Comparative study on anti-tumor immune response of autologous cytokine-induced killer (CIK) cells, dendritic cells-CIK (DC-CIK), and semi-allogeneic DC-CIK

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[Abstract] Background and Objective: Cytokine-induced killer (CIK) cells and autologous dendritic cells-CIK (DC-CIK) cells co-cultured with autologous dendritic cells (DCs) and CIK cells are commonly used for immunotherapy recently. We compared the anti-tumor immune response of CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells to explore a more effective anti-tumor adoptive immunotherapy approach. Methods: Peripheral monocytes were isolated from patients with renal carcinoma, lung cancer, or maxillary squamous cell carcinoma and their healthy adult children. Isolated cells were cultured and induced as DCs and CIK cells in vitro. CIK cells from patients were co-cultured with autologous DCs and DCs from their children respectively, generating DC-CIK cells and semi-allogeneic DC-CIK cells. The anti-tumor activities of autologous CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells were measured by LDH assay. Intracellular staining was used to test the secretion of cytokines. Flow cytometry was applied for detecting the phenotype changes of these three types of cells. Cell proliferation and cell apoptosis were detected by 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) and Annexin V/PI respectively. Results: Compared with autologous CIK cells and DC-CIK cells, semi-allogeneic DC-CIK cells significantly enhanced the anti-tumor activity and IFN-γ secretion, reduced IL-4 secretion, increased the ratio of CD3+CD56+c cells and CD3+CD8+c cells, decreased the number of CD4+CD25+c cells, promoted cell proliferation, and lessened cell apoptosis. Conclusions: Semi-allogeneic DC-CIK cells had a stronger anti-tumor effect than did autologous CIK cells and DC-CIK cells. Our results provided experimental evidence for clinical application of DC-CIK cells.

Keywords: Dendritic cells, cytokine-induced killer cells, semi-allogeneic DC-CIK cells, Immunotherapy

Cytokine-induced killer (CIK) cells, stimulated by multiple cytokines, are a group of heterogeneous cells with anti-tumor activity [1]. CIK cells express two types of membrane proteins, CD3 and CD5, and display both powerful anti-tumor ability of T lymphocytes and major histocompatibility complex (MHC) with restricted killing tumor cell capacity of natural killer cells. CIK cells have the advantages of high cytotoxicity, fast proliferation, and independent of multi-drug resistance of tumor cells [2,3]. Dendritic cells (DCs), as main antigen presenting cells (APC), present tumor antigen to T lymphocytes, generate cytotoxic T lymphocyte (CTL) response, and induce anti-tumor immune response [5]. In previous study, through co-culturing autologous DCs with CIK cells, autologous DCs significantly increased the proliferation ability of CIK cells and enhanced their anti-tumor activity [6]. Treatment with autologous DC-CIK cells has displayed well clinical application prospect for many types of cancers, such as chronic myeloid leukemia, liver cancer, colon cancer, kidney cancer, and breast cancer [6,7]. In this study, we co-cultured...
DCs from healthy adult children of cancer patients with autologous CIK cells from patients to generate semi-allogeneic DC-CIK cells. In addition, we compared the anti-tumor abilities of autologous CIK cells, DC-CIK cells, and semi-allogeneic DC-CIK cells to explore the most effective anti-tumor adoptive immunotherapy approach.

Materials and Methods

**Experimental materials**

Leukemia cell line (K562), renal cancer cell line (ACHN), and liver cancer cell lines (HepG2 and Hep3B) were purchased from Shanghai Chinese Academy of Science Committee. Recombinant human interleukin (IL)-4, granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-2 were purchased from Biosource. Fetal calf serum (FCS) and RPMI-1640 were purchased from Gibco. Mouse anti-human monoclonal antibodies, APC-CD3, PE-CD4, FITC-CD8 and CD56, were purchased from BD Biosource. LDH assay kit was purchased from Promega. Brefeldin A (BFA) was from Ebioscience. Membrane broken medium was purchased from Ebioscience. 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes. Annexin V apoptosis kit was purchased from Bender MedSystems. Flow cytometry was purchased from Becton Dickinson.

