Original Research

Comparison of cytotoxic T lymphocyte responses against pancreatic cancer induced by dendritic cells transfected with total tumor RNA and fusion hybrided with tumor cell

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Abstract

Pancreatic cancer (PC) is a deadly human malignancy. Dendritic cell (DC)-based immunotherapy with whole tumor antigens demonstrates potential efficiency in cancer treatment. Tumor RNA and tumor fusion hybrid cells are sources of whole tumor antigens for preparing DC tumor vaccines. However, the efficacy of these sources in eliciting immune responses against PC has not yet to be directly compared. In the present study, patient-derived PC cells and DCs were fused (DC–tumor hybrids) and primary cultured PC cell-derived total RNA was electroporated into autologous DCs (DC–tumor RNA). The antitumor immune responses induced by DC–tumor hybrids and DC–tumor RNA were compared directly. The results showed that both RNA and hybrid methodologies could induce tumor-specific cytotoxic T lymphocyte (CTL) responses, but pulsing DCs with total tumor RNA could induce a higher frequency of activated CTLs and T-helper cells than fusing DCs with autologous tumor cells. In addition, DC–tumor RNA triggered stronger autologous tumor cell lysis than DC–tumor hybrids. It could be concluded that DCs pulsed with whole tumor RNA are superior to those fused with tumor cells in priming anti-PC CTL responses. Electroporation with total tumor RNA may be more suitable for DC-based PC vaccination.

Keywords: Dendritic cell, tumor total RNA, hybrid, pancreatic cancer, cytotoxic T cell

***Experimental Biology and Medicine* 2015; 240: 1310–1318. DOI: 10.1177/1535370215571884**

Introduction

Pancreatic cancer (PC) was the fourth leading reason of cancer-related deaths in the United States and caused the death of 38,460 Americans in 2013.[1] This disease has an extremely poor prognosis, with an overall five-year survival rate of less than 5%.[2] The high mortality rate and poor prognosis of PC patients can partially be attributed to the lack of effective therapies. The current therapeutic options for PC are limited to surgical resection and systemic chemotherapy; however, neither strategy can completely cure it.[3] Therefore, the development of effective treatment strategies is urgently needed.

Dendritic cells (DCs) are potent antigen-presenting cells involved in the initiation of antitumor immune responses.[4] The activities of cytotoxic T lymphocytes (CTLs), which are directly responsible for killing tumor cells *in vivo*, are mainly directed by DCs. Therefore, immunotherapy with DC-based tumor vaccines has emerged as an alternative therapeutic approach for cancer.[5] In clinical practice, antigen selection is crucial in designing an effective vaccine. Only one of the many tumor-associated antigens (TAAs), such as MART-1/Melan-A,[6] is usually used to load DCs. However, evidence on the clinical effects of one TAA is lacking because a single antigen may be easily lost on the tumor cell surface and a wide spectrum of target antigens may be desirable to induce powerful immune responses on the contrary.[7] Moreover, most tumor antigens are probably unique for each patient,[8] and individual tumor antigens may have arisen from numerous mutations during tumor development. Hence, loading DCs with whole tumor antigens has recently become popular. Because of lacking defined antigens, PC displays weak antigenicity and high heterogeneity.[9] Loading antigens from whole PC tumors can generate a broad T-cell immune response to TAAs. This method may reduce the possibility of PC escape from immune recognition when vaccinating with a single repertoire of tumor antigens.

Most recent studies on DC-based cancer vaccines have focused on the advancement of strategies that can effectively deliver exogenous tumor antigens to DCs for the cross-priming of CD8+ T-cells through the endogenous major histocompatibility complex (MHC) class I processing and presentation pathway. These strategies include loading

with peptides, proteins, whole tumor cell antigens, or nucleic acids.[10] Each strategy has inherent advantages and disadvantages. DC–tumor cell fusion hybrids can trigger the expression of molecules needed for immune stimulation with the presentation of a potential repertoire of tumor antigens during combination. These hybrids can elicit antitumor responses in animal models[11] and in human clinical trials.[12] However, data to clearly assess the effects of vaccines for PC treatment are lacking. The use of tumor-derived RNA as total antigen is also common. This method is attractive because it is more straightforward than the use of exogenously provided peptides and proteins.[13] Several reports have described the use of tumor-extracted RNA as the source of tumor antigens for the preparation of DCs and have indicated the potential of this method for antigen specific or polyvalent tumor vaccination in the absence of identified tumor antigens.[14,15] This finding is particularly important in PC, which has a few specific tumor antigenicity.[16] To date, little is known about the relative efficacy of whole tumor antigen vaccination based on tumor cell hybrid or RNA transfection in PC.

The present study compared the efficiency of DCs fused with autologous tumor cells or pulsed with total tumor RNA derived from the same patients in stimulating specific anti-PC immune responses and the results showed that pulsing DCs with total tumor RNA induced a higher frequency of activated CTLs and T-helper cells than fusing DCs with autologous tumor cells. In addition, the former triggered stronger autologous tumor cell lysis than the latter. Therefore, using total tumor RNA as a source of tumor antigens is a valuable alternative and is more effective than using tumor cell hybrids.

Materials and methods

Ethics statement

The study was approved by the Ethics Committee of Shenyang General Hospital of PLA. All tissue harvesting was performed after obtaining written informed consent.

