RESEARCH ARTICLE

Combination Doxorubicin and Interferon-α Therapy Stimulates Immunogenicity of Murine Pancreatic Cancer Panc02 Cells via Up-regulation of NKG2D ligands and MHC Class I

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Abstract

Background: Pancreatic adenocarcinoma is a malignant gastrointestinal cancer with significant morbidity and mortality. Despite severe side effects of chemotherapy, the use of immunotherapy combined with chemotherapy has emerged as a common clinical treatment. In this study, we investigated the efficacy of the combined doxorubicin and interferon-α (IFN-α) therapy on murine pancreatic cancer Panc02 cells *in vitro* and *in vivo* and underlying mechanisms. Materials and Methods: A Panc02-bearing mouse model was established to determine whether doxorubicin and interferon-α (IFN-α) could effectively inhibit tumor growth *in vivo*. Cytotoxicity of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) was evaluated using a standard LDH release assay. To evaluate the relevance of NK cells and CD8 T cells to the combination therapy-mediated anti-tumor effects, they were depleted in tumor-bearing mice by injecting anti-asialo-GM-1 antibodies or anti-CD8 antibodies, respectively. Finally, the influence of doxorubicin+interferon-α (IFN-α) on the ligands of NK and T cells was assessed by flow cytometry. Results: The combination therapy group demonstrated a significant inhibition of growth of Panc02 *in vivo*, resulting from activated cytotoxicity of NK cells and CTLs. Depleting CD8 T cells or NK cells reduced the anticancer effects mediated by immunochemotherapy. Furthermore, the doxorubicin+IFN-a treatment increased the expression of major histocompatibility complex class I (MHC I) and NKG2D ligands on Panc02 cells, suggesting that the combined therapy may be a potential strategy for enhancing immunogenicity of tumors. All these data indicate that the combination therapy using doxorubicin and interferon-α (IFN-α) may be a potential strategy for treating pancreatic adenocarcinoma.

Keywords: Pancreatic cancer - interferon-α - doxorubicin - combination therapy

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Introduction

Pancreatic adenocarcinoma is the fifth leading cause of cancer-related deaths worldwide and has a low survival rate (Jemal et al., 2010; Ma et al., 2014). Currently, surgical resection, chemotherapy and radiation therapy are widely used as clinical treatments. However, surgical resection can only be performed on a small number of pancreatic cancer patients because pancreatic adenocarcinomas are usually at an advanced stage when diagnosed (Sener et al., 1999). Unfortunately, even after resection, recurrence occurs in a majority of patients. Additionally, even though traditional chemotherapy and radiation therapy have improved the overall survival rate, their severe side effects, including an impaired immune system, limit their clinical use (Neoptolemos et al., 2004). Hence, this current situation creates the need for new therapeutic approaches. Among them, immunotherapy is being investigated for treating pancreatic cancer, and this type of treatment mainly strives to induce efficient immune responses against pancreatic cancer cells (Uram and Le 2013). However, single strategies alone have not produced a significant effect, and the combined use of existing treatments has attracted the attention of scientists.

The combination of chemotherapy and immunotherapy is a novel strategy in treating pancreatic cancer, and this combination might not only directly kill tumor cells but also activate an immune response to clear target cancer cells (Gabrilovich 2007). Therefore, in this present study, the effect of immunochemotherapy (doxorubicin and IFN-α) was investigated.

Doxorubicin (DOX) is a traditional chemotherapeutic agent. It has been reported that in addition to its direct tumoricidal activity, doxorubicin also promotes antitumor immunity (Bandyopadhyay et al., 2010; Mattarollo et al., 2011; Nugroho et al., 2012). Furthermore, cytokines,

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such as IL-2 and IFN-α, are usually combined with chemotherapeutics in treating cancer (Savage et al., 1997 , Ma et al., 2005). However, the effect of doxorubicin combined with IFN-α on pancreatic adenocarcinomas has not yet been investigated.

