RESEARCH ARTICLE

5-Fluorouracil and Interleukin-2 Immunochemotherapy Enhances Immunogenicity of Non-Small Cell Lung Cancer A549 Cells through Upregulation of NKG2D Ligands

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Abstract

Background: The aim of this study was to investigate the anti-cancer effects and mechanisms of immunochemotherapy of 5-fluorouracil (5-FU) and interleukin-2 (IL-2) on non-small cell lung cancer (NSCLC) A549 cells. Materials and Methods: In order to detect whether 5-FU+IL-2 could effectively inhibit tumor growth in vivo, we established an A549-bearing nude mouse model. The cytotoxicity of natural killer (NK) cells was evaluated using a standard chromium release assay. To evaluate the relevance of NK cells in 5-FU+IL-2-mediated tumor inhibitory effects, we depleted NK cells in A549-bearing mice by injecting anti-asialo-GM-1 antibodies. Effects of 5-FU+IL-2 on the expression and promoter activity of NKG2D ligands (MICA/MICB) in A549 cells in vitro were also assessed. Results: In A549-bearing nude mice, combination therapy significantly inhibited tumor growth in comparison with monotherapy with 5-FU or IL-2 and enhanced the recognition and lysis of tumor cells by NK cells. Further study of mechanisms showed that NK cells played a vital role in the anticancer immune response of 5-FU+IL-2 immunochemotherapy. In addition, the combination therapy synergistically stimulated the expression and promoter activity of MICA/MICB. Conclusions: 5-FU and IL-2 immunochemotherapy significantly inhibited tumor growth and activated NK cytotoxicity in vivo, and these effects were partly impaired after depleting NK cells in tumor-bearing mice. Combination treatment of 5-FU and IL-2 upregulated the expression and the promoter activity of MICA/MICB in A549 cells, which enhanced the recognition of A549 cells by NK cells. All of the data indicated that immunochemotherapy of 5-FU and IL-2 may provide a new treatment option for patients with lung cancer.

Keywords: 5-FU - IL-2 - immunochemotherapy - NK cell - MICA/B

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Introduction

Non-small cell lung cancer (NSCLC) is a heterogeneous disease that is difficult to treat, and remains the leading cause of cancer-related mortality worldwide (Holt et al., 2011; Stella et al., 2013). In recent years, despite recent advances in surgery, irradiation, chemotherapy, and targeted therapy, the five-year survival rate of patients with advanced stage NSCLC is very low (Xu et al., 2014). In traditional, chemotherapy has been widely used in the treatment of NSCLC, but their severe side effects and drug resistance limited its use in clinic. Therefore, alternative therapeutic approaches which can effectively treat lung cancer attract the scientists’ attentions.

Immunotherapy, which utilizes the immune system to control and eradicate cancer, that are refractory to conventional therapies (Wang et al., 2014). Recently, immunotherapies used in patients with lung cancer has made breakthrough (Zheng et al., 2013; Xu et al., 2014). Notably, one of attractive immunotherapy candidates used in combination with chemotherapy is Interleukin 2 (IL-2). IL-2, a glycoprotein produced by activated T cells (Du et al., 2012), is one of the most successful cytokines applied in tumor immunotherapy for stimulating potent cellular immune response (Ye et al., 2014). In addition, IL-2 enhances the proliferation and cytolytic activities of T and NK cells (Gaffen et al., 2004). All of these properties underly the potential of IL-2 in immnotherapy.

5-Fluorouracil (5-FU) has been widely used in the treatment of various human cancers, including NSCLCs, colorectal cancers, and gastric cancers (Takiuchi et al., 1998; Macdonald et al., 2001). Nevertheless, drug resistance of 5-FU has become a apparent problem in clinical use (Das et al., 2013), which promotes the combination therapy with immunomodulatory factors. It has been reported that continuous infusional 5-FU and subcutaneous IL-2 in treating metastatic renal cancer exhibited good effects (Savage et al., 1997). However, the relative mechanism of this immunochemotherapy has not yet been investigated.

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Lei Zhao et al
In the present study, the effect and the mechanism of immunochemotherapy of 5-FU and IL-2 were investigated. Our results suggested that 5-FU+IL-2 treatment suppressed A549 growth in vivo by stimulating NK cytotoxicity, which were based on stimulating the promoter activity and upregulation the expression of NKG2D ligands (MICA and MICB) on A549 cells. These data indicated that the anti-lung cancer activity of 5-FU combined with IL-2 is mediated by NK cytotoxicity.