Patients

Six HLA-A2+ PC patients (three males and three females, median age 44.2 years, range 30–56 years) were included in this study. According to the TNM classification of American Joint Committee on Cancer (AJCC),[17] there were two stage II patients and four stage III patients. The location of tumor was divided into head (four cases) and body/tail (two cases). All patients underwent surgical resection and were pathologically diagnosed with invasive ductal adenocarcinoma (poorly differentiated for all six cases). Portions of tumor and normal tissues were placed in RPMI-1640 medium (Hyclone, South Logan, UT, USA) supplemented with 50 mg/mL streptomycin and 50 U/mL penicillin (Hyclone) for primary cell culture.

Generation of human DCs

DC generation was performed as previously described by Lee *et al.*[18] A concentrated leukocyte fraction was isolated from the peripheral blood monocyte cells (PBMCs) that processed 200 mL of blood during each collection. The leukapheresis products were separated by density-gradient centrifugation over polysucrose sodium diatrizoate (Sigma, St Louis, MO, USA), and cells were re-suspended in serum-free AIM-V medium (Gibco, Burlington, Canada). Cells were incubated in a humidified incubator for 2 h at 37°C to allow plastic adherence. The non-adherent fraction was removed, and the adherent cells were cultured for seven days 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, USA) at 37°C under 5% CO_2. Maturation of DCs was induced by adding 10 ng/mL of tumor necrosis factor-α (TNF-α) (Roche Molecular Biochemicals, Mannheim, Germany) for 24 h starting at day 6 after the seeding of the immature DC culture. The mature DCs were harvested. Then, inverted phase contrast microscopy (1 × 70, OLYMPUS, Japan) and electron microscopy (transmission electron microscope, HX-30, TOSHIBA, Japan) were used for the morphological characterization of DCs after Giemsa (Sigma) staining.

Preparation of autologous tumor cells and tumor cell lines

Autologous tumor cells were obtained as previously described.[19] 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) with gentle agitation at room temperature for 4–6 h. After filtration and gradient centrifugation using 75% Ficoll–Hypaque, supernatants at a cell concentration of 1×10^6/mL were obtained. After cultured for seven days, the immunohistochemistry technique was used to detect the expression of MUC1 (MUC1 mAb from DPC-Biermann, Bad Neuheim, Germany).

The human PC cell lines Capan-2 (HLA-A2+), AsPC-1 (HLA-A2−), Panc-1 (HLA-A2+), and MiaPaCa-2 (HLA-A2−),[20] as well as the leukemia cell line K562, were obtained from the American Type Culture Collection (Manassas, VA, USA). 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 seven days and maintained in logarithmic phase growth at 37°C in a humidified atmosphere supplemented with 5% CO_2.

DC-tumor cells fusion and DC electroporation with tumor RNA

DCs and auto tumor cells were prestained with fluorescein isothiocyanate (FITC)-conjugated CD86 mAb (BD Pharmingen, San Diego, CA, USA) and phycoerythrin (PE)-conjugated MUC1 mAb, respectively, according to the manufacturer's instructions. After staining, DCs were mixed with tumor cells (irradiated 5000 cGy) immediately at a ratio of 2:1 and were suspended in a 5% glucose solution containing 0.1 mM $Ca(CH_3COO)_2$, 0.5 mM $Mg(CH_3COO)_2$, and 0.3% bovine serum albumin (BSA).

The pH of the fusion medium was adjusted to 7.2–7.4 with L-histidine (all chemicals from Sigma). After centrifugation, the pellets were re-suspended in the same fusion medium without BSA at a concentration of 1×10^7 cells/mL and electrofusion was carried out using a custom-designed concentric fusion chamber connected to a pulse generator (ECM 2001, BTX Instrument, San Diego, CA, USA). After fusion, the cells were grown overnight in a culture medium containing GM-CSF. Fusion efficiency was assessed by FACS Caliber flow cytometer (Becton Dickinson, Mountain View, CA, USA) and Cell Quest software (BD Biosciences, San Jose, CA, USA). The hybrid cells (dual color) were gated and sorted using FACSCalibur cell sorter. After the trypan blue dye exclusion, the sorted and alive DC-irradiated tumor cell hybrids were used for 24 h later. DC-irradiated normal tissue-cell hybrids were used as controls for some experiments.[21]

Total cellular RNA was extracted from autologous PC cells using TRIzol Reagent (Sigma) according to the manufacturer's instructions. Only RNA exhibiting a ratio of 28 S:18 S >1 was subjected to further analysis. Viable DCs were counted by trypan blue dye exclusion and re-suspended to a final concentration of 1×10^7 cells/mL in the low-conductance medium. Subsequently, 0.5 mL of the cell suspension was mixed with an appropriate amount of total tumor RNA (about 20 μg) in a 0.2 cm cuvette. These cells were then electroporated at a voltage of 300 V/cm and a capacitance of 150 μF in an electroporation volume of 200 μL using a Gene Pulser II (Bio-Rad, Hercules, CA, USA). The mean pulse duration was 10 ms.[22] DCs electroporated with normal tissue RNA were used as controls for some experiments. *In situ* cellular immune staining for MUC1 was applied to evaluate the RNA transduction efficiency in auto DCs after electroporation for 48 h.

