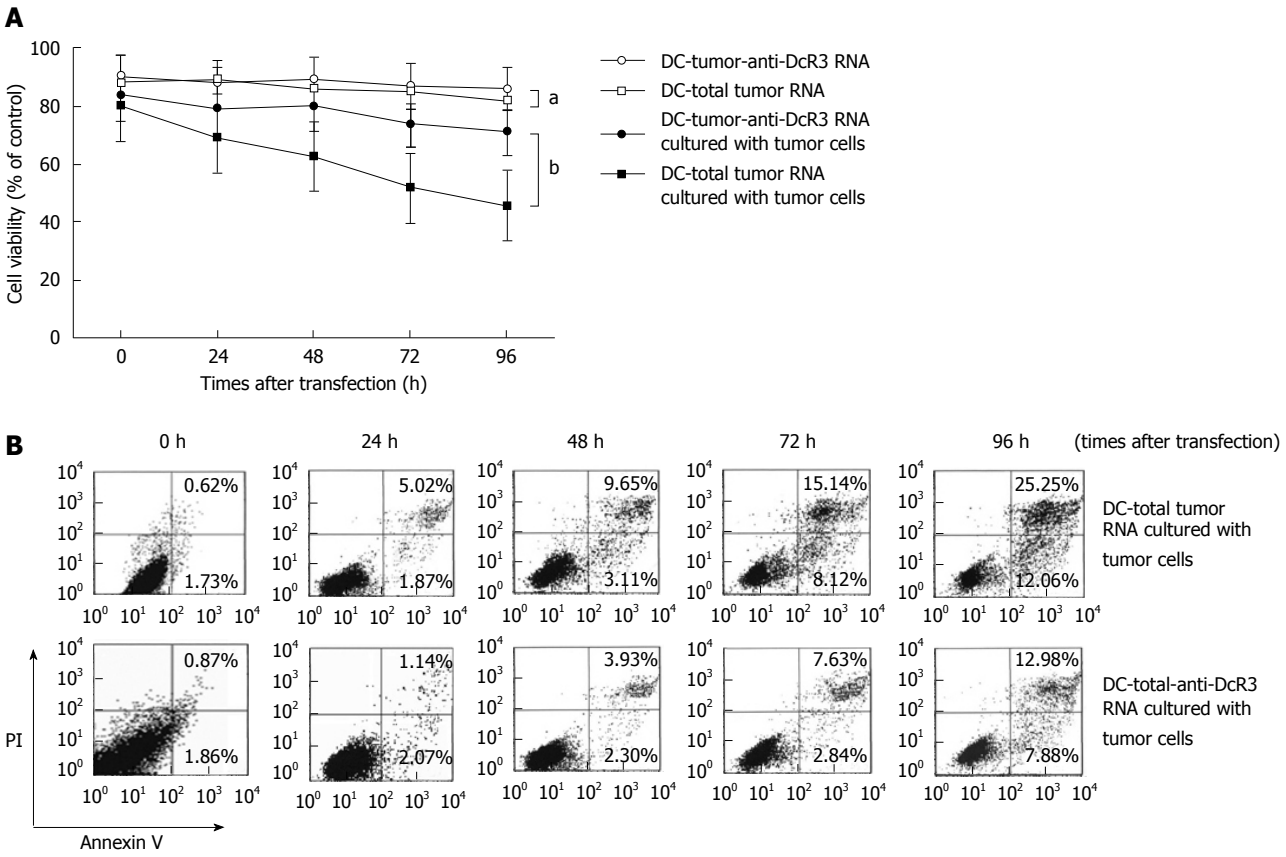

Figure 4 Effects of anti-DcR3 monoclonal antibody secreting dendritic cells on cell viability and apoptosis in tumor RNA-loaded dendritic cells. A: Dendritic cells (DCs) secreting anti-DcR3 mAb improved the viability of RNA-loaded DCs. The viabilities of DC-total tumor RNA and DC-tumor-anti-DcR3 RNA, cultured with or without autologous tumor cells, were measured using MTT assay after transfection for 0-96 h. The viability of DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA did not change significantly at the designed time points, with approximately 85% survival throughout ($^{a}P > 0.05$). Within the same period, the viability of DC-total tumor RNA cultured with tumor cells was evidently lower than that of DC-tumor-anti-DcR3 RNA and decreased in a time-dependent manner ($^{b}P < 0.01$). Three representative experiments were run, and the data are shown as mean ± SD; B: DC-total tumor RNA and DC-tumor-anti-DcR3 RNA were co-cultured with autologous pancreatic cancer (PC) cells (1×10^{6}) for 0, 24, 48, 72 and 96 h. Cells stained with annexin V-FITC and propidium iodide were analyzed by flow cytometry. The percentage of annexin V-positive apoptotic cells in DCs transfected with total tumor RNA markedly increased in a time-dependent manner, whereas the apoptotic cells increased slowly in DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA. mAb: Monoclonal antibody.