Materials and Methods

Cell culture
Human NSCLC cells (A549) was obtained from ATCC (Manassas, Virginia, USA) and cultured in RPMI-1640 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% CO2.

Experimental animals
Nude mice were purchased from the Academy of Military Medical Science, housed in a rodent facility at 22±1°C with a 12 h light-dark cycle and provided with continuous standard rodent chow and water for acclimatization. All of the procedures, involving animals and their care in this study, were in accordance with protocols approved by the Ethics Committee of Jilin University.

In vivo treatments
A549 cells (0.1 ml, 5×10^7 cells) was transplanted subcutaneously into the right axilla of each nude mouse. When the tumor grew to 100-300 mm3, mice were randomly divided into four groups: a model group administered with normal saline, 5-FU-treated group (35 mg/kg of body weight), IL-2-treated group (500 IU/ml) or 5-FU (10 μM) and IL-2 (50 IU/ml) for 24 h, then the cells were collected, stained with anti-human MICA/MICB PE antibody (eBioscience, San Diego, CA, USA) and analyzed using flow cytometry.

Isolation of NK cells
NK cells were prepared by using DX5 MicroBeads (Miltenyi Biotech, CA, USA), respectively, as previously described (Martinez J, et al., 2010). In brief, single-cell suspensions from the spleens of mice were purified using Mouse CD49b (DX5) MicroBeads. The NK cell population was detected using flow cytometry, and the purity was determined to be greater than 90% (data not shown).

Cytotoxicity Assay
The cytotoxic activity of the NK cells was evaluated using a standard chromium release assay. Target cells (A549 cells) were labeled with 100 μCi Cr radioactive chromium as sodium chromate for 2 hours. After washing, 1×10^5 target cells per well were incubated with effector cells (NK cells isolated from the spleens of mice) at different effector to target cell ratios (E:T ratio) over 4 hours. The supernatant was collected and counts per minute were determined (Packard, Dreieich, Germany). Maximum release was obtained by incubating the target cells with an anionic detergent (0.1% IGEPAL). The negative control (spontaneous release) was represented by target cells without effector cells. Cytotoxicity calculations were performed using the following formula:

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\text{Cytotoxicity (\%)} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})} \times 100\% 
\]

Construction of Recombinant Plasmids (MICA/MICB Promoter and pGL3-Basic Vector)
The primers were designed according to the promoter region of MICA/MICB. The upstream primer and downstream primer of MICA promoter was 5'-ACGGATCCACCGCGTTGTCTGT CCTG-3' and 5'-TGCCAGCCGAAAGCAAGGACCC-3' respectively; The upstream primer and downstream primer of MICB promoter was 5'-ACGGATCCACCGCGTTGTCTGT CC-3' and 5'-AGGCGACGGCCAGAAACGAGC-3' respectively. The primers were synthesized by Meiji Co, Ltd. (Shanghai, China). PCR to amplify MICA/MICB promoter (the complete genome of A549 cell served as the
Activity analysis of promoter

1×10^6 A549 cells per well were inoculated in 24-well plate, one day after inoculation A549 cells were transfected with 0.79 μg reporter gene plasmid and 0.01μg internal control plasmid (pRL-TK) per well by Lipofectamine 2000 (Invitrogen, Vienna, NY, USA). The mole ratio of reporter gene plasmid and internal control plasmid was 50: 1. At the same time, the pGL3-Basic and pGL3-Control were transfected as negative and positive control respectively. The cell culture supernatant were abandoned 24 h after transfection, then 100μL passive lysis buffer was added per well to lyse the cells for 15 min. The cell samples were collected respectively and the relative activity of promoter was measured by Glomax 96 well plate determinator (Promega, WI, USA).

The cell culture medium was changed 24 h after A549 cells were transfected with MICA/ MICB, at the same time, IL-2 (50 IU/ml) alone, 5-FU (10 μM) alone or 5-FU+IL-2 (10 μM+50 IU/ml) was added and incubated for 8 h. Finally, the relative activity of promoter was measured according to the above mentioned method.

Statistical analyses

The data in this article were analyzed using a one-way analysis of variance (ANOVA), followed by Dunnett’s test to identify any 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 of less than 0.05 were considered statistically significant.