In this study, the effect and underlying mechanism of a combined therapy using doxorubicin and IFN-α were investigated. Our results suggested that the doxorubicin+IFN-α treatment suppressed Panc02 cell growth *in vivo* by stimulating NK cytotoxicity and CTL activity, which resulted from an up-regulation in the expression of the CD8 T ligand and NKG2D ligands in the Panc02 cells. These results emphasize the therapeutic potential of doxorubicin and IFN-α immunochemotherapy in treating pancreatic cancer.

Materials and Methods

Cell culture

The murine pancreatic carcinoma cell line Panc02 was cultured in RPMI medium supplemented with 10% fetal calf serum (Gibco, Vienna, NY, USA), 100 U/ml of penicillin and 100 U/ml of streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

Experimental animals

Male C57BL/6 mice (purchased from the Academy of Military Medical Science) were housed in a rodent facility at 22±1°C with a 12h light-dark cycle and were provided with a continuous supply of standard rodent chow and water while acclimating to the environment. All of the procedures in this study were conducted in accordance with protocols approved by the Ethics Committee of Jilin University.

In vivo treatments

Eight-week-old male mice were used for the experiments. A total of 2.5×10^4 Panc02 cells were injected into the head of the pancreas as previously described (Khallouf et al., 2012). Five days after tumor cell implantation, mice were treated with one of the following regimens: vehicle control (0.2ml phosphate-buffered saline (PBS) i.*p*. from day 5 to day 9], doxorubicin alone (2.5mg/kg; i.*p*. from day 5 to day 9), murine IFN-α alone [5×10^2 IU/g, injected subcutaneously (SC) on days 5, 7, and 9], or doxorubicin (2.5mg/kg; i.*p*. from day 5 to day 9) with murine IFN-α (5×10^2 IU/g, SC on days 5, 7, and 9). On day 21, mice were sacrificed by cervical dislocation. Then, the tumors were harvested, and the tumor volume was measured.

The tumor volume (TV) was measured and calculated using the following formula: $TV=1/2\times a\times b^2$, where a and b are the long and short diameters, respectively, of the tumor in each mouse.

Depletion of NK cells or CD8 T cells in vivo

To deplete the NK cells or CD8 T cells in tumor-bearing mice, the neutralizing monoclonal antibody anti-asialo-GM-1 antibody (Wako Pure Chemical Industries) and antibody clone YTS 169.4, (AbDSerotec) were used to deplete the NK cells and CD8 T cells, respectively. Anti-asialo-GM-1 antibody (50μl) (Wako Pure Chemical Industries) or antibody clone YTS 169.4 were administered by intraperitoneal injection 3 days prior to drug treatment, followed by an injection every four days for three weeks. Control mice received nonimmune antibodies with the corresponding IgG isotype.

Flow cytometry analysis

Panc02 cells were treated with doxorubicin (0.05 μM), IFN-α (50 IU/ml) or doxorubicin (0.05 μM) and IFN-α (50 IU/ml) for 48 h. Then, the cells were collected, stained with anti-Mult-1 (eBioscience), anti-Pan-Rae (R&D Systems), and anti-MHC class I H-2Db (BD Bioscience) and analyzed using flow cytometry.

Cytotoxicities of NK cells and CTLs

The cytotoxic activities of the NK cells and CTLs were determined using a CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, WI, USA) as previously described (Ke et al., 2014). Briefly, for CTL activity, splenic lymphocytes from each group were treated with Panc02 cells at the effector:targetor (E:T) ratios of 40:1, 20:1, 10:1. After incubating for 4h, the cytotoxicity was measured. The detection protocol for NK cytotoxicity was similar to the protocol used for CTL cytotoxicity detection except that YAC-1 cells were used instead of Panc02 cells.

Statistical analyses

The data in this study were analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett's test to identify differences between either the control and drug-treated groups or the isotype-antibody control and antibody-treated groups. The results are presented as the means ± standard deviation (SD). P-values less than 0.05 were considered statistically significant.