DISCUSSION

Tumor cells can produce several immunomodulatory molecules to induce immunosuppressive TME and inhibit the function of tumor-associated DCs[9,30]. Therefore, new DC-based strategies for producing tumor vaccines are necessary to abrogate immunosuppressive molecules in tumor tissue-induced mechanisms for suppressing the activation of CTL responses that can treat established cancers[8].

DcR3 is one of the candidate target tumor-derived factors[31]. As we initially demonstrated, most cultured primary tumor cells showed a DcR3-positive expression in the cytoplasm of PC patients, which is consistent with the findings of Zhou *et al*[17]. Tumor cells engineered to release high amounts of DcR3 are able to protect themselves from apoptosis, consequently resulting in a decreased immune response and suggesting that DcR3 is involved in the immune evasion of malignant tumors[19,32]. As a powerful immunomodulatory factor, DcR3 can suppress actin polymerization in mitogen-stimulated T cells, prevent the formation of pseudopodia, down-regulate the activation of DCs and macrophages, induce abnormal aggregation of T cells after antigen stimulation, reduce the interaction between T cells and DCs, inhibit T cell chemotaxis, and induce T cell apoptosis[19,33]. Therefore, neutralizing the DcR3 protein secreted by tumor cells is particularly important in cancer immunotherapy.

Different methods have been developed for neutralizing immunosuppressive factors released by tumor cells. The effects of systemic administration of antibodies have been examined by numerous studies. Their findings showed that mAbs can improve special immune responses when administered systemically[34]. Human studies, however, have revealed that the side-effects of mAbs are unavoidable when delivered systemically, and one major concern is induction of autoimmunity[35-37]. Thus, our present study shows an alternative strategy of delivering mAb by transfecting DCs with the RNA that encodes both PC tumor-antigens and a defined immunosuppressive molecule, namely humanized anti-DcR3 mAb. Using this strategy, DCs were used both to produce anti-DcR3 mAb and