**Preparation of autologous CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells**

Fifty millilitre of peripheral blood was collected from one patient with renal cancer, two patients with lung cancer, and one patient with maxillary squamous cell carcinoma and their children, respectively. Peripheral blood mononuclear cells (PBMC) were isolated by lymphocyte separation medium. Cells were cultured in RPMI-1640 containing 5% FCS and adhered for 2 h. Suspended cells were removed and induced to CIK cells. Adherent cells were cultured using DC medium (RPMI-1640 containing 5% FCS, 100 ng/mL of GM-CSF, and 30 ng/mL of IL-4). After 24 h, mouse anti-human CD3 monoclonal antibody (final concentration was 150 ng/mL), 1500 units/mL of IL-2, and 1.5 ng/mL of IL-1α were added to CIK cells and cultured at 37°C in 5% CO₂ incubator. On the sixth day, another 500 ng of TNF-α were added to DCs to induce maturation. On the 7th day, CIK cells and autologous DCs or allogeneic DCs were mixed according to a ratio of 20:1 and cultured for another 7 days.

**Tumor cell culture**

Purification of human renal carcinoma cells and primary culture: renal carcinoma tissues were excised from patients and digested by type IV collagenase to produce single cell suspension. Highly purified renal carcinoma cells were isolated using a method established previously by our lab. Isolated cells as well as K562, ACHN, HepG2, and Hep3B were cultured in RPMI-1640 containing 10% FCS at 37°C in 5% CO₂ incubator.

**Phenotype analysis**

Autologous CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells were incubated with CD3, CD4, CD8, CD25 and CD56 antibodies for 30 min. Cells were washed by PBS, fixed by 2% of paraformaldehyde, and analyzed by flow cytometry.

**Proliferation assay**

CIK cells cultured were collected on day 7, washed and resuspended with RPMI-1640, and incubated for 10 min at 37°C after adding CFSE (final concentration was 1 μmol/L). Cells were washed twice by PBS. Labeled T lymphocytes and DCs were mixed according to the ratio of 20:1 and cultured on a 48-well plate. Medium was changed every day (half volume). After 6 days, cells were collected and analyzed by flow cytometry.

**Cellular apoptosis detection**

Autologous CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells were collected, resuspended with PBS, and double stained with Annexin V/PI for flow cytometry analysis.

**Cytotoxicity detection**

Autologous CIK cells, autologous CD-CIK cells, and allogeneic DC-CIK cells were mixed respectively with leukemia cell line (K562), primary renal carcinoma cells, ACHN cells, and live cancer cell lines (HepG2 and BEL7402) according to the ratios of 30:1, 10:1 and 3:1. CIK cells were as negative control. LDH assay was used to measure the cytotoxicity of CTL.

**Intracellular factor detection**

Anti-CD3 and -CD28 antibodies (1 μg/mL) were added to T lymphocytes and cells were incubated for 1 h. Then BFA was added and cells were cultured for another 5 h. T
lymphocytes were collected, washed once by PBS, and resuspended with 100 μL of PBS. FITC-anti-CD8 and PE anti-CD4 monoclonal antibodies were added and cells were incubated on ice for 30 min. Cells were washed once by PBS, fixed by 2% paraformaldehyde on ice, then washed once by PBS, and incubated for 10 min at room temperature after membrane broken medium was added. APC-anti-IFN-γ and APC-anti-IL-4 monoclonal antibodies were added directly to membrane broken medium and cells were incubated on ice for 30 min. Then cells were washed once by PBS, fixed by 2% paraformaldehyde, and analyzed by flow cytometry.

Statistical analysis

Two independent samples were calculated by t test. SPSS10.0 software was applied for data analysis. A value of \( P < 0.05 \) was considered as significantly different.

Results

Anti-tumor ability

We tested the anti-tumor activities of autologous CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells. The results showed that all three types of cells had anti-tumor effect on five types of tumor cells, including ACHN, primary renal carcinoma cells, BEL7402 cells, HepG2 cells, and K562 cells. Additionally, semi-allogeneic DC-CIK cells displayed the strongest anti-tumor activity under the ratio of 3:1, 10:1 and 30:1 (effective cells: tumor cells) (Figure 1).