Phenotypic analysis of DC by flow cytometry

FITC-conjugated mAbs were purchased from BD Pharmingen (San Diego, CA, USA). After three washes in cold PBS supplemented with 0.5% of BSA, DCs were fixed with 2% paraformaldehyde in PBS. The following mAbs were used: FITC-anti-CD80, FITC-anti-CD83, FITC-anti-CD86, and FITC-anti-HLA-DR. The stained cells were analyzed using flow cytometry.[23]

Assay for DC viability

DC viability was determined by the 3 -(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell proliferation assay. The DCs transfected with RNA were seeded at a density of 3000 cells per well in 96-well tissue culture plates. The cells were treated in sequence with MTT at designated times (0, 24, 48, 72, and 96 h). The resulting formazan crystals were dissolved in dimethyl sulfoxide (Sigma), and the absorbance was measured at 490 nm. Cell viability was expressed as the percentage of exposed cells to controls. The DCs without the fusion with tumors and without transfection with RNA were used as controls. Experiments were replicated for three times.

Analysis of cytokines

After subjecting to different treatments, DCs (1×10^6/mL) were cultured in 24-well round bottom plates. The final volume of each well was adjusted to 1 mL with the complete medium. The supernatants were harvested on day 3. The cytokines interleukin (IL)-12p70, interferon-γ (IFN-γ), IL-10, and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Woburn, MA, USA). The supernatant of primary tumor cells were used as the control group and results were obtained from triplicate wells.

Induction of CTLs from PBMCs

CTLs were generated following the protocol described by Heiser *et al.*[24] The T cell-enriched non-adherent fraction of PBMCs was used for CTL generation. Non-adherent PBMCs were cultured in serum-free medium supplemented with 20 U/mL human IL-2 and 10 ng/mL human IL-7 (R&D Systems). The cells were stimulated weekly for at least four times with DC–tumor cell hybrids (DC–tumor hybrid) or DCs transfected with autologous total tumor–mRNA (DC–tumor RNA) at a stimulator:effector ratio of 1:10. After 16 days of culture, the cells were harvested without further separation for cytotoxicity assays.

In vitro cytotoxicity assay

Autologous primary cultured pancreatic cells, K562 cell line, PBMCs, and PC cell lines (Capan-2, AsPC-1, Panc-1, and MiaPaCa-2) were used as the target cells.

The target cells (2×10^6) were incubated in 1 mL of RPMI 1640 with 100 μCi $NaCrO_4$ solution (Isotope Products, Beijing, China) in 5% CO_2 for 1 h at 37°C. After three washes, 5×10^3 ^{51}Cr-labeled target cells and serial dilutions of effector CTL cells at various E:T ratios were incubated in 200 μL of RPMI 1640 in 96-well U-bottom plates. The plates were incubated at 37°C for 6 h in 5% CO_2. Then, 50 μL of the supernatant was removed, and ^{51}Cr release was measured with a gamma counter (Beckmann, Heidelberg, Germany). The spontaneous release was <15% of the total release by detergent in all assays. The percentage of specific lysis was calculated as ([experimental cpm – spontaneous cpm]/[maximum cpm – spontaneous cpm]) × 100. All determinations were made in triplicate, and the standard deviations (SDs) of the means from the triplicate wells were <5% of the mean.

CD4+ and CD8+ T-cell responses induced by DC pulsed with whole tumor antigens

The multiantigen specific CD4+ and CD8+ T-cell responses were measured by cytokine release assay as described.[25] CD4+ and CD8+ T cells were isolated from proliferating peripheral blood lymphocytes that were cultured after three cycles of re-stimulation *in vitro* using an autoMACS™ instrument (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were then incubated with CD4 or CD8 microbeads (Miltenyi Biotec) for 15 min at 4°C and then washed prior to separation. Separation was performed using an autoMACS column (Miltenyi Biotec).