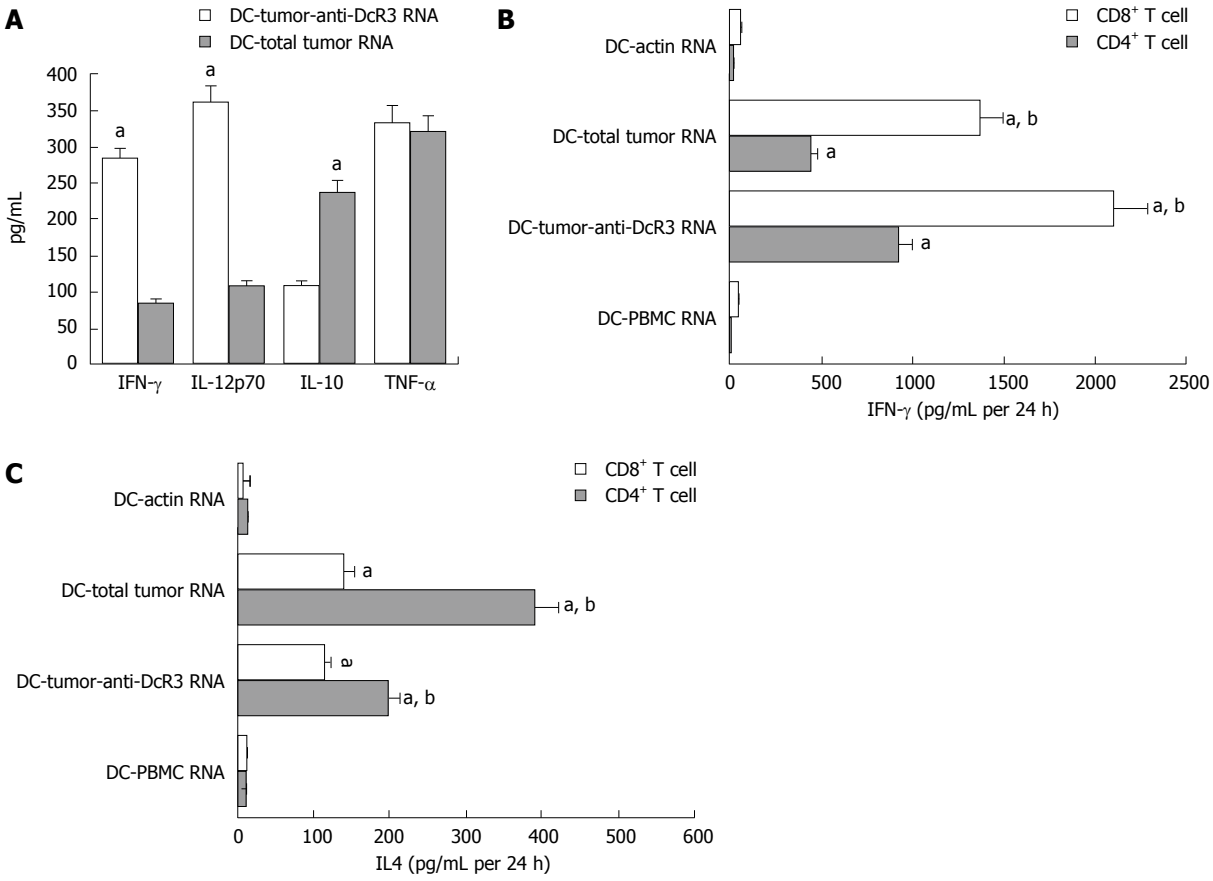

Figure 5 T cell responses induced by anti-DcR3 monoclonal antibody secreting dendritic cells. The T cells were co-cultured with RNA-dendritic cells (DCs) for 24 h. Then, the supernatants were collected, and the cytokine levels were measured by ELISA assay. A: ELISA test showed the cytokines of IL-12p70 and IFN-γ secreted by T cells pulsed by DC-tumor-anti-DcR3 RNA were higher than those secreted by T cells pulsed by DC-total tumor RNA without significant change of TNF-α. Meanwhile, IL-10 level was lower in DC-tumor-anti-DcR3 RNA than in DC-total tumor RNA ($^aP < 0.05$); B: $CD4^+$ T cells and $CD8^+$ T cells incubated with DCs encoding whole tumor antigens could produce extremely higher IFN-γ levels compared with those incubated with DCs as control or DCs treated with normal tissues ($^aP < 0.01$). Furthermore, the $CD4^+$ T and $CD8^+$ T cells incubated with DCs co-transfected total tumor RNA and anti-DcR3 mAb mRNA produced higher IFN-γ levels than those incubated with DC-total tumor RNA ($^bP < 0.01$); C: The $CD4^+$ T and $CD8^+$ T cells incubated with DCs loaded with whole tumor antigens showed a positive expression of IL-4, whereas a negative or weak expression of IL-4 was observed in DC-actin RNA and DC-PBMC RNA cells ($^aP < 0.01$). Moreover, decreased levels of IL-4 production were detected in $CD4^+$ T cells stimulated by DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA compared with DCs transfected with total tumor RNA individually ($^bP < 0.01$). The experiment was repeated three times representatively, and results are shown as mean ± SD. IL: Interleukin; ELISA: Enzyme-linked immunosorbent assay; PBMC: Peripheral blood monocyte cell; mAb: Monoclonal antibody.

as vehicles for delivery to the site of T cell activation considering their well-documented function as antigen-presenting cells.

Our results showed that the supernatant of cultured DCs co-transfected with total tumor RNA and anti-DcR3 H+L mRNA could specifically bind the recombinant human DcR3 protein and generate a band with molecular weight that was slightly greater than 30 kDa, which was in line with the theoretical molecular weight of DcR3 protein. In addition, the DcR3 level in the supernatant of autologous PC cells co-cultured with DC-tumor-anti-DcR3 RNA was significantly lower than that of the control group. These data suggest that this approach is feasible and can generate sufficient anti-DcR3 mAb to neutralize the DcR3 secreted by PC cells *in vitro*.