Results

Combined therapy of doxorubicin and IFN-α effectively suppresses the growth of Panc02 in vivo

The combined effect of doxorubicin and IFN-α on Panc02-bearing mice was first investigated. As shown in Figure 1A, the tumor volumes were markedly reduced in the doxorubicin+IFN-α treatment group (Figure 1A), and this reduction was greater in the combination treatment group than in the doxorubicin or IFN-α monotherapy treated groups.

Considering that either doxorubicin or IFN-α has immune regulatory activity, the cytotoxicity of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells were measured. The results indicated that both the CTL activity and NK cytotoxicity were up-regulated after doxorubicin and IFN-α administration (Figures 1C and 1D). Specifically, at the ratio of 40:1 (effector:targetor), the activity of the CTLs and NK cells increased to 72.2% and 65.1%, respectively. These results suggest that the doxorubicin+IFN-α-induced tumor inhibitory effect may result from the stimulation of the immune response in tumor-bearing mice.

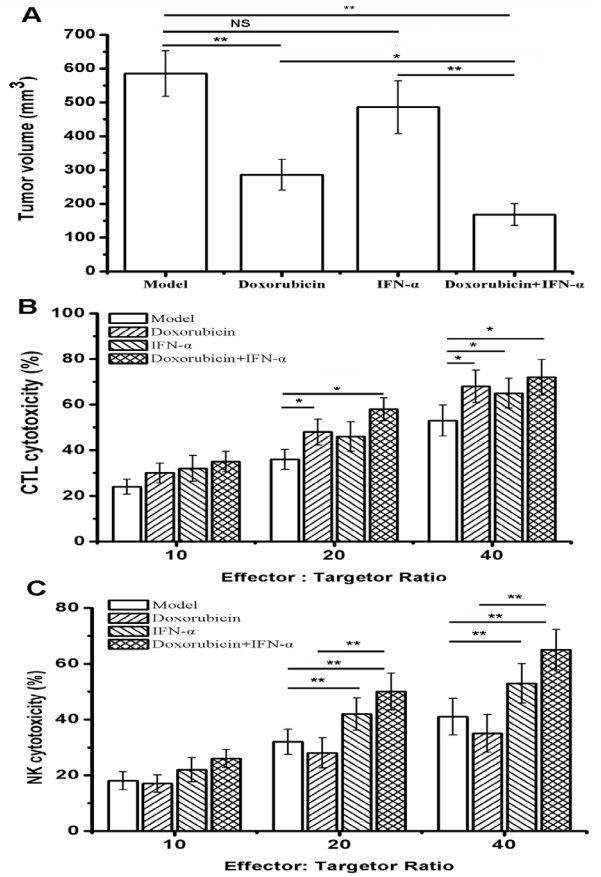

Figure 1. Doxorubicin combined with IFN-α prevents Panc02 tumor growth *in vivo* by stimulating the immune response. A) The tumor volume of each group was measured, and doxorubicin+IFN-α significantly suppressed the increase in tumor volume. **B) C)** The cytotoxicity of CTLs (B) and NK cells (C) was up-regulated in the combined therapy group. (n=10; the data represent the means±SD; **p*<0.05, ***p*<0.01, NS (not significant), *p*>0.05)

Depletion of CD8+T cells or NK cells impairs the anti-tumor effects of doxorubicin+IFN-α combination therapy in Panc02-bearing nude mice

The finding that the cytotoxic activities of both the CTLs and NK cells were up-regulated in the combination therapy *in vivo* strongly suggested that CD8 T cells and NK cells are necessary for doxorubicin+IFN-α-induced tumor suppression. To confirm this hypothesis, CD8 T cells were depleted in tumor-bearing mice *via* intraperitoneal injection of anti-CD8 T cell antibody. As shown in Figure 2, treatment with this antibody almost completely abolished the doxorubicin+IFN-α-induced suppression of Panc02 growth *in vivo*. Similar results were obtained in NK cell-depleted tumor-bearing mice. Taken together, these results indicate that CTL activity and NK cytotoxicity are necessary for the anti-Panc02 activity of doxorubicin+IFN-α.