Figure 1 Cytotoxicity effects of CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells

Cytotoxicity against different tumor cell lines (K562, ACHN, HepG2, and BEL7402) and primary renal cell carcinoma (RCC) cells (patient 1) was measured by LDH assay.
Intracellular factor

Next, we detected the secretion of IFN-γ and IL-4 of autologous CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells. The secretion of IFN-γ were 5.5%, 22.1%, and 35.3% respectively in these three types of anti-tumor cells from renal carcinoma patient, while that of IL-4 was 37.4%, 36.5%, and 25.4%, respectively (Figure 2A and 2B).

Figure 2 Intracellular cytokine production of CIK, autologous DC-CIK, and semi-allogeneic DC-CIK cells
A, IFN-γ production of CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells from RCC patient; B, IL-4 production of CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells from RCC patient.

Phenotype

The cell phenotype test showed that cells with CD3 positive were increased in autologous DC-CIK cells and semi-allogeneic DC-CIK cells, while the ratios of CD56+ cells, CD4+ cells, and CD4+CD25+ cells were decreased (Figure 3). In semi-allogeneic DC-CIK cells, the ratios of CD3+CD56+ cells and CD3+CD8+ cells significantly increased, which were similar to those of semi-allogeneic DC-CIK cells from other types of tumors (Table 1).

Table 1 Phenotype analysis of CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells from lung cancer patients and squamous cell carcinoma of maxillary sinus patient

<table>
<thead>
<tr>
<th>Patient</th>
<th>CIK cells</th>
<th>Autologous DC-CIK cells</th>
<th>Semi-allogeneic DC-CIK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3+CD56+</td>
<td>CD3+CD8+</td>
<td>CD3+CD56+</td>
</tr>
<tr>
<td>Lung cancer patient 1</td>
<td>9.9 ± 2.3</td>
<td>10.3 ± 1.7</td>
<td>17.3 ± 3.7</td>
</tr>
<tr>
<td>Lung cancer patient 2</td>
<td>12.5 ± 4.3</td>
<td>10.1 ± 1.8</td>
<td>15.3 ± 2.8</td>
</tr>
<tr>
<td>Squamous cell carcinoma of maxillary sinus patient</td>
<td>10.2 ± 1.8</td>
<td>9.8 ± 2.0</td>
<td>11.1 ± 2.4</td>
</tr>
</tbody>
</table>

CIK, cytokine-induced killer; DC-CIK, dendritic cells-CIK.

* P < 0.05, vs. CIK cells.

Cellular proliferation

We used CFSE assay to examine the proliferation capacity of autologous CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells. As showed in Figure 4A, the proliferation rates of these three types of cells from renal carcinoma patients were 5.78%, 13.1%, and 34.2%, respectively. Data from patients with lung cancer and maxillary squamous carcinoma also revealed that the proliferation capacity of autologous DC-CIK cells and semi-allogeneic DC-CIK cells were higher than that of
autologous CIK cells, and the proliferation capacity of semi-allogeneic DC-CIK cells was the highest (Figure 4B).

**Cellular apoptosis**

We applied Annexin V/PI double staining to test the cellular apoptosis of autologous CIK cells, autologous DC-CIK cells and semi-allogeneic DC-CIK cells. As showed in Figure 5, autologous CIK cells cultured for 14 days generated 10.2% of apoptotic cells, while autologous DC-CIK cells and semi-allogeneic DC-CIK cells generated 6.6% and 6.2% of apoptotic cells respectively.

**Discussion**

With the characteristics of high anti-tumor activity, broad spectrum of tumor sensitivity and sensitive to multiple-drug resistant tumors, CIK cells are considered to be safe and effective heterogenic cells. DCs are the most effective antigen presenting cells. Mature DCs present tumor antigen through type II histocompatibility antigen (MHC-II), resisting the immune escape of tumor cells[4]. Previous studies have shown that co-cultured CIK cells and DCs generate anti-tumor effective cells (DC-CIK cells)[8,9]. In this study, we compared the autologous CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells. We found that semi-allogeneic DC-CIK cells significantly enhanced the anti-tumor activity, increased the proliferation capacity of CIK cells, improved the ratios of CD3+CD56+ cells and CD3+CD8+ cells, promoted the secretion of IFN-γ, and maintained the activity of CIK cells. The establishment of semi-allogeneic DC-CIK cells provided a basis for a new, safe, and effective immunotherapy.

