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Erlotinib enhances the CIK cell-killing sensitivity of lung adenocarcinoma A549 cells

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ABSTRACT. We examined the effects and molecular mechanism of the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor erlotinib on NKG2D ligand expression in human lung adenocarcinoma A549 cells and the cytotoxicity of cytokine-induced killer cells. Flow cytometry was used to detect NKG2D ligand expression in A549 cells under effects of erlotinib and EGFR downstream molecules, including LY294002 (phosphoinositide 3-kinase inhibitor), SB203580 (mitogen-activated protein kinase inhibitor), and STAT21 (signal transduction and transcription 3 inhibitor) after 24 h. A lactate dehydrogenase release assay was used to detect, at different effector-to-target ratios, the A549 cell killing activity of cytokine-induced killer cells before and after treatment with 10 μM erlotinib. Erlotinib suppressed MICA expression in A549 cells and upregulated MICB and UL16 binding protein 1 expression. EGFR downstream molecules mitogen-activated protein kinase and signal transduction and transcription 3 inhibitor did not affect the expression of NKG2D ligands in A549 cells. The phosphoinositide 3-kinase inhibitor reduced MICA expression in A549 cells, while erlotinib enhanced the killing sensitivity of cytokine-induced killer cells in A549 cells. The anti-lung carcinoma effects of

EGFR tyrosine kinase inhibitor were associated with the sensitivity of lung cancer cells to enhanced immune cell killing.

Key words: Cytokine-induced killer cells; NKG2D ligands; EGFR tyrosine kinase inhibitor; Lung cancer

INTRODUCTION

Chemotherapy and targeted drugs are the principal means of treating advanced non-small cell lung cancer. For patients with epidermal growth factor receptor (EGFR) mutations, EGFR tyrosine kinase inhibitors (EGFR TKI) can prolong the survival time of patients. Unfortunately, in the Asian population, EGFR mutations account for only 20-30% of advanced non-small cell lung cancerin patients (Han et al., 2005). Determining an effective population to further develop the efficacy of EGFR TKI requires further study. The surface expression of the NKG2D ligands on tumor cells results in an attack targeted against the body's immune cells (Bae et al., 2012a). Previous studies (Mei et al., 2009; Inagaki et al., 2009; Morisaki et al., 2011; Huang et al., 2011) have shown that chemotherapy and targeted drug therapy can induce the surface expression of NKG2D ligands on tumor cells, which can enhance the killing activity of immune cells and improve anti-tumor effects. However, the myelosuppression of conventional chemotherapeutic drugs has limited the combination of chemotherapy and immune cell therapy. In a previous study, investigating the effects of the EGFR TKI erlotinib on NKG2D ligand expression on the surface of A549 cells and cytotoxicity of cytokine-induced killer (CIK) cells, human lung adenocarcinoma A549 cells were found to express wild-type EGFR (Rho et al., 2011). These results provide a theoretical and experimental basis for the interaction between erlotinib and immune cells in the treatment of lung cancer.

MATERIAL AND METHODS

Materials

The following materials were used in this study: recombinant human interleukin-2 (Liaoning Weixing Biological Products Institute, Liaoning, China); interferon-γ (Shanghai Clone Bio-Tech Co., Ltd., Shanghai, China); lymphocyte separation medium (Tianjin Haoyang Biological Products Co., Ltd., Tianjin, China); RPMI 1640 (Gibco, Grand Island, NY, USA); tetrazolium blue (Sigma, St. Louis, MO, USA); CD3 mAb (ProSpec-Tany TechnoGene, Rehovot, Israel); FITC-CD4/PE-CD8/PerCP-CD3, FITC-CD56, PE-CD3, PerCP-CD3, NKG2D-PE, FITC-IgG1, PE-IgG1, MICA, MICB, ULBP1, ULBP2, ULBP3 monoclonal antibodies, and flow cytometry apparatus (BD Biosciences, Franklin Lakes, NJ, USA); lactate dehydrogenase release assay kit (Promega Corporation, Madison, WI, USA); erlotinib (Roche, Basel, Switzerland), LY294002, SB203580 (Sigma), and signal transduction and transcription 21 (STAT21) (Biomol, Farmingdale, NY, USA). The drugs were dissolved in dimethyl sulfoxide and stored at -20°C. Drugs were thawed before use. The RPMI 1640 culture medium containing 10% fetal bovine serum was diluted to the desired concentration. The final concentration of dimethyl sulfoxide was <0.1%. Human lung adenocarcinoma A549 cells were routinely passaged and stored in our center.