Doxorubicin combined with IFN-α treatment increases the expression of MHC Class I in Panc02 Cells

Doxorubicin and IFN-α administration significantly inhibited tumor growth *in vivo*, in part, by stimulating the cytotoxicity of CTLs. Moreover, the expression of MHC class I on tumor cells is necessary for recognition by CD8 T cells. Hence, the effect of doxorubicin+IFN-α on the

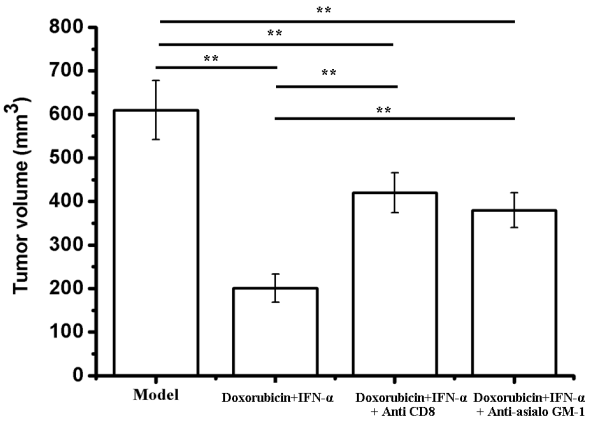

Figure 2. NK cells and CD8 T cells are necessary for the anti-Panc02 effects of doxorubicin and IFN-α combination therapy. The selective depletion of CD8 T cells or NK cells by intraperitoneal injection of anti-CD8 antibody or an anti-asialo-GM-1 antibody, respectively, abrogated the doxorubicin+IFN-α-induced anti-tumor effects. (n=10; the values represent the means±SD; ***p*<0.01)

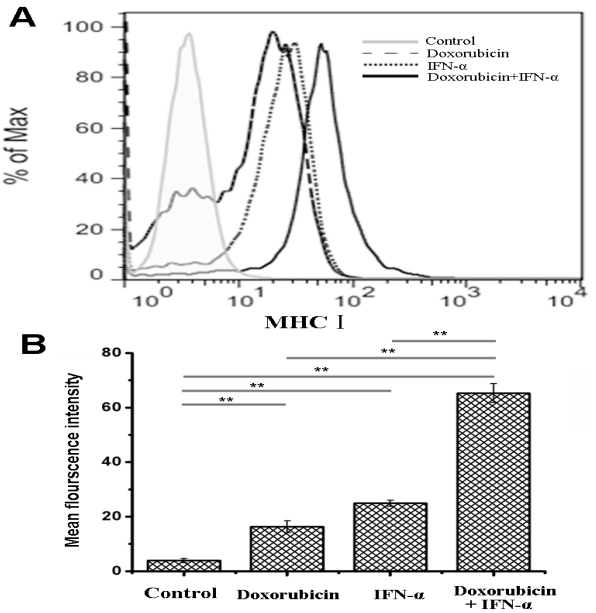

Figure 3. The combination therapy of doxorubicin and IFN-α enhances the expression of MHC I on Panc02 cells. Panc02 cells were cultured without (control) or with doxorubicin (0.05μM), IFN-α (103 IU) or doxorubicin+IFN-α (0.05μM and 103 IU, respectively) for 48 hours. The expression of MHC class I molecules in Panc02 cells was detected with flow cytometry using Anti-mouse MHC class I and presented as the mean fluorescence intensity (MFI). Controls include unstained cells and isotype controls of the corresponding IgG isotype. Peak chart of the flow cytometry analysis A) and corresponding MFI **B)** are shown (n=3; the values represent the means±SD, ***p*<0.01)

expression of MHC class I proteins in Panc02 cells *in vitro* was investigated. As shown in Figure 3A, although both doxorubicin alone and IFN-a alone increased the expression of MHC class I proteins in Panc02 cells, doxorubicin+IFN-a significantly increased the MHC class I protein expression compared with either the control or drug monotherapies. Therefore, the combination therapy-mediated MHC class I protein up-regulation may explain