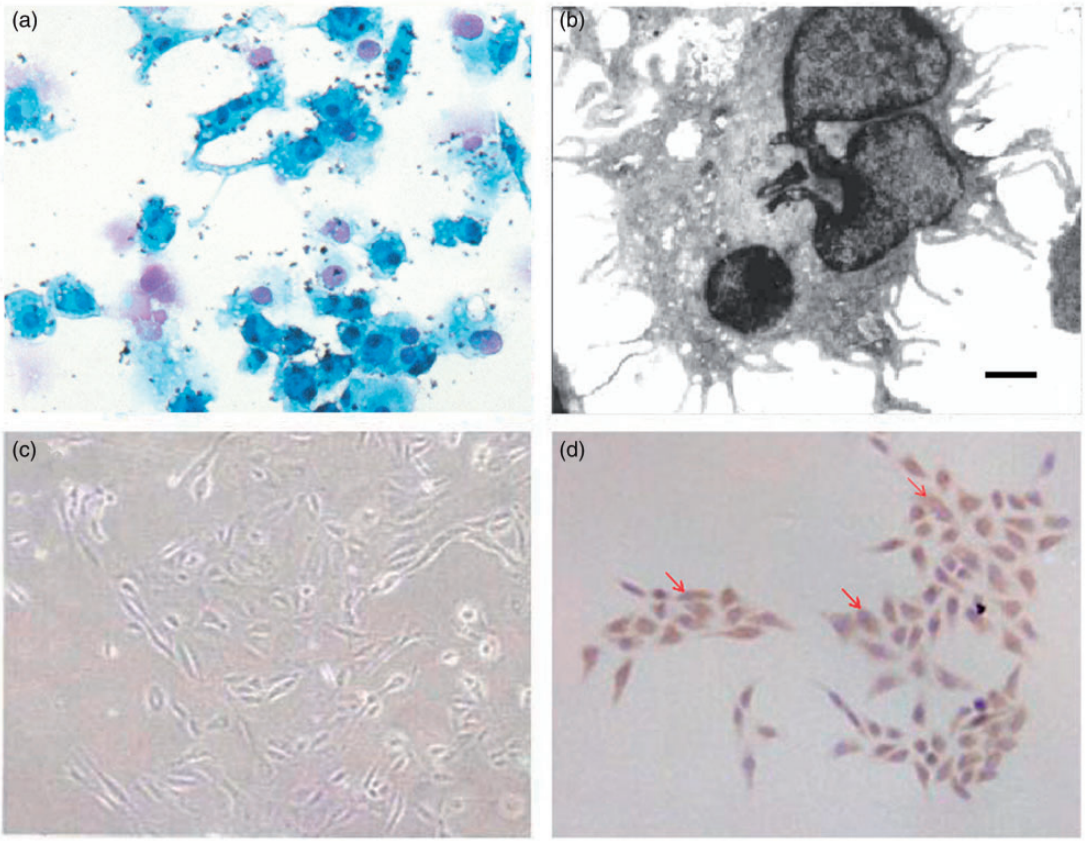

Figure 1 The characteristics of dendritic cells and primary tumor cells (derived from patient F). (a, b) Cultured for seven days, cells showed a typical morphology of DC (a: Giemsa staining, magnification 400 ×; b: transmission electron microscopy, bar represents 4 μm). (c) Morphology of the purified primary tumor cells (cultured for seven days, magnification 200×). (d) Over 90% of the cultured primary tumor cells (red arrows) showed a MUC1-positive expression (magnification 200 ×) (A color version of this figure is available in the online journal.)

The column was placed in the magnetic field, and magnetically labeled cells were retained in the column and then flushed out as positively selected cells when the magnetic field was turned off. The purity of the sorted populations was determined by flow cytometry. The positively selected CD4+ and CD8+ T cells (5×10^4) were stimulated with DCs (naked DCs, DC–tumor RNA, DC–normal tissue RNA, DC–tumor cell hybrid, DC–normal cell hybrid, 5×10^3) in a total volume of 200 μL of the complete medium in 96-well round bottomed plates for 24 h. The supernatants were collected, and the IFN-γ levels were measured using human IFN-γ ELISA kits (Endogen). Each assay was performed on duplicate samples.

Statistical analyses

The quantitative results were expressed as mean ± SD. Statistical analyses, including ANOVA and *post hoc* test, were performed using StatView 5.0 software (Abacus Concepts, Inc., Berkeley, CA). Statistical significance was considered at $P < 0.05$.

Results

Generation of DCs and primary tumor cells

DCs and primary tumor cells were cultured from all six PC patients. For DCs, irregularly shaped cells became predominant after further induction with TNF-α on day 7 (Figure 1(a) and (b)). After purification, most cultured primary tumor cells were fusiform and semi-polygonal in morphology (Figure 1(c)). Through the cell immunohistochemistry of anti-MUC1 mAb, over 90% of the cultured primary cells were identified as positive (Figure 1(d)). Otherwise, the stromal cells were not stained.

Efficiency of DC-tumor cells fusion and DCs electroporation with tumor RNA

As shown in Figure 2, DCs and irradiated tumor cells were clearly distinct. After the fusion, the percentage of dual-positive cells in the total cell population was approximately 45% for all six patients. The dual-colored cells were then gated and sorted as purified fusion hybrids. The dual-colored cell percentage in the purified hybrids cell population was >95% (data not shown).

Through using *in situ* cellular immune staining, >95% of auto DCs could be verified by the positive expressions of MUC1 after electroporation with tumor RNA for 48 h (data not shown).

Phenotype and cell viability changes in DCs pulsed with whole tumor antigens

As shown in Figure 3(a), the DCs in both groups (DC–tumor RNA and DC–tumor hybrids) exhibited positive expression

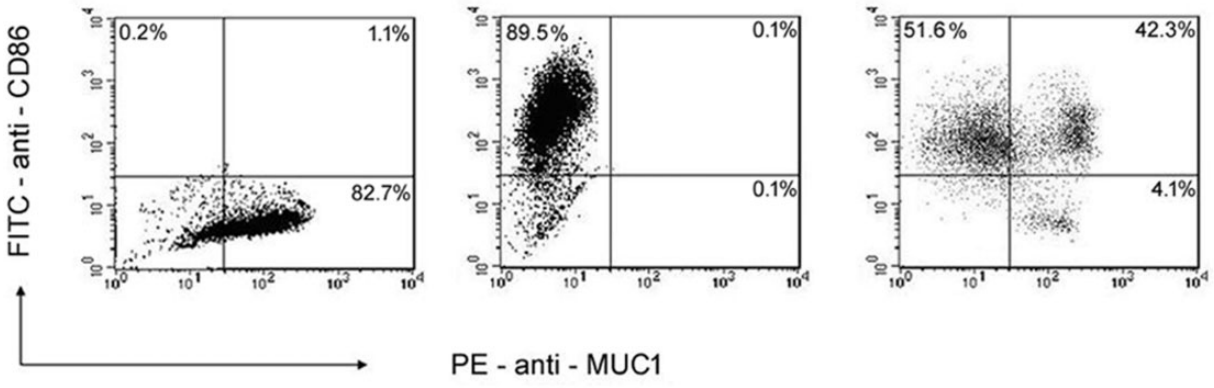

Figure 2 FACS analysis of DC–tumor fusion hybrid. DCs and tumor cells (from patient C) were stained with PE-anti-CD86 and FITC-anti-MUC1, respectively. After electrofused for 24 h, cells were collected and subjected to FACS analysis. The amount of double-positive hybrid cells population was 42.3%