Results

5-FU+IL-2 immunochemotherapy effectively suppresses the growth of A549 in nude mice

As the Figure 1 shown, 5-FU+IL-2 exhibited a significant inhibitory effects in A549-bearing mice. During the treatment, the tumor volume of each mouse was measured every three days. On day 21, mice were killed and tumor weight were measured. The obtained results indicated that the tumor volumes were remarkably decreased in the 5-FU+IL-2 treatment group (Figure 1A). Similar results were obtained in the tumor weights (Figure 1B), and the tumor inhibitory rate was increased to 75.2% in combination therapy group, whereas that in the monotherapy with 5-FU or IL-2 were 60.3% and 36.8%, respectively.

5-FU+IL-2 immunochemotherapy activates cytotoxic activity of NK cells

Considering that NK cells played a predominant role in innate immune system to eliminate cancer cells, and the nude mice were deficient of T cells. Therefore, we suspect that activating NK cells was the main contributor in inhibiting A549 growth in vivo. Then we detected both the the percentage of NK cells (defined as DX5+ cells) in spleens and the specific lysis activity of NK cells against A549 cells. As the Figure 2A indicated, the number of NK cells in spleens was 5.28% in the 5-FU+IL-2 treatment group, whereas that in the 5-FU or IL-2 treated alone group were 3.67% and 4.89%, respectively. These data illustrated that the number of NK cells were upregulated.
more in combination therapy than that in the monotherapy group. Simultaneously, the NK cytotoxicity against A549 cells was also increased in the 5-FU+IL-2-treated group (Figure 2B). Taken together, 5-FU+IL-2 prevented tumor growth in vivo through stimulating NK cells.

Depletion of NK cells impairs the antitumor effects of 5-FU+IL-2 immunochemotherapy in A549-bearing nude mice

Both the significant antitumor effects and upregulation of NK cytotoxicity in combination therapy in vivo strongly suggested that NK cells were crucial for 5-FU+IL-2-induced lung cancer suppression. To confirm this hypothesis, NK cells were depleted in A549-bearing nude mice via intraperitoneal injection of an anti-asialo-GM1 antibody. As shown in Figure 3, treatment with this antibody almost completely abolished the 5-FU+IL-2-induced suppression of A549 tumor growth. Therefore, these results indicated that NK cytotoxicity is vital to the anti-lung cancer activity of 5-FU and IL-2.

5-FU+IL-2 immunochemotherapy increases the expression of MICA/MICB in A549 cells

The expression of MHC class I-related chain molecule (including MICA and MICB) expressed on tumor cells is essential for recognition by NK cells. Therefore, we evaluated the effects of different treatments on the expression of MICA/MICB in A549 cells in vitro. When the cells treated with 5-FU, IL-2, or with 5-FU+IL-2 for 24 hours, the MICA expression was detected using flow cytometry. As the Figure s 4A and C indicated, combination of 5-FU and IL-2 treatment significantly upregulated expression of MICA/MICB. The results of mean fluorescence intensity (MFI) showed that the MFI in 5-FU+IL-2 combination therapy was 36.88, whereas that in the 5-FU or IL-2 treatment group was 12.9 and 17.12, respectively. Likewise, MFI showed in Figure 4D indicated the combination therapy significantly increased the expression of MICB in A549 cells.

5-FU+IL-2 immunochemotherapy activates the promoter activity of MICA/MICB

Since the expressions of both MICA and MICB were upregulated in the 5-FU+IL-2 treatment group, then we detect the promoter activity of MICA and MICB in 5-FU+IL-2 group. As shown in Figure 5A, the MICA promoter activity was increased to 1.45-, 1.79 - and 2.72-, fold at the 5-FU, IL-2 and 5-FU+IL-2 group, respectively, compared with the control group. Similar results obtained in the MICB promoter, which was remarkably increased
in the 5-FU and IL-2 combination treated group (Figure 5B). Hence, combination therapy of 5-FU and IL-2 would stimulate the promoter activity of MICA and MICB firstly, then enhancing the protein expression of these two proteins.

Discussion

Lung cancer is the most common malignant cancer and has been associated with high fatality rates in humans. Chemotherapy in the clinical management of lung cancer cause significant side effects. Interleukins which could stimulate the immune response has been applied to treat cancer, such as IL-7, IL-12 (Ahamed et al., 2014; Yuan et al., 2014). Therefore, combination with immunotherapy was developed to decrease the toxicity of chemotherapy in treating lung cancer and the relative mechanism would lay a foundation in clinic use.