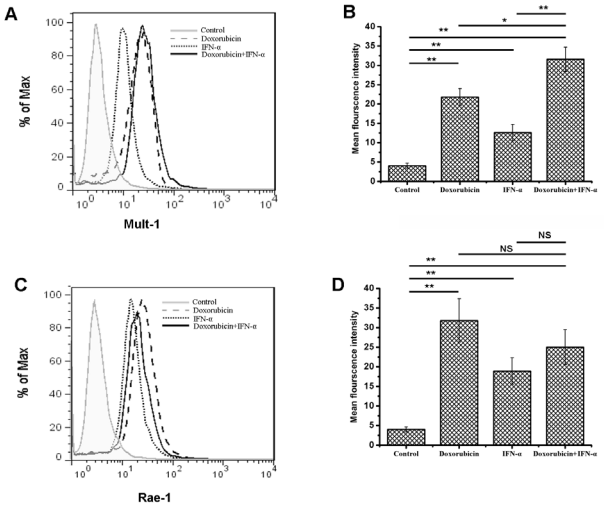

Figure 4. Doxorubicin combined with IFN-α increases the expression of NKG2D ligands (Mult-1 and Rae-1) on Panc02 cells. Panc02 cells were cultured as previously described. After 48h, the cells were harvest, then the expression of Mult-1 or Rae-1 in Panc02 cells was detected with flow cytometry using anti-mouse Mult-1 (A, B) or Rae-1 (B, D), respectively. Peak chart of the flow cytometry analysis (A,C) and corresponding MFI (B, D) are shown. (n=3; the values represent the means±SD, *p<0.05, **p<0.01, NS (not significant), p> 0.05)

the enhanced recognition of Panc02 cells by CD8 T cells.

Doxorubicin and IFN-α combination therapy up-regulates the expression of NKG2D ligands (Mult-1and Rae-1) in Panc02 cells

The doxorubicin and IFN-α immunochemotherapy *in vivo* results suggested that NK cells are also necessary in the combined therapy-mediated immune response. Therefore, we measured the expression levels of the ligands of the killing-activating receptors Mult-1 and Rae-1, which are the NKG2D ligands, in Panc02 cells. To evaluate the effects of the different treatments on the expression of Mult-1 and Rae-1, we treated Panc02 cells with doxorubicin, IFN-α, or doxorubicin+IFN-α. The combined therapy significantly increased the expression of Mult-1, the elevated level of which was higher than that for doxorubicin alone or IFN-α alone. As for Rae-1 expression, although the doxorubicin+IFN-α treatment increased Rae-1 expression, this increase was smaller than that observed with the doxorubicin alone treatment. All of the data indicated that the main effect of the combination therapy on increasing the expression of the NKG2D ligands results from doxorubicin.

Discussion

Pancreatic adenocarcinoma is the most common malignant cancer, and it has been associated with a high fatality rate in humans. Chemotherapy causes significant side effects when used in the clinical management of diseases. Therefore, a strategy using chemotherapy combined with immunotherapy was developed. In clinical trials, the combined therapy strategy has produced some promising results. It has been reported that the adjuvant combination therapy approach including 5-FU and IFN-a has resulted in an overall 5-year survival rate of 55% in a phase II clinical trial (Picozzi 2003). In this study, doxorubicin combined with IFN-α exhibited an undeniable antitumor effect in a Panc02-bearing mouse model, which was better than that observed in the mono-treatment group. Next, the mechanism underlying the effects of this combined therapy was further elucidated.