One advantage of the local release of anti-DcR3 mAb that is provided *via* DC mRNA transfection is its secretion over a relatively short time span at the precise site where T cell activation is needed[8]. We previously showed that mRNA subunits have a brief half-life of less than 24 h after DC transfection[2], implying that mRNA translating into protein is an instantaneous event in DC. Here, we confirmed that for anti-DcR3 mAb, most target proteins were freed in 12 h after mRNA transfection, but the extra secreting of target could last for 24-48 h. Considering that human PC cells showed unregulated production of DcR3 within 12 h, we found that the time course of anti-DcR3 mAb release by DCs is ideal for obstructing the DcR3 expressed by PC cells[8].

In the present study, we found that DCs transfected with total tumor RNA alone or together with anti-DcR3 mAb mRNA could induce tumor-specific cytotoxic T cells to recognize and lyse tumor RNA-loaded DCs and tumor cells effectively. In comparison, no damage in K562 cells were found, which implies that the two manners can not only be PC-specific but also eliminated the possibility of NK cell activity [2]. These data also indicate that CTLs induced by DC-tumor-

anti-DcR3 RNA were more powerful at indicting lysis than the CTLs induced by DC-total tumor RNA. These results demonstrate that use of DCs co-transfected with RNA encoding humanized anti-DcR3 mAb and whole PC tumor-antigens may be a superior strategy for designing a DC-based tumor vaccine. In addition, it is known that, like Fas-Fc antibody, DcR3 can block apoptosis in Jurkat cells[21]. The more superior cytolytic function might be due to blockade of activation-induced cell death (AICD) in CTLs induced by DC-tumor-anti-DcR3 RNA rather than better CTL response generation due to superior priming of CTL precursors by engineered DCs. This should be addressed in future studies.

An HLA allele that matches the target cells and tumor-specific CTLs is necessary. All PC patients that were included in this study were HLA-A2+, and we are concerned with the function of HLA-A2 among different HLA alleles. We found that CTLs induced by DC-tumor-anti-DcR3 RNA and DC-total tumor RNA could deliver potent cytotoxicity towards Capan-2 cells. However, owing to HLA-A2 mismatching, AsPC-1 PC cells with HLA-A2- could not be lysed by HLAA2+ CTLs. This result indicates that HLA-A2 may be a key allele for presenting antigens, and PC-specific CTL immune response may be limited to MHC class I antigens.

The induction of autoimmunity is one potential problem that may limit the application of PC tumor and anti-DcR3 mAb RNA-transected DC vaccines, partly because RNAs loaded with both normal antigens and tumor antigens share the same antigen presentation pathway when they are delivered to DCs[26,38], and partly because RNA-encoding anti-DcR3 mAb in DCs may improve the risk of inducing autoimmunity just like systemic administration of neutralizing immunosuppressive factor antibody in TME[34]. Here, we adopted tissue cell enrichment by primary tumor cell culture, and found that CTLs stimulated by DC-total tumor RNA lysed tumor cells (autologous primary cultured tumor cells and PC cell lines) but not PBMCs (normal tissue cells). Meanwhile, using mRNA-transfected DCs to locally deliver anti-DcR3 mAb, no increase in non-specific background immune responses against control target cells or the normal tissue PBMCs *in vitro* was detected. On the basis of these results, we anticipate that local delivery of anti-DcR3 mAb by utilizing DCs transfected with RNA will bypass the adverse effects of autoimmune responses triggered by systemic delivery of mAb. At the same time, vaccine-induced anti-tumor immune response increased in patients. These findings indicate that harmful autoimmunity with pathological results may not be an issue with this method.

The mechanism by which DCs engineered to secrete anti-DcR3 mAb augments CTL response remains to be fully elucidated. In our study, we found that the cell viability of both DC-tumor-anti-DcR3 RNA and DC-total tumor RNA did not change significantly at the designated time points, with approximately 85% survival when cultured alone (without DcR3 influence). On the contrary, when co-cultured with autologous tumor cells (with DcR3 influence), the viability of DC-total tumor RNA was evidently lower than that of DC-tumor-anti-DcR3 RNA. Furthermore, when co-cultured with tumor cells for 0-96 h, apoptotic cells in DC-total tumor RNA evidently increased, whereas apoptotic cells in DC-tumor-anti-DcR3 RNA were found to increase slowly and mildly. Similar to the findings of You *et al*[39], results suggested that enhancement of CTL responses induced by anti-DcR3 mAb DCs may partly be due to its powerful DcR3 blocking capability, which down-regulated the apoptosis of DCs induced by DcR3 and increased DC viability at the site of T-cell activation in the process of whole tumor-antigen delivery.