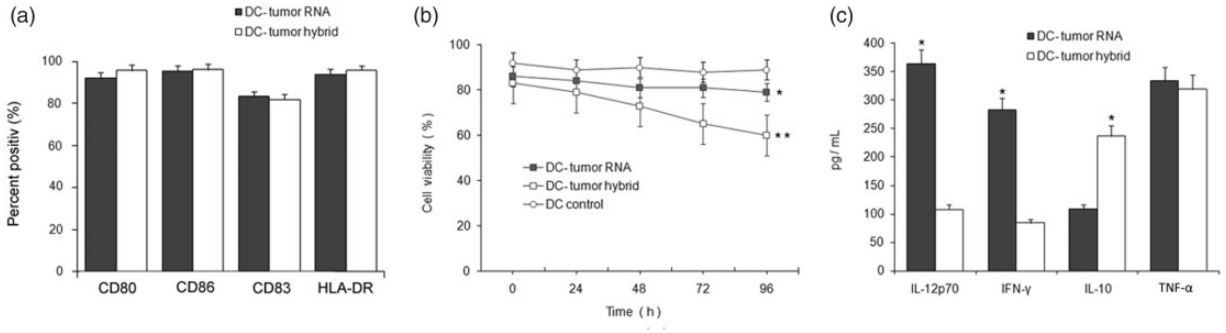

Figure 3 Phenotypic expression, cell viability, and cytokine expression in DC–tumor RNA and DC–tumor hybrid. (a) Both DC–tumor RNA and DC–tumor hybrid showed high expression levels of CD80, CD86, CD83, and HLA-DR. There were no significant change between the two methodologies.(b) The MTT assay showed that no significant difference in cell viability was observed between DC–tumor RNA (■) and DC control (○) from 0 to 96 h (*$P > 0.05$). But at the same period, the viability of DC–tumor hybrid cells (□) was obviously lower than that of the DC control (○) and decreased in a time-dependent manner (**$P < 0.01$). (c) ELISA test showed the cytokines of IL-12p70 and IFN-γ secreted by DC–tumor RNA were higher than those secreted by DC–tumor hybrids without significant change of TNF-α. Meanwhile, IL-10 level was lower in DC–tumor RNA than in DC–tumor hybrids (*$P < 0.05$). Results were all shown as the mean ± SD of all six patients for A, B, and C

of co-stimulatory molecules, including CD86, CD80, CD83, and HLA-DR, after loading the whole tumor antigens in different ways. Moreover, the flow cytometry test revealed that both total tumor RNA and tumor hybrid cell pulsing did not alter the four phenotypic surface molecules in matured DCs (*$P > 0.05$).

The results of the MTT test showed that the viability of DC–tumor RNA did not change significantly, with approximately 80% survival rate throughout the experiment (*$P > 0.05$). By contrast, the viability of DC–tumor hybrid cells was lower than that of DC control and decreased in a time-dependent manner (**$P < 0.01$) (Figure 3(b)).

Effects of total tumor RNA or tumor cell fusion pulsing on DC cytokine production

Measurement of IL-12p70, IFN-γ, TNF-α, and IL-10 production by ELISA showed that there were no significant releasing of all the four cytokines in the culture supernatant of the primary tumor cells (data were not shown). But DCs could secrete high levels of cytokines after pulsing with whole tumor RNA (Figure 3(c)). The IL-12p70 and IFN-γ secreted by DC–tumor RNA were higher than those secreted by DC–tumor hybrids (*$P < 0.01$). No significant difference in TNF-α level was observed between DC–tumor RNA and DC–tumor hybrids ($P > 0.05$). Simultaneously, the IL-10 level was lower in DC–tumor RNA than in DC–tumor hybrids (*$P < 0.05$).

CTL responses induced by DC–tumor RNA and DC–tumor hybrids

Auto-tumor cells were more effectively recognized and lysed by T lymphocytes induced by DC–tumor RNA and DC–tumor hybrids compared with T cells treated with DC–normal tissue RNA or DC–fusion normal cells (*$P < 0.05$; Figure 4(a) from six patients) in a dose-dependent manner. The lysis induced by DC–tumor RNA was more effective and powerful than that induced by DC–tumor hybrids (**$P < 0.05$). No differences were found between DC–normal tissue RNA and DC–fusion normal cells ($P > 0.05$).

No lysis of natural killer-sensitive K562 cells or normal PBMCs was observed (Figure 4(b) from all six patients). However, a significant lysis against PC cell lines was occurred. As shown in Figure 5(b), the effector T cells (HLA-A2+; from six patients) stimulated by DC–tumor RNA and DC–tumor hybrids lysed the Capan-2 and Panc-1 cell lines, which endogenously expressed the HLA-A2 antigen effectively. By contrast, the AsPC-1 and MiaPaCa-2 cell lines (HLA-A2−) were not recognized and