It has been well established that both NK cells and CTLs are vital in the cell-mediated immunity response to eliminate target tumor cells. Notably, tumor cells often exhibited low or no expression of MHC molecules and then escape the cytotoxicity of CTLs (Sande et al., 2005). NK cells derived from the bone marrow are an important and necessary effector population for eliminating the malignant tumor cells that do not express MHC or express mutant forms of MHC molecules (Siddle et al., 2013; Kärre 2002; Stojanovic and Cerwenka 2011). In this study, the combined therapy of doxorubicin and IFN-α not only significantly suppresses tumor growth *in vivo* but it also stimulates the immune system, including CTL activity and NK cell cytotoxicity. To further investigate the role of doxorubicin+IFN-α-induced CTL and NK cytotoxicity in inhibiting pancreatic adenocarcinoma, CD8 T or NK cell depletion *via* pretreatment with an anti-CD8 T or anti-asialo-GM-1 antibody, respectively, in Panc02-bearing mice almost completely abolished the immunochemotherapy-induced suppression of cancer cells and the stimulation of NK cytotoxicity. Based on these results, we concluded that doxorubicin+IFN-α-enhanced CTL and NK cytotoxicity is necessary in treating Panc02-bearing mice.

Doxorubicin is an antineoplastic drug that is broadly used in cancer treatments (Tacar et al., 2013). Doxorubicin could be involved in the NK cell-mediated killing of tumor cells by stimulating the expression of the ligands for the NK group 2D (NKG2D) and DNAX accessory molecule-1 (DNAM-1)-activating NK cell receptors (Gasser et al., 2005; Soriani et al., 2009). This drug has indeed been shown to induce an "immunogenic type" of tumor cell death, leading to the stimulation of dendritic cell antigen-presenting function (Casares et al., 2005). Then, we detected the ligand of CD8 T cells, which is a major population of CTLs, and the ligands of NK cells expressed on Panc02 cells after combined treatment *in vitro*.

In the doxorubicin+IFN-α treatment group *in vitro*, the MHC-I molecule, which is the ligand of CD8 T cells, and Rae-1 and Mult-1, which are the ligands of the activating receptor NKG2D on NK cells, were more highly expressed on the Panc02 cells. The expression of MHC class I molecules in tumor cells is essential for recognition by CD8 T cells (Gromme and Neefjes 2002). In this study, the combination treatment up-regulated the MHC class I expression, thus making the Panc02 cells sensitive to CD8 T cells. Additionally, the doxorubicin+IFN-α treatment also increased the Rae-1 and Mult-1 expression levels. NKG2D, a stimulatory receptor, is expressed on the surface of NK cells and subsets of T cells (Raulet 2003). NKG2D ligands consist of the Rae-1 family, H60 and Mult-1 in mice (Diefenbach et al., 2000; Carayannopoulos et al., 2002). Moreover, NKG2D ligands are widely

expressed in cancer cell lines (Pende et al., 2002; Salih et al., 2003; Zhao et al., 2014) and are generally absent in healthy tissues. Therefore, it is possible that the doxorubicin+IFN-α treatment increased the expression of the ligands of CD8 T and NK cells first, then activated the immune system-mediated anti-tumor effect.

IFN-α is a pluripotent cytokine that is already used in cancer therapy (Gutterman 1994). IFN-α can not only enhance the generation of CD8+ memory cells, but it can also activate macrophages and natural killer (NK) cells (Marrack et al., 1999; Pfeffer et al., 1998). In the present study, the combined treatment group increased the expression of the ligands of CD8 T and NK cells, and the activity of the CD8 T and NK cells also increased after IFN-α administration, then the immune response was significantly enhanced to recognize and delete tumor cells.

In conclusion, our study provides more insight into the mechanism underlying the effects of doxorubicin used in combination with IFN-α. This combined therapy enhances the recognition of Panc02 cells by CTLs and NK cells by up-regulating MHC I molecules, Rae-1 and Mult-1. All of these results illustrate the potential use of the combination therapy of doxorubicin and IFN-α in treating pancreatic adenocarcinoma in the clinic.

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