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Original article

Docetaxel enhances CD3+ CD56+ cytokine-induced killer cells-mediated killing through inducing tumor cells phenotype modulation

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ABSTRACT

Pretreatment with chemotherapeutic agents could sensitize human lung adenocarcinoma cells to the lyses of cytokine-induced killer (CIK) cells, however, the mechanism still unclear. We hypothesized that chemotherapeutic agents could induced immunogenic modulation (IM) and calreticulin (CRT) expression and enhanced the cytokine-induced killer (CIK) cells-mediated killing. Here, using docetaxel, one of the most widely used cancer chemotherapeutic agents, as a model, we examined the molecular and immunogenic consequences of chemotherapeutic agent exposure in lung adenocarcinoma cell SPC-A1 cells. Our results showed that the human lung adenocarcinoma cells displayed an increased sensitization to lyses of CD3+ CD56+ CIK cells after treatment with nonlethal/sublethal doses of docetaxel in vitro. Docetaxel treatment of tumor cells did not induce ATP or high-mobility group box 1 (HMGB1) secretion, or cell death. However, calreticulin (CRT) exposure was observed. Enhanced killing by CIK cells was mediated largely by CRT membrane translocation, as determined by functional knockdown of CRT, or CRT blocking antibody. This study provides evidence that the pretreatment with chemotherapeutic agents can sensitize tumor cells to the lyses of CD3+ CD56+ CIK cells in vitro through inducing immunogenic modulation and upregulating in target cells.

1. Introduction

Lung cancer is still the leading cause of cancer-related death worldwide, non-small cell lung cancer (NSCLC) accounts for more than 85% of all lung cancer cases [1,2]. Despite advances in surgery, chemotherapy and radiotherapy, the prognosis of the patients with advanced Lung cancer remains poor. Therefore, new and effective treatment modalities, such as immunotherapy, are urgently needed. Adoptive cell immunotherapy might be used in combination with standard therapies-as adjuvant postsurgical treatment and as palliative treatment-to improve survival and quality of life of lung cancer patients [3]. Adoptive cytokine-induced killer cells (CIKs) transfer showed promising antitumor effect on various malignant tumors, including NSCLC [4].

CIK cells are generated in vitro from peripheral blood lymphocytes (PBLs) after stimulated with interferon-γ (IFN-γ), monoclonal antibody against CD3 and interleukin (IL)-2 [4]. These cells mediate highly efficient non–MHC-restricted killing of tumor cell targets. The greatest lytic activity in CIK cell cultures is found in the population of cells coexpressing CD3 and CD56 [5]. The molecular structures that involved in tumor recognition and killing by CIK cells is not completely understood. Previous observations suggested a possible involvement of the NKG2D and lymphocyte function-associated antigen-1 (LFA-1) molecules [6,7]. In clinical studies, the success of adoptive CIK cell transfer is still unsatisfactory. Therefore, it is urgent to find a substantial enhancement of adoptive cell transfer efficacy to improve clinical effects of cancer patients.

Anticancer therapies such as chemotherapy aim for direct killing of tumor cells. However, recent studies have demonstrated that these modalities may have immunomodulatory effects. The group of Kroemer found that malignant cells dying upon exposure to doxorubicin (Dox) can elicit strong antitumor immune responses, which is mediated by calreticulin (CRT) exposure and ATP, high-mobility group box 1 protein (HMGB1) secretion on apoptotic cells [8]. Such a cell death modality has been designated "immunogenic cell death" (ICD). CRT is a major molecular determinant that makes the difference between ICD and non- ICD. In addition, researchers have also reported that a novel mechanism – "immunogenic

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modulation" (IM), complementary to ICD, whereby anticancer therapies alter the surface phenotype of surviving cancer cells, including the exposure of CRT, to render them more sensitive to antigen-specific CD8+ cytotoxicity T cell (CTL) – mediated killing [9,10]. Docetaxel (DTX), is one of the conventional anticancer agents, exhibits broad antitumor activity. Ppretreatment nonlethal/sublethal dose of DTX can induce phenotype modulation of tumor cells, upregulate CRT expression on the outer leaflet of the plasma membrane. CRT is a ubiquitous endoplasmic reticulum multifunctional Ca^{2+} binding chaperone and has been implicated in a variety of diverse functions. Although CRT mainly localizes in intracellular compartments, it can also appear at the surface of various types of cells and modulate modulate cell adhesion and migration through interaction with integrins [11], extracellular matrix proteins fibrinogen [12] and laminin [13,14]. Ecto-CRT on dying or apoptotic cells can serve as an "eat me signal", rendering the cells recognized and attacked by phagocytosis more efficiently. Ecto-CRT also can promote tumor cell-CTL interaction and then enhance the cytotoxicity of CTL [9,15]. Studies have demonstrated that CIK cells originate in vitro from CD56-CD8+ T cell progenitors [16] and pretreatment low-dose of DTX could enhance the cytotoxicity of CIK cells [17]. Herein, we hypothesized that immunogenic modulation of tumor cell phenotype by DTX could enhance productive interactions between CIK cells and cancer cells. In this study, we used lung adenocarcinoma cell SPC-A1 as a model to investigate the effects of DTX on molecules that have been implicated in enhancing T cell-mediated tumor cell killing through diverse mechanisms, including calreticulin (CRT), the adhesion/costimulatory molecule ICAM-1 were evaluated.