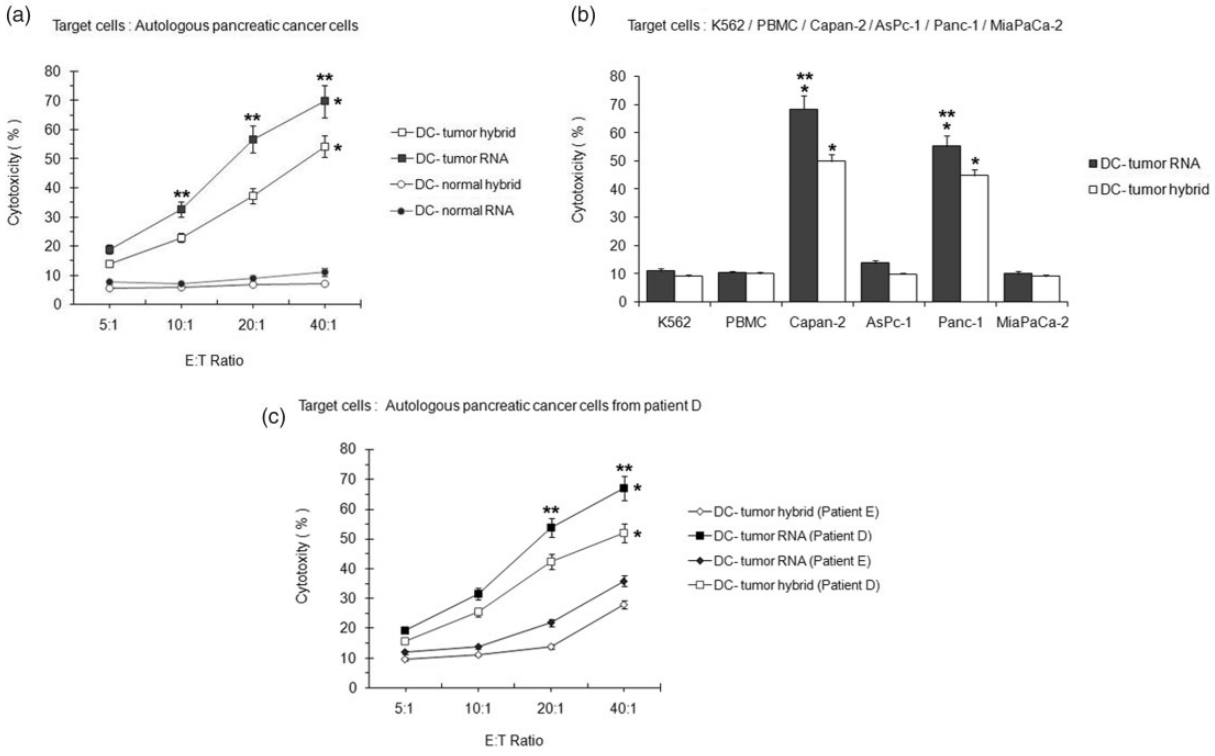

Figure 4 CTL induced by DC–tumor RNA and DC–tumor hybrids. Non-adherent PBMCs stimulated with DC–tumor RNA (■), DC– tumor hybrid (□), DC–normal RNA (•), and DC–normal hybrid (○) for 16 days were used as effector cells. Primary tumor cells (a and c), K562, PBMCs, Capan-2, AsPC-1, Panc-1 and MiaPaCa-2 cell lines (B) were used as target cells. (a) CTLs induced by DC–tumor RNA and DC–tumor hybrid could lyse auto tumor cells effectively whereas T cells activated by DC–normal RNA or DC–normal hybrid cells could not (*$P < 0.05$). Moreover, CTLs induced by DC–tumor RNA could produce a more powerful killing activity toward the tumor cells than the DC–tumor hybrids with the E:T ratio increasing from 10:1 to 40:1 (**$P < 0.05$). Experiments repeated for three times (n = 6). (b) At the E:T ratio of 40:1, CTLs could lyse the Capan-2 and Panc-1 cell lines (HLA-A2+) but not the AsPC-1 and MiaPaCa-2 cell lines (HLA-A2−) to a significant extent (*$P < 0.05$), which was more efficiently induced by DC–tumor RNA than by DC–tumor hybrid cells (**$P < 0.05$). Whereas no lysis against K562 or PBMCs was induced by either DC-tumor RNA or DC-tumor hybrid cells. Columns were shown as mean ± SD and triplicate samples were adopted (n = 6). (c) The lytic of CTLs induced by DC–autologous tumor RNA was more significant than DCs encoding allogeneic tumor antigen (*$P < 0.05$). Meanwhile, compared with allogeneic tumor cells fusion hybrid, the CTLs induced by DC-autologous tumor hybrid showed a more efficient response in recognizing and lysing autologous tumor cells (*$P < 0.05$). Furthermore, DC–tumor RNA showed a greater activity in inducing CTL responses than DC–tumor hybrid cells with the E:T ratio increasing from 20:1 to 40:1 (**$P < 0.05$).The reaction was repeated thrice, and the data were shown as mean ± SD

lysed (*$P < 0.05$). The cytotoxicity of CTLs induced by DC–tumor RNA showed a more powerful lysis against the HLA-A2-matched tumor cell line (Capan-2) compared with that induced by DC–tumor hybrids (**$P < 0.05$).

As shown in Figure 4(c), DCs encoding autologous tumor antigens or allogeneic tumor antigens stimulated remarkable lytic activity against target tumor cells. However, the lytic activity of CTLs induced by DC loaded with auto-tumor total RNA was more significant than DCs loaded with the allogeneic tumor antigen (*$P < 0.05$). Meanwhile, compared with allogeneic tumor cells fusion hybrid, the CTLs induced by DC-autologous tumor hybrid cells showed a more efficient response in recognizing and lysing autologous PC cells (*$P < 0.05$). Furthermore, DC–tumor RNA showed a greater activity in inducing target cell lyse CTL responses than DC–tumor hybrid cells with the E:T ratio increasing from 20:1 to 40:1 (**$P < 0.05$).