After chemotherapy and radiotherapy, the patients with advanced cancer often appear WBC reduction, anemia and other serious side effects. Therefore, repeated blood is not allowed. The number of monocytes per mL in peripheral blood of patients is less than healthy adults. In addition, the number of DCs induced by monocytes is reduced and the biological activity of DCs decreases[9]. However, the number and biological activity of DCs induced by monocytes in peripheral blood from healthy children of patients are normal. Moreover, DCs stimulate the proliferation and anti-tumor activity of CIK cells through secreting cytokines such as IL-12 and IFN-γ[9]. DCs with high activity and
Apoptosis of CIK cells, autologous DC-CIK cells, and semi-allogeneic DC-CIK cells

The apoptosis was quantified on day 3 with an annexin V and propidium iodide viability assay.

Thus, the effect of immune system is limited. In addition, the damage caused by radiotherapy and chemotherapy also reduce immune system functions. As a danger signal, semi-allogeneic DCs from healthy children of patients can stimulate immune response in patients\textsuperscript{[13,14]}, induce cytokines secretion\textsuperscript{[13,14]}, and enhance anti-tumor ability. Secondly, the originating from hemizygous produce higher level of co-stimulatory factors, IL-12, and other cytokines, enhancing the cytotoxicity and proliferation rate of CIK cells\textsuperscript{[8,9]}.
increase of Th1 type cytokine secretion improves the anti-tumor response. Allogeneic DCs stimulate stronger Th1 type immune response and more Th1 type cytokines secreted by immune cells, showing stronger anti-tumor ability[19]. Our results confirmed that semi-allogeneic DC-CIK cells increase IFN-γ secretion and decrease IL-4 secretion.

Thirdly, semi-allogeneic DCs increase the ratios of CD3+CD56+ cells and CD4+CD8+ cells (effective cells)[16] and reduce the production of CD4+CD25+ cells (inhibitory cells)[17], enhancing the anti-tumor ability.

Previous clinical studies and in vitro experimental data have shown that application of cellular immunotherapy associated with allogeneic DCs shows no side effects and self immune response[18–22]. Compared with allogeneic DCs, semi-allogeneic DCs from children of patients express MHC class I molecules [28], which prevent the risk of anti-host immunologic rejection due to re-injection of allogeneic cells, and enhance the safety of the treatment. Thus, application of semi-allogeneic DC-CIK cells is a more safety and effective cellular immunotherapy[20,24–26].

In conclusion, compared with autologous CIK and autologous DC-CIK cells, semi-allogeneic DC-CIK cells not only significantly enhance the immune response, but also display better security in clinical application, providing a new method for adoptive cellular immunotherapy. Semi-allogeneic DC-CIK cells show broad clinical application and play an important role in anti-tumor immunotherapy.

Acknowledgments

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Stem-like cancer cells are inducible by increasing genomic instability in cancer cells


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The existence of cancer stem cells (CSCs) or stem-like cancer cells (SLCCs) is regarded as the cause of tumor formation and recurrence. However, the origin of such cells remains controversial with two competing hypotheses: CSCs are either transformed from tissue adult stem cells or dedifferentiated from transformed progenitor cells. Compelling evidence has determined the chromosomal aneuploidy to be one of the hallmarks of cancer cells, indicating genome instability plays an important role in tumorigenesis, for which CSCs are believed to be the initiator. To gain direct evidence that genomic instability is involved in the induction of SLCCs, we utilized multiple approaches to enhance genomic instability and monitored the percentage of SLCC in cultured cancer cells. Using side population (SP) cells as a marker for SLCC in human nasopharyngeal carcinoma (NPC) and CD133 for human neuroblastoma cells, we found that DNA damage inducers, UV and mitomycin C were capable of increasing SP cells in NPC CNE-2 and neuroblastoma SK-N-CH cells. Likewise, either overexpression of a key regulator of cell cycle, Mad2, or knock down of Aurora B, an important kinase in mitosis, or Gdh1, a key E3 ligase in cell cycle, resulted in a significant increase of SP cells in CNE-2. More interestingly, enrichment of SP cells was observed in recurrent tumor tissues as compared with the primary tumor in the same NPC patients. Our study thus suggested that, beside transformation of tissue stem cells leading to CSC generation, genomic instability could be another potential mechanism resulting in SLCC formation, especially at tumor recurrence stage.