2. Materials and methods

2.1. Cell line

Human lung adenocarcinoma cell line SPC-A1 was purchased from the American Type Culture Collection and cultured in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin and grown at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Reagents

Recombinant human interferon-γ (IFN-γ), recombinant human interleukin-2 (IL-2) and CD3 monoclonal antibody were purchased from Peprotech (Rocky Hill, NJ, USA). Anti-ICAM-1-FITC, CRT-PE, anti-ICAM-1 and CRT blocking antibody were obtained from BD Co. (BD, USA). High-mobility group box 1 (HMGB1) ELISA kit was from Shino-Test Corporation (Shanghai, China), ATP bioluminescence kit was from Kaiji (Nanjing, China) and MTT kit were form Sigma-Aldrich (St. Louis, MO). Anti-CD56 microbeads was from Miltenyi Biotec. siRNA heteroduplexes specific for CRT (sense strand: 5′-CCGCUGGGUCGAAUCCAAATT-3′), an unrelated control (5′-GCCGGUAUGCCGGUUAAGUTT-3′) were constructed by Genepharma (Shanghai, China). The HiPerFect transfection reagent was from Qiagen (Valencia, CA).rCRT was produced by our laboratory. Doxorubicin was from Sigma-Aldrich. Preparations of DTX were diluted to 1 mg/mL in phosphate-buffered saline (PBS). The drug was then further diluted in sterile PBS to a working stock of 10 mg/mL.

2.3. Analysis of immunogenic cell death

SPC-A1 cells were treated with indicated treatments for 72 h. Cells were harvested and assessed for viability by PI staining and cell-surface expression of CRT (see below). Supernatant fluids were analyzed for high-mobility group box 1 (HMGB1) protein by ELISA, and for ATP by bioluminescence, according to the manufacturer.

2.4. Generation of CIK cells

CIK cells were prepared as described previously [18,19]. Briefly, human peripheral blood mononuclear cells (PBMCs) were obtained after the centrifugation of fresh blood on a density gradient, using the Ficoll-Hypaque technique. Cells were then resuspended in complete RPMI medium. IFN-γ was added at 1000 U/mL for 24 h. The next day, IL-2 and anti-CD3 antibody were added at 300 U/mL and at 50 ng/mL, respectively. Thereafter, cells received both recombinant IL-2 (500 U/mL) and fresh media every 3–5 days. Cells were harvested on day 14 with apparent viability above 90%. At the end of the expansion, CD56 + CIK cells were immunoselected with anti-CD56 microbeads according to the manufacturer's instructions.

2.5. Flow cytometry analysis

Cell-surface staining of tumor cells was performed using the primary labeled monoclonal antibodies (mAb) ICAM-1-FITC, CRT-PE. The appropriate isotype-matched controls were purchased from BD Biosciences. Stained cells were acquired on a FacsCalibur flow cytometer using CellQuest software (BD Biosciences).

2.6. RNA interference knockdown and manipulation of surface CRT

SPC-A1 cells were transfected by siRNA at a final concentration of 100 nM using HiPerFect Reagent. To restore ecto-CRT expression, cells were exposed to rCRT in PBS on ice for 30 min, followed by three washes.

2.7. Cytotoxic assay

MTT assay was used to estimate the antitumor cytotoxic activity of CIK cells. The SPC-A1 cells were cultured with DTX at various concentrations (20 and 200 ng/mL) for 72 h and subsequently used as targets. The SPC-A1 cells (5×10^3 cells/well) were incubated with CIK cells (E:T ratio of 40:1) to constitute a final volume of 200 μL/well on the 96-well plate for 48 h at 37 °C, 5% CO_2.

For masking experiments, anti-ICAM-1 and CRT blocking were used. CIK cells were preincubated 1 h at room temperature either in the absence or in the presence of 0.02 mg/mL of specific or isotype-matched control and, after washing, used in the cytolytic assay tested.

The percentage of specific lysis was determined according to the formula: percent specific lysis = [(experimental-spontaneous)/(maximum-spontaneous)] × 100. Maximal release was obtained with 1% Triton X-100 detergent.

2.8. Statistical analysis

The results are shown as mean ± standard error of the mean (SEM) of triplicate determinants. Statistical analysis of standard deviations (SDs) and *P*-values were done by using the Student's *t*-test and analysis of variance. A P-value < 0.05 was taken as statistically significant. Statistical analysis was conducted using the SPSS software 16.0 (SPSS, Chicago, IL, USA).

3. Results

3.1. Tumor cells treated with DTX significantly increased sensitivity to CD3+ CD56+ CIK killing

At the end of the culture, CD3+ CD56+ CIK cells were purified by CD56+ selection (mean purity > 90%) (Fig. 1A). All the following experiments were performed with the use of such purified CD3+ CD56+ CIK cells to directly test their antitumor function.