5-FU is widely used in chemotherapeutic regimens. Although 5-FU-based chemotherapy improves the overall survival of patients, the response rate is extremely low. In order to overcome this challenge, 5-FU combined with immunotherapy attracted the scientists’ attention. IL-2, secreted by activated T cells, could promote the proliferation and cytolytic activity of CTLs and NK cells to eliminate cancer cells (Fearon et al., 1990; Makedonas et al., 2010). Moreover, it has been reported that immunotherapy with IL-2 in combination with 5-FU is superior to 5-FU chemotherapy in enhancing survival rate (Savage et al., 1997). In this study, we found that in the 5-FU+IL-2-treated group, the tumor growth of A549 was significantly inhibited, which is better than 5-FU or IL-2 alone treated group. Considering that IL-2 played a vital role in activating immune system, the relative immunoregulatory mechanisms were studied further and the result would provide a basis for the combination therapy of 5-Fu and IL-2 in clinic.

NK cells and CTLs played crucial roles in cell-mediated immunity in innate and adaptive immunity and eliminated target tumor cells. Notably, tumor cells often exhibit low MHC expression, thereby escaping from CTL-mediated immunologic surveillance (Sandel et al., 2005). Bone marrow-derived NK cells are an important effector population required to eliminate malignant tumor cells in the absence of MHC expression or the presence of mutant MHC molecules (Siddle et al., 2013; Kärre, 2002). Therefore, NK cells may play more important roles than CTLs in the treatment of lung cancer. It has been reported that IL-7 and IL-12 exhibited anti-tumor activity via stimulating CTLs Interlens which could stimulate the immune response has been applied to treat cancer, such as IL-7, IL-12 (Ahamed et al., 2014; Yuan et al., 2014). Moreover, we think that drugs or interleukins which could activate NK cells would be more potential in treating cancer. Our results further indicated that both the percentage and the specific lysis activity of NK cells were enhanced in the 5-FU+IL-2-treated group, indicating that the activation of NK cells was the main contributor in 5-FU+IL-2-induced tumor suppression. Moreover, NK cell depletion via pretreatment with an anti-asialo-GM-1 antibody in A549-bearing nude mice nearly completely abolished in the 5-FU+IL-2-induced suppression of lung cancer cells. Based on these results, we concluded that enhanced NK cytotoxicity is crucial and that NK cells may be a unique target in A549-bearing mice.

In addition, there are a lot of receptors expressed on NK cells, including activation and inhibitory receptors. Among NK activating receptors, natural killer group 2, member D receptor (NGK2D) is a C-type lectin-like transmembrane glycoprotein recognizing self-molecules (referred as NKG2D ligands; NKG2DLs) that emerged as a pivotal signaling pathway supporting cancer immune surveillance (Diefenbach et al., 1999; Moretta et al., 2001). MHC class I chain-related A and B (MICA and MICB) or UL16 binding proteins are NKG2D Ligands (Ljunggren et al., 2008; Kim et al., 2008; Nauch et al., 2008). To further confirm the role of 5-FU+IL-2 immunochemotherapy played in MICA/MICB, the promoter activity was investigated. We cloned MICA/ MICB promoter and detected the promoter activity after incubation with 5-FU+IL-2. Results showed that 5-FU+IL-2 immunochemotherapy could significantly enhance the activity of MICA/MICB promoter. As described in the theory of Gasser (Gasser et al., 2005; Gasser et al., 2006): 5-FU+IL-2 immunochemotherapy induced DNA damage, which activated stress response molecules (ATM and ATR) and a variety of effector molecules downstream. Then some transcription factors were excited, which up-regulated the transcriptional level of MICA/MICB. The fact that 5-FU+IL-2 increased activity of MICA/MICB promoter consistent with the increased expression of MICA/MICB in A549 cells.

In conclusion, our study provides more insight into the mechanism of action of IL-2 in combination with 5-FU. This immunochemotherapy enhances the recognition of A549 cells by NK cells and up-regulates the activity of MICA/MICB at the level of transcription and protein expression. All of the results presage the potential use of immunochemotherapy of 5-FU and IL-2 in treating lung cancer in clinic.

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References