Methods

CIK cell preparation

CIK cells were prepared according to the methods described in a previous study (Linn et al., 2012) and cultured for 14 days. Flow cytometry was used to detect the CIK cell phenotype.

A549 cell culture

A549 cells were seeded into 100-mL flasks. The cell culture medium was RPMI 1640 containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. The culture was placed in a 5% CO_2 incubator at 37°C for incubation. Cells were cultured to cover 70-80% of the flasks. Trypsin was used for digestion, counting, and passage.

Flow cytometry analysis of NKG2D ligand expression in A549 cells before and after adding erlotinib

A549 cells in logarithmic growth phase were seeded in 100-mL flasks at a concentration of 5 x 10^4/mL. After 24 h of culture, erlotinib was added. When the final concentrations were equivalent to 5 and 10 μM, the empty drug group was used as the control group. After a 24-h culture, the A549 cells before and after the use of erlotinib were collected and washed with phosphate-buffered saline. The cells were counted and placed into different tubes. At a cell concentration of 1 μg/10^6, MICA, MICB, ULBP1, ULBP2, and ULBP3 monoclonal antibodies (mAbs) were added at 4°C for 30 min. After washing with phosphate-buffered saline, the fluorescein isothiocyanate-labeled goat anti-mouse IgG1 secondary antibody was added at 4°C for 30 min. After phosphate-buffered saline washing, the sample was analyzed. The same IgG1 antibody was used as a negative control. Flow cytometry was used to analyze positive cells among 1 x 10^4 cells, and the percentages were calculated. These experiments were repeated in triplicate

Effects of downstream molecules of epidermal growth factor signaling pathways on NKG2D ligand expression in A549 cells using flow cytometry

A549 cells in logarithmic growth phase were seeded in 100-mL flasks at a concentration of 5 x 10^4/mL. After 24 h of culture, 30 μM STAT21, 15 μM LY294002, and 25 μM SB203580 were added. The empty drug group was used as a control. After a 24-h culture, the A549 cells were collected. Flow cytometry was used to detect NKG2D ligand expression on the surface of A549 cells.

CIK cell-killing activity

A 4-h lactate dehydrogenase release assay was conducted (Mei et al., 2007) according to Cytotox 96 Non-Radioactive Cytotoxicity Assay instructions. CIK cell-killing activity before and after the use of erlotinib at a concentration of 10 μM in A549 cells was observed in different effector to target ratios. When the effector target ratio was 20:1, NKG2D mAb was incubated with CIK cells for 15 min. A549 cells, before and after the use of erlotinib, at a con-

centration of 10 μM, were added to measure the killing rate. The experiment was repeated 3 times.

Statistical analysis

The SPSS10.0 statistical software was used for analysis (SPSS, Inc., Chicago, IL, USA). Data are reported as means ± standard deviation. The NKG2D ligand expression rate on the surface of A549 cells and the CIK cell-killing rate before and after drug treatment were compared using the paired sample *t*-test. In different effector target ratios, the cytotoxicity of CIK cells between groups was compared using one-way analysis of variance, with $P < 0.05$ indicating statistical significance.

RESULTS

CIK cell phenotype

Figure 1 shows the flow cytometry results: CD3+ >90%, CD3+CD8+ >80%, CD3+CD56+ >30%, CD3+CD4+ <25%, NKG2D >80%.

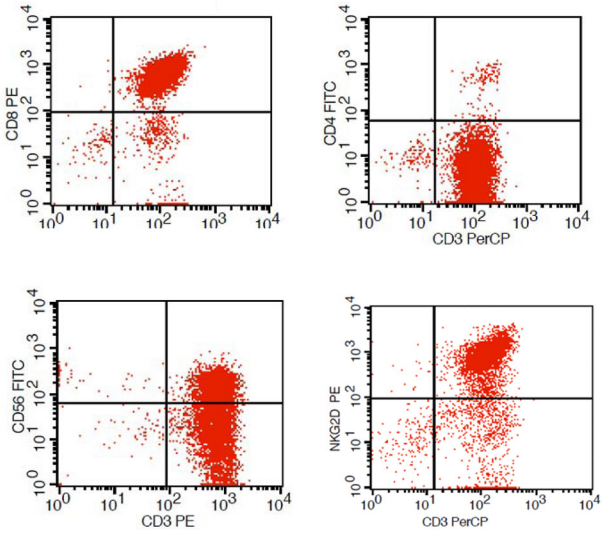

Figure 1. Phenotype of CIK cells.

