Preliminary report

Antitumor activity of cytokine-induced killer cells against human lung cancer

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Abstract

Lung cancer is the leading cause of cancer-related death among men and women in the world. Despite the aggressive treatment with surgery, radiation and chemotherapy, the long term survival for lung cancer patients remains low. In this study, the anti-tumor activity of cytokine-induced killer (CIK) cells against human lung cancer was evaluated in vitro and in vivo. Although CD3+CD56+ CIK cells were rare in fresh human peripheral blood mononuclear cells, they could expand more than 1000-fold on day 14 in the presence of anti-CD3 antibody plus IL-2. At an effector-target cell ratio of 30:1, CIK cells destroyed 98% of NCI-H460 human lung cancer cells, which was determined by the 51Cr-release assay. In addition, CIK cells at doses of 3 and 30 million cells per mouse inhibited 57% and 77% of NCI-H460 tumor growth in nude mouse xenograft assay, respectively. This study suggests that CD3+CD56+ CIK cells may be used as an adoptive immunotherapy for patients with lung cancer.

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1. Introduction

The development of adoptive cell immunotherapy for the treatment of cancer has received considerable attention. The goal of immune cell-based cancer therapy is to eliminate cancer cells through the transfer of ex vivo expanded and activated immune cells. Immune cells such as dendritic cells [1], lymphokine-activated killer cells [2], natural killer cells [3], cytotoxic T cells [4], and cytokine-induced killer (CIK) cells [5] have been explored for the active immunotherapy of cancer. Among them, CIK cells were known to have several attractive advantages; 1) It is very easy to generate large number of CIK cells ex vivo and they are readily expandable from peripheral blood mononuclear cells of cancer patients [6]; 2) CIK cells have strong anti-tumor effects [7]; 3) Cytotoxicity is MHC-unrestricted; 4) CIK cells are the final effector cells,
which are able to directly kill cancer cells; 5) More importantly, the toxicity is minimal and no graft-versus-host reaction is observed [8]. Allogenic spleen cells with bone marrow cells could induce graft-versus-host disease (GVHD) in recipient mice by showing body weight loss and death within 9 days after transplantation [9]. However, CIK cells did not induce GVHD, even when injected with 10 times the amount of CIK cells compared to spleen cells [8]. This reduction in GVHD was known to be related with IFN-γ production of CIK cells. IFN-γ+ CIK cells produced no GVHD, but IFN-γ−/− CIK cells caused acute lethal GVHD [8,10].

CIK cells showed promising antitumor effects on various cancers, such as hepatoma [11], leukemia [12], and liver [11], renal [4], and gastric cancer [13], in preclinical and clinical studies. In this study, we examined the antitumor activity of CIK cells against human non-small cell lung cancer. We generated CIK cells from human peripheral blood mononuclear cells, characterized their phenotypes, and evaluated the anti-tumor activity of CIK cells in vitro and in vivo nude mouse xenograft model.

2. Materials and methods

2.1. Cells

Human lung cancer cells, such as NCI-H460, A549, NCI-H556, NCI-H441, and NCI-H23, were obtained from the American Type Culture Collection (Manassas, VA, USA). Cell lines were grown in the RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin.

Cytokine-induced killer (CIK) cells were generated from peripheral mononuclear cells (PBMC) of healthy volunteers. After informed consent, 10–20 ml of blood was drawn from volunteers in evacuated tubes with heparin (Vacutainer, Becton Dickinson). The PBMC were obtained from buffy coats of PBMC by Ficoll-Hypaque density centrifugation and washed three times with PBS [11]. All blood draws and PBMC isolation experiments were finished within 1.5 h. Next, the PBMC were re-suspended at 1×10⁶ cells/ml in Lymphomedia (Lymphotech, Japan) containing 10% fetal bovine serum (Invitrogen, CA, USA) and cultured with immobilized anti-CD3 antibody (5 μg/ml, BD Pharmingen, NJ, USA) and recombinant human IL-2 (700 U/ml, R&D systems, MN, USA)
for 5 days. Following this, the cell suspension was further incubated in a new Lymphomedia containing recombinant human IL-2 only (170 U/ml) for 9 days. Furthermore, fresh IL-2 and medium were replenished every third day. The viability of CIK cells was usually 85–95%.