CD4+ and CD8+ T-cell responses induced by DC–tumor RNA and DC–tumor hybrids

As shown in Figure 5, the CD4+ T and CD8+ T cells incubated with DCs loaded with whole tumor antigens produced significantly higher IFN-γ levels than those incubated with DC alone or DC groups treated with normal tissues (*$P < 0.01$). Moreover, the CD4+ T and CD8+ T cells incubated with DC–tumor RNA produced higher IFN-γ levels than those incubated with DC–tumor hybrids (**$P < 0.01$).

Discussion

The present study showed that electroporation with total tumor cell RNA and pulsing with tumor fusion hybrid cells were both effective in eliciting antitumor immune responses in an HLA-restricted manner. But the RNA electroporation probably resulted in a potent tumor vaccine in activating T cells, such as Th1 and CTL against PC cells. This result was evidenced by the high percentage of tumor cells killing and IFN-γ secretion of CD4+ and CD8+ T cells. High expressions of cytokines, such as IL-12 and IFN-γ, might be the reason that why RNA-electroporated DCs could induce an enhanced CTL responses.

Tumor RNA transfection and DC/tumor cell fusion hybrid are two strategies for tumor-antigen delivery to DCs.[10] To ensure the utility of the comparison for the two types of strategies, the same number of cells was used to derive the tumor antigen with both methodologies.

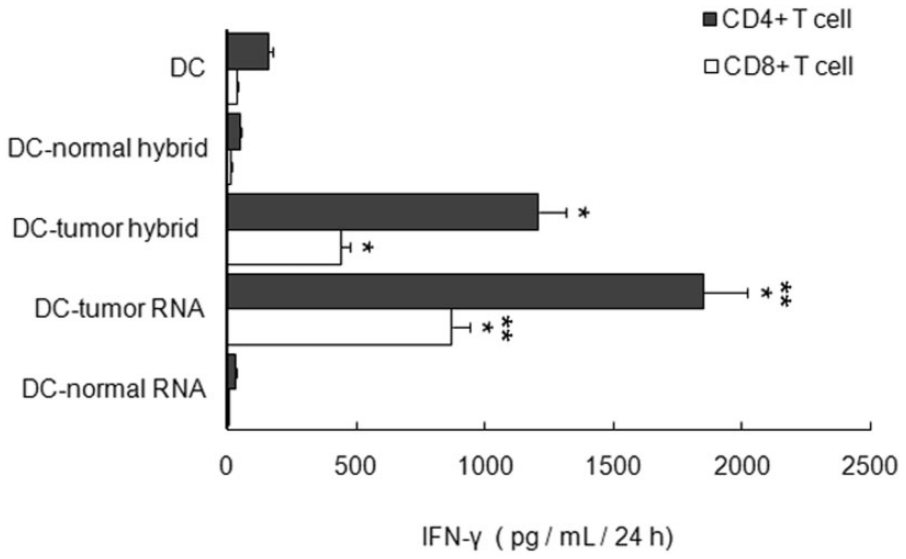

Figure 5 CD4+ and CD8+ T-cell responses induced by DC–tumor RNA and DC–tumor hybrid cells. The CD4+ T and CD8+ T cells incubated with DCs encoding whole tumor antigens could produce extremely higher IFN-γ levels than those incubated with DC alone or DCs treated with normal tissues (*$P < 0.01$). Furthermore, the CD4+ T and CD8+ T cells incubated with DC–tumor RNA could secrete more IFN-γ than those incubated with DC–tumor hybrids (**$P < 0.01$). The tests repeated for three times and results were shown as mean ± SD of all six patients

In humans, MUC1 is overexpressed in several cancer types, including breast, prostate, and PC.[26–28] Ideno *et al.*[29] found that MUC1 staining is positive in all 179 resected intraductal papillary mucinous neoplasms (IPMNs) and 180 resected pancreatic ductal adenocarcinoma cells without IPMNs but is negative or only weakly expressed in adjacent normal pancreatic tissues. This finding was confirmed by many other studies using microarrays, serial analysis of gene expression, and immunohistochemical staining.[30–32] In this study, the MUC1 expression in PC cells was used as a biomarker to indicate the efficiency of tumor antigens' loading in DCs. After the fusion hybridization with autotumor cells, about 45% of the cell population showed a CD86 and MUC1 dual-positive. Meanwhile, after RNA electroporation, over 95% of auto DCs were verified by the positive expressions of MUC1 protein using *in situ* cellular immune staining.

Our results indicated that RNA electroporation could be achieved with a relatively limited cell death compared with DC/tumor fusion hybrid cells, which may lead to apoptosis induced by some cytokines secreted from tumor cells.[33] Unlike the tumor cell fusion hybrid, transfected RNA may only function in the cytoplasm by reducing damage to DCs and thus maintaining the activity of DCs and the function of antigen presentation cells.[34] The results suggested that RNA electroporation was superior to DC-tumor cell fusion which might be due to its relative high DCs viability in the process of whole tumor-antigen delivering.