Erlotinib regulated expression of NKG2D ligands in A549 cells

Figure 2 shows NKG2D ligand expression in A549 cells prior to treatment with erlotinib, which were as follows (blue curve represents the isotype control mAb; red curve represents the original expression): MICA 66.62 ± 1.42%, MICB 32.75 ± 2.37%, ULBP1 9.80 ± 2.40%, ULBP2 4.09 ± 1.94%, and ULBP3 34.96 ± 4.22%. Erlotinib at 5 μM (green curve) and 10 μM (black curve) was incubated with A549 cells for 24 h. Expression levels of MICB and ULBP1 on the surface of A549 cells were significantly enhanced compared with those before the use of erlotinib ($P < 0.05$). MICA expression decreased compared with that before the use

of erlotinib ($P < 0.05$). ULBP2 and ULBP3 showed no significant changes. Between 5 and 10 μM erlotinib, the NKG2D ligand expression showed no significant difference ($P > 0.05$).

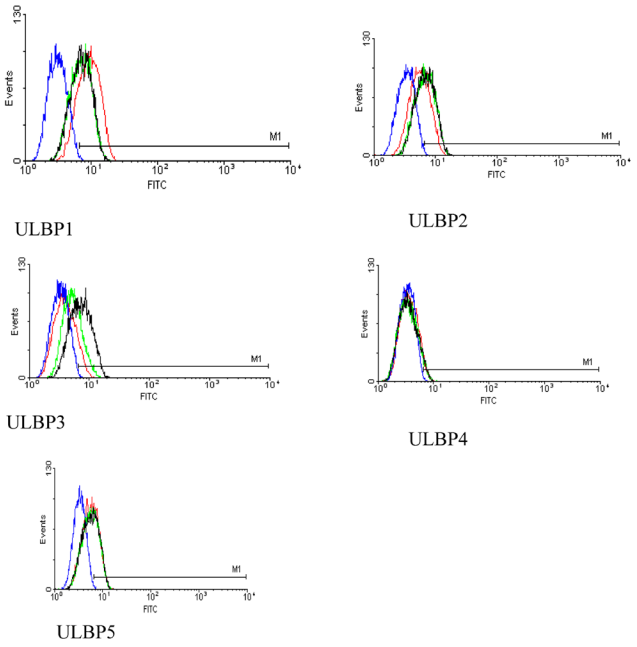

Figure 2. Analysis of NKG2D ligands expression in A549 cells following treatment with erlotinib.

Effects of EGFR downstream molecules on NKG2D ligand expression in A549 cells

Flow cytometry results showed that when STAT21 (STAT3 inhibitor) and SB203580 [mitogen-activated protein kinase (MAPK) inhibitor] were cultured with A549 cells after 24 h, NKG2D ligand expression on the A549 cell surface showed no significant change ($P > 0.05$; Table 1). LY294002 [phosphoinositide 3-kinase (PI3K) inhibitor] decreased surface MICA expression in A549 cells ($P < 0.05$), but did not affect the expression of other ligands.

Table 1. Expression of NKG2D ligands in A459 cells by treatment with inhibitors of EGFR downstream molecules cells (%, means ± SD, N = 3).

Group	MICA	MICB	ULBPI	ULBP2	ULBP3
Control	66.62 ± 1.41	32.75 ± 2.37	9.80 ± 2.40	4.09 ± 1.94	34.96 ± 4.22
STAT21	67.37 ± 0.54	31.59 ± 0.41	10.58 ± 0.86	4.19 ± 0.19	32.86 ± 0.35
LY294002	42.90 ± 3.49*	32.40 ± 0.30	9.58 ± 1.22	4.40 ± 0.07	34.65 ± 0.35
SB203580	66.08 ± 0.28	31.79 ± 0.19	9.15 ± 0.39	4.36 ± 0.10	34.97 ± 0.40

*$P < 0.05$ *vs* control.

Erlotinib enhanced the lethal sensitivity of CIK cells against A549 cells

When the effector to target ratios were 10:1, 20:1, and 30:1, the cytotoxicity values of CIK cells against A549 cells were 11.08 ± 1.22, 36.22 ± 0.91 and 45.73 ± 2.00%, respectively.