2.2. Phenotype analysis

Cells were obtained from CIK cultures for phenotype analysis with the appropriated monoclonal antibodies, including CD3-FITC, CD4-FITC, CD8-PE, CD56-APC, and CD56-PE. One million CIK cells were washed once in PBS containing 1% bovine serum albumin (BSA) and re-suspended in 100 μl of PBS/BSA buffer. The cells were incubated with various conjugated monoclonal antibodies for 20 min at 4 ºC, washed twice in PBS, and re-suspended in 400 μl of PBS. A flow cytometric analysis was performed on a FACSCalibur flow cytometry (BD Biosciences), and the data were analyzed using the WinMDI statistical software (Scripps, La Jolla, CA, USA). Forward and side scatter parameters were used to gate on live cells [14].

2.3. 51Cr release assay

The human cancer cell lysis by CIK cells was measured in a 4 h 51Cr release assay [15]. Briefly, two million target cells were labeled with 100 μCi of sodium chromate (Dupont-NEN, Boston, MA, USA) for 4 h at 37 ºC. The labeled cells were washed three times in PBS and re-suspended in 10 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum. The labeled cells were plated in round bottom 96-well plates at 1 × 10⁴ cells/100 μl per well in triplicate. CIK cells were added at specified E:T ratios (1:1, 3:1, 10:1, and 30:1) and incubated for 4 h. The supernatant was removed and the radioactivity measured in a gamma counter. The percentage of specific lysis was calculated according to the following equation:

\[
\text{%cytotoxicity} = \frac{[(\text{sample} - \text{spontaneous})/(\text{maximum} - \text{spontaneous})] \times 100}{100}
\]

Spontaneous release was obtained by incubating target cells in medium alone, whereas maximal release was obtained after treatment with 2% Nonidet P-40 (Sigma, St Louis, MO, USA).

2.4. Nude mouse xenograft assay

Nude mice (6–8 weeks old) were obtained from the Charles River (Japan) and were housed under specific pathogen-free conditions according to the guidelines of the Animal Care Committee at the KRBIB. To eradicate NK cells, the study mice received 200 cGy of whole body irradiation on day-1. On day 0, six million NCI-H460 cells were injected subcutaneously into irradiated nude mice. Following this, CIK cells were injected intravenously twice in a week at dose ranges from 0.3 to 30 million cells per mouse. The tumor volumes were estimated by the formula length (mm) × width (mm) × height (mm)/2. On day 18, the mice were sacrificed and the tumor weights were measured. To determine the animal toxicity, the body weights of the animals were measured [16].

2.5. Statistics

The in vivo results represent samples from six mice per experiment, and the in vitro results represent the mean values of four samples. Standard deviations (SD’s) and p-values were calculated using the Student’s t-test and ANOVA (Prisim, GraphPad Software, USA) [17].

The cytotoxicity of CIK cells. Cytotoxicity of CIK cells cultured for 14 days were assessed against NCI-H460, A549, NCI-H596, NCI-H441, and NCI-H23 target cells. The target cells were labeled with 51Cr and incubated for 4 h with CIK cells at effector-to-target ratios of 1–30:1.

Fig. 2. The cytotoxicity of CIK cells cultured for 14 days were assessed against NCI-H460, A549, NCI-H596, NCI-H441, and NCI-H23 target cells. The target cells were labeled with 51Cr and incubated for 4 h with CIK cells at effector-to-target ratios of 1–30:1.

Fig. 3. Inhibition of the NCI-H460 tumor growth by CIK cells in nude mouse xenograft models. Irradiated nude mice (n=6) were implanted subcutaneously with six million NCI-H460 lung cancer cells. CIK cells at doses from 0.3 to 30 × 10⁶ cells/mouse were injected intravenously twice in a week. Adriamycin (ADR) was injected intravenously at 2 mg/kg. Tumor volumes were estimated by the formula length (mm) × width (mm) × height (mm)/2. Statistical significance was determined using the Student’s t-test versus PBS-treated control group (*p<0.01).
3. Results

CD3⁺CD56⁺ CIK cells are rare in fresh human PBMC, but they will markedly expand and be generated from T cell precursors. After two weeks of culturing ten million PBMCs, the absolute number of total cells increased up to 2500+/−150 million CIK cells. During the generation period, cell number was maintained at approximately one million cells per milliliter. The viability of expanded cell populations on day 14 was 85–95%. First, we examined the phenotypes of cultured cell population with fluorescence-activated cell sorting analyses. Cell population showed 92% CD3⁺CD56⁻, 7% CD3⁻CD56⁺, and 38% CD3⁺CD56⁺ (Fig. 1A). Fresh PBMC is known to include less than 5% of CD3⁺CD56⁺ cells. In addition, most cells were CD8⁺, but not CD4⁺ (Fig. 1B–D).