The whole total tumor and anti-DcR3 mAb RNA electroporation likely acted as a powerful tumor vaccine which could effectively activate antigen-specific T cells against PCs, just like Th1 cells[2]. This interpretation is supported by our observations of a high percentage of killing of tumor cells and IFN-γ secretion of T cells. High expressions of cytokines, such as IL-12 and IFN-γ, and low expression of cytokine, such as IL-10, might be the reason why DC-tumor-anti-DcR3 RNA exhibited enhanced capability of inducing CTL responses. Th1 cells and Th2 cells are two important T regulatory (Treg) cells in the body. Transformation of Treg cells from Th1 to Th2 is a unique phenomenon in malignant tumors. Development of Th2 cells promotes long-term retention of cancer cells in the host body and protects them from immune surveillance and attack. Th1 cells and Th2 cells can both stimulate IFN-γ production, whereas Th2 cells preferentially induced production of IL-4 compared to Th1 cells[39,40]. In this study, we clearly demonstrated that CD4+ T cells induced by DCs co-transfected with total tumor RNA and anti-DcR3 mAb mRNA markedly decreased IL-4 secretion and increased IFN-γ production, indicating that DCs engineered to secrete anti-DcR3 mAb could increase the number of Th1 cells and decrease Th2 cells. Ojima *et al*[41] and Chen *et al*[2] reported that DCs that were loaded with TAA could induce antigen-specific CD4+Th1 cells and such CD4+ Th1 cells played a key role in the priming phase of CD8+ CTLs. In the present study, we showed that both DC-tumor-anti-DcR3 RNA and DC-total tumor RNA can activate not only tumor-specific CD4+ T cells but also CD8+ T cells assessed by IFN-γ release. Besides, CD4+ T cells and CD8+ T cells incubated with DC-tumor-anti-DcR3 RNA could produce more IFN-γ compared with those incubated with DC-total tumor RNA. These results indicate that adjusting the Th1/Th2 cytokine network and promoting the recovery of CD8+ anti-tumor cellular immunity may be the other two mechanisms for engineering DCs to secrete anti-DcR3 mAb to augment CTL response.

In summary, our results demonstrate that DCs engineered to secrete anti-DcR3 antibody can augment

CTL responses against PC *in vitro*. The observed immune-enhancing effects may be partly due to their capability of down-regulating DC apoptosis and adjusting the Th1/Th2 cytokine network. Therefore, use of DCs engineered to secrete anti-DcR3 antibody vaccine may be an attractive and promising therapeutic strategy for a patient with PC.

COMMENTS

Background

Pancreatic cancer (PC) is considered as a highly destructive human malignant tumor without effective treatments. Dendritic cell (DC) tumor vaccines have emerged as an alternative treatment manner for advanced PC. But tumor cells can produce some immunosuppressive molecules to inhibit the function of tumor-associated cells, such as T lymphocytes and DCs.

Research frontiers

DcR3, a soluble protein secreted by tumor cells, is overexpressed in carcinoma originating from the gastrointestinal tract system, including PC. DcR3 is regarded as an important immunosuppressive factor in immune effector cells' defect and it is particularly important to neutralize DcR3 protein secreted by tumor cells in cancer immunotherapy.

Innovations and breakthroughs

A new strategy of delivering mAb by transfecting DCs with the RNA encoding both anti-DcR3 mAb and the whole tumor-antigens was shown by this study. Furthermore, the DCs engineered to secrete anti-DcR3 mAb could augment cytotoxic T lymphocyte responses against PC *in vitro* and the immune-enhancing effects may be partly due to their capability of down-regulating apoptosis of DCs and adjusting the Th1/Th2 cytokine network.

Applications

This study lays a good foundation for further investigation of tumor DC vaccine targeting DcR3 protein against PC.

Terminology

As a tumor necrosis factor receptor superfamily's member, DcR3 works as decoy receptor for Fas ligand, LIGHT and TNF-like molecule 1A to neutralize their cytotoxic and regulatory functions.

Peer-review

This manuscript has shown that DCs engineered to secrete anti-DcR3 antibody can stimulate cytotoxic T lymphocyte responses against pancreatic cancer cells *in vitro*. It provided important contribution to development of immunotherapy for pancreatic cancers. The proposed method is convincing.

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