In the present study, we found that both DC–tumor hybrids and DC–tumor RNA could induce tumor-specific cytotoxic T cells to recognize and lyse autologous tumor cells effectively. Whereas K562 cells were not damaged, which suggest that the two strategies may not only be PC specific, but also simultaneously excluded the possibility of NK cell activity. The study data also demonstrated that the CTLs induced by DC–tumor RNA could produce a more effective lysis than that induced by DC–tumor hybrids. Moreover, the CTL responses induced by DC–autologous tumor RNA were superior to the CTLs induced by DC–allogeneic tumor RNA, demonstrating that DC–autologous tumor RNA might be a more excellent strategy.[35] Of course, it was also possible that tumor cells irradiation partly accounted for the difference results between the two approaches.[21]

CD4+ T cells plays an important role in priming of antitumor CD8+ CTLs in most of the tumors.[36] Ojima *et al.*[37] pointed out that DCs transduced with the TAA gene can elicit tumor-specific CD4+ T cells and that the CD4+ T cells had a critical function in the priming phase of CD8+ CTLs. The present study showed that DCs encoding whole tumor antigens with the two methods can activate not only tumor-specific CD8+ T cells but also CD4+ T cells judged by IFN-γ secretion. Moreover, the CD4+ T and CD8+ T cells incubated with DC–tumor RNA produced significantly higher IFN-γ levels than those incubated with DC–tumor hybrids.

One important problem that potentially limits the application of DC-whole tumor antigens PC vaccines is the induction of autoimmunity by encoding normal tissue antigens, which presented on DCs in the same antigen processing and presentation pathway as the tumor antigens.[9] Pancreatic tumor tissue is often surrounded by stromal and inflammatory tissues that reduce cellular homogeneity by 20–80%.[38] Of course, tumor cells do express a lot of autologous antigens as well as potential tumor antigens, but minimizing the effect of normal tissue on the homogeneity of tumor samples and avoiding the occurrence of autoimmunity induced by normal tissue–encoded antigens are still necessary. This study adopted tissue cell enrichment by primary tumor cell culture. The data showed that the cellular homogeneity of the tumor samples derived from PC patients was >90%. Moreover, the CTLs stimulated by DC–tumor RNA or DC–tumor hybrids lysed the tumor cells (PC cell lines and autologous primary cultured tumor cells) but not normal tissue cells (PBMCs).

Furthermore, DC–normal RNA and DC–normal hybrid cells all failed to stimulate tumor-specific CTL responses. This result indicated that harmful autoimmunity with pathological consequences may not be initiated by both RNA electroporation and tumor cell fusion hybrid approaches.

HLA allele matching between tumor-specific CTLs and target cells is necessary. As we know, all six patients evolved in this study were HLA-A2+ and the role of HLA-A2 among different HLA alleles is what we care about. We found that tumor-specific CTLs induced by DC-tumor hybrids and DC-tumor RNA could exert powerful cytotoxic activity against HLA-A2+ Capan-2 and Panc-1 cells. But for the reason of HLA-A2 mismatching, HLA-A2- AsPC-1 and MiaPaCa-2 PC cell lines could not be lysed by tumor-specific CTLs derived from HLA-A2+ PC patients. This result indicated that the HLA-A2 might be one important allele to present antigens and the immune response of PC-specific CTLs may be restricted to MHC class I antigens.

This study focused on why RNA electroporation DCs could induce a more potent activity in activating T cells than DC–tumor hybrid cells. The activation and polarization of naive T cells require some specific signals from DC, such as the engagement of a T cell receptor with a peptide-MHC on the DC and the interaction between the expression of co-stimulatory molecules (e.g. CD80 and CD86) on the DC surface with ligands (e.g. CD28). A third signal is provided by cytokines secreted by the DCs (e.g. IL-12), which promote T cell differentiation and polarization toward specific effector T cell phenotypes.[39] The present study revealed that the strategies of RNA electroporation and tumor cell fusion hybrid effectively delivered the whole tumor antigens to DCs. Meanwhile, both types of methods did not change the expression of co-stimulatory molecules on the DC surface, indicating that the approaches did not impair signals 1 and 2. Unlike RNA electroporation, tumor cell fusion hybrid significantly increased IL-10 production and suppressed IL-12 and IFN-γ secretion. This finding suggested that the limited T cell responses induced by DC–tumor fusion hybrids might be due to the disordered inflammatory cytokine environment. Th1 cytokines (IL-12, IFN-γ, and TNF-a) have critical functions in establishing long-lasting antitumor immunity; thus, the therapeutic efficacy of cancer vaccines largely depends on their capability to induce a switch to a Th1-type response.[40] However, a major obstacle in developing this type of vaccine is the suppression of the immune system by cytokines, such as IL-10, in the same microenvironment.[41] In the present study, the higher potency of the total tumor RNA transfection DC vaccine than the DC-tumor fusion hybrid vaccine may be partly due to the higher levels of IL-12p70 and IFN-γ accompanied with lower levels of IL-10 released by the DCs in the former vaccine than in the latter.

The results of the present study showed that pulsing DCs with total tumor RNA as antigen delivery is a promising alternative to tumor cell fusion hybrid because it induces better Th1 and CTL responses against PC cells. These findings suggest that RNA electroporation may be a preferred method of loading DCs with whole tumor antigens. Further investigation should be carried out to evaluate the efficacy of this strategy in clinical applications.

Authors' contributions: All authors participated in the design of experiments and interpretation and analysis of the results.

ACKNOWLEDGEMENTS

This work was supported by the grant of national natural science foundation of China (81071982).

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(Received September 13, 2014, Accepted December 21, 2014)