Next, we examined the cytotoxicity of CIK cells in an in vitro condition by 4 h-Cr⁵¹ release assay. CD3⁺CD56⁺ cells have been demonstrated to have strong cytotoxicity in a non-MHC-restricted manner. At an effector-target cell ratio of 30:1, CIK cells destroyed 98%, 61%, 36%, 22%, and 61% of NCI-H460, NCI-H441, A549, NCI-H596, and NCI-H23 human lung cancer cell lines, respectively (Fig. 2). We also examined the cytotoxicity of fresh PBMC to these target cells, and observed that fresh PBMC destroyed less than 4% of all human lung cancer cell lines.

Finally, we evaluate the antitumor activity of CIK cells in nude mouse xenograft assay. Nude mice were irradiated to...
eradicate intrinsic NK cells. Preliminary experiments revealed that one hundred million CIK cells did not show any observable toxicity in irradiated-nude mice. Mice did not show hair ruffling, lowered morbidity, and weight loss. Thus, we injected CIK cells intravenously at dose levels under one hundred million cells. Six million NCI-H460 cells were injected subcutaneously and they grew to a tumor volume of 316 mm³ 18 days after implantation (Fig. 3). CIK cells were injected intravenously at doses of 0.3, 3 and 30 million cells per mouse, and they inhibited in vivo tumor growth by 32%, 67%, and 77%, respectively. Adriamycin (ADR), used as a reference drug, strongly inhibited the growth of NCI-H460 tumors. On day 18, all tumors were isolated from nude mice and weighed, which demonstrated the strong anti-tumor effect of CIK cells against NCI-H460 (Fig. 4A and B). The body weights of tumor-bearing nude mice were examined to assess the toxicity. Control and CIK cell-injected nude mice showed a body weight gain of 121–125%; whereas, adriamycin-treated mice showed a body weight gain of 110%, which shows the typical characteristics of a cytotoxic anti-cancer drug (Fig. 5).

4. Discussion

Lung cancer is the leading cause of cancer-related death among men and women in the world and in Korea [18]. Despite the aggressive treatment with surgery, radiation and chemotherapy, the long term survival for lung cancer patients remains low [19]. The mean survival for patients with advanced stage treated with platinum-based chemotherapy is a disappointing 8–10 months [20,21]. As a result, new therapeutic approaches to improve patient mortality and morbidity are urgently needed.

Lung tumors appear to have fewer tumor-infiltrating lymphocytes, suggesting that immune response of cancer patients does not properly work to eradicate cancer cells [22]. The development of immunotherapy for the treatment of cancer has received considerable attention. The goal of immunotherapy is to eliminate cancer cells through the activation of host immunity by using biological response modifying polysaccharides, cytokines, monoclonal antibody, cytokines, and ex vivo activated immune cells. Adjuvant immunotherapy with IFN-α, IL-2, thymosin, and Bacille Calmette–Guerin (BCG) showed promising effect on lung cancer [23,24]. Anti-HER2 antibody therapy had been recently applied to human antitumor therapy of lung cancer, although in vivo activity was disappointing [25]. Anti-ganglioside fucosyl GM-1 antibody appeared to decrease formation of metastatic lung cancer via antibody-dependent cell-mediated cytotoxicity [26]. Cell immunotherapy had been also attempted for the treatment of lung cancer. Dendritic cells pulsed with lung cancer cell lysates could induce tumor cell-specific immune response and showed promising clinical outcomes in patients with lung cancer [27]. Dendritic cells pulsed with alpha-galactosylceramide induced NKT cell expansion and exerted a strong antitumor activity in patient with lung cancer [28]. Except from dendritic cells, there were a few, or no, reports on cell immunotherapy with lymphokine-activated killer cells, natural killer cells, cytokotic T cells, and CIK cells against lung cancer.

Here, we provide good preclinical evidence that CIK cell immunotherapy can be used in patient with lung cancer. CIK cells from human PBMC could expand more than 1000 times in the presence of anti-CD3 antibodies and IL-2 for 14 days. CIK cells could break down various lung cancer cells in a non-MHC-restricted manner, and the adoptive transfer of CIK cells was highly effective in preventing NCI-H460 lung tumor growth in the nude mouse xenograft model. This study suggested that CIK cells could be good candidates for cell immunotherapy in patients with lung cancer.

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References