The cytotoxicity values of CIK cells against A549 cells after addition of 10 μM erlotinib were 18.24 ± 0.96, 48.49 ± 0.78 and 56.67 ± 2.11%, respectively. Using the same effector to target ratio, the A549 cell-killing activity of CIK cells was significantly enhanced after erlotinib addition ($P < 0.05$). When the effector to target ratio was 20:1, NKG2D mAb closed CIK cell surface NKG2D receptor, the cytotoxicities of CIK cells on A549 cells before and after erlotinib use were 7.29 ± 0.73 and 7.99 ± 0.11%, respectively. The difference was not statistically significant ($P > 0.05$).

DISCUSSION

The NKG2D-NKG2D ligand pathway plays an important role in anti-tumor immunity, with NKG2D as the main activating receptor of NK cells that induces anti-tumor effects (Bae et al., 2012a; Morisaki et al., 2012). There are 2 categories of NKG2D ligands (Bae et al., 2012a), including major histocompatibility complex class I chain-related molecule A or B (MICA, MICB) and human cytomegalovirus glycoprotein UL16 binding proteins (ULBP1, ULBP2, ULBP3). NKG2D ligands are widely expressed on the surface of tumor cells. The NKG2D receptor on the surface of immune cells and NKG2D ligands on the surface of tumor cells combine to stimulate the anti-tumor immune response.

CIK cells are heterogeneous cells cultivated by the co-culture of human peripheral blood mononuclear cells and a variety of cytokines *in vitro*. Compared with natural killer cells, the low expression of CIK cells inhibits immunoglobulin receptors. CIK cells express the NKG2D receptor (Mei et al., 2011) and play a role in anti-tumor effects (Franceschetti et al., 2009). CIK cells can be easily amplified *in vitro* for clinical applications (Laport et al., 2011; Mesiano et al., 2012). CIK cells are closely related to the cytotoxicity of tumor cells and tumor cell surface expression of NKG2D ligands (Mesiano et al., 2012). Studies have shown that chemotherapy drugs and molecular-targeted therapy can induce NKG2D ligand expression on the tumor cell surface and enhance the cytotoxic activity of immune cells through a variety of signaling pathways (Tang et al., 2008; Xu et al., 2011; Bae et al., 2012b). EGFR is overexpressed on the surface of various tumor cells and regulates the molecular activity of downstream signaling pathways, including the Ras-Raf-MAPK and PENT-PI3K-AKT pathways. In addition, JAK/STAT3 participates in the EGFR signaling pathway. These pathways are associated with tumor cell differentiation, proliferation, apoptosis, invasion, and angiogenesis. EGFR-targeted drugs have become a hot spot in the treatment of lung cancer in recent years. Lung adenocarcinoma A549 cells express wild-type EGFR. In this study, we used A549 cells.

NKG2D ligand expression is regulated by numerous factors. MICA expression is actively regulated by the STAT3, PI3K, and MAPK molecules (Molinero et al., 2003; Boissel et al., 2006; Bedel et al., 2011). In this study, we used the STAT3 inhibitor STAT21 and the MAPK inhibitor SB203580, which did not affect NKG2D ligand expression. However, the PI3K inhibitor LY294002 downregulated MICA expression on the surface of A549 cells. This indicated that erlotinib-induced MICA downregulation of A549 cells was associated with EGFR/PI3K signal suppression. MICB and ULBP1 upregulation was not associated with these molecules, indicating that EGFR signaling pathway regulation of MICB and ULBP1 expression requires further analysis.

Although MICA downregulation of A549 cells treated with erlotinib was observed, the killing activity of CIK cells increased significantly. When the NKG2D receptor on the CIK cell surface was blocked, the A549 cell killing activity of CIK cells showed no difference

before and after treatment with erlotinib. The results indicate that the killing of CIK cells on A549 cells occurs through a combination of NKG2D receptor and NKG2D ligands. Erlotinib mainly increased activated ligand MICB and ULBP1 expression on the surface of A549 cells as well as enhanced sensitivity of A549 cells for CIK cell killing.

Based on our results, EGFR TKIs *in vivo* may stimulate the anti-tumor effects of immune cells. EGFR TKIs combined with CIK cells may be useful for adoptive immunotherapy of lung cancer.

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