Antitumor activities of human autologous cytokine-induced killer (CIK) cells against hepatocellular carcinoma cells in vitro and in vivo

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INTRODUCTION
Cytokine-induced killer (CIK) cells are the major histocompatibility complex-unrestricted cytotoxic lymphocytes and generated by incubation of peripheral blood mononuclear cells (PBMC) in the presence of various types of cytokines such as CD3 monoclonal antibody, interleukin-2 (IL-2), interleukin-1 (IL-1) and interferon-gamma. CIK cells are the population of heterogeneous effector cells possessing enhanced cytotoxicity and a higher proliferation rate as compared with lymphokine-activated killer (LAK) and tumor infiltrating lymphocytes (TIL) cells. The high anti-tumor activity of CIK cells is mainly due to the high proliferation of double CD3+ and CD56+ positive cells. Some reports indicated that CIK cells, other than LAK and TIL cells, can be efficiently employed as an adjuvant in anticancer immunotherapeutic strategy for the eradication of residual cancer cells and prevention or delay of tumor relapse.

Hepatocellular carcinoma (HCC) is the malignant transformation of hepatocytes and is a common complication of chronic hepatitis mainly caused by hepatitis B virus (HBV) and hepatitis C virus (HCV) infection in China. The mechanisms underlying the malignant transformation of hepatocytes are not well defined. Many driving factors including the persistent hepatitis B or C virus infections, host immunological and genetic factors might have been involved in the process. For example, a failure to mount an efficient immune response to HCC cells, either because of selective defects or immune tolerance in the host immune system or because of tumor interference with a function(s) of immune cells, could account for the inability of HCC patients to block the pathogenesis of HCC occurrence. In attempt to characterize the anticancer function of CIK cells and develop an alternative adoptive immunotherapy for the patients with primary HCC, we evaluated the proliferation rate and phenotype of CIK cells from healthy donors and HCC patients. We first characterized the anticancer function of CIK cells and developed an alternative adoptive immunotherapeutic strategy for HCC patients.

AIM: To characterize the anticancer function of cytokine-induced killer cells (CIK) and develop an adoptive immunotherapy for the patients with primary hepatocellular carcinoma (HCC), we evaluated the proliferation rate, phenotype and the antitumor activity of human CIK cells from healthy donors and HCC patients in vitro and in vivo.

METHODS: Peripheral blood mononuclear cells (PBMC) from healthy donors and patients with primary HCC were incubated in vitro and induced into CIK cells in the presence of various cytokines such as interferon-gamma (IFN-γ), interleukin-1 (IL-1), IL-2, and monoclonal antibody (mAb) against CD3. The phenotype and characterization of CIK cells were identified by flow cytometric analysis. The cytotoxicity of CIK cells was determined by 51Cr release assay.

RESULTS: The CIK cells were shown to be a heterogeneous population with different cellular phenotypes. The percentage of CD3+/CD56+ positive cells, the dominant effector cells, in total CIK cells from healthy donors and HCC patients, significantly increased from 0.1-0.13% at day 0 to 19.0-20.5% at day 21 incubation, which suggested that the CD3+ CD56+ positive cells proliferated faster than other cell populations of CIK cells in the protocol used in this study. After 28 day in vitro incubation, the CIK cells from patients with HCC and healthy donors increased by more than 300-fold and 500-fold in proliferation cell number, respectively. CIK cells originated from HCC patients possessed a higher in vitro antitumor cytotoxic activity on autologous HCC cells than the autologous lymphokine-activated killer (LAK) cells and PBMC cells. In in vivo animal experiment, CIK cells had stronger effects on the inhibition of tumor growth in Balb/c nude mice bearing BEL-7402-producing tumor than LAK cells (mean inhibitory rate, 84.7% vs 52.8%, P<0.05) or PBMC (mean inhibitory rate, 84.7% vs 37.1%, P<0.01).

CONCLUSION: Autologous CIK cells are of highly efficient cytotoxic effector cells against primary hepatocellular carcinoma cells and might serve as an alternative adoptive therapeutic strategy for HCC patients.

MATERIALS AND METHODS
Reagents and cell lines
RPMI1640 and DMEM medium, recombinant human IL-1a, IL-2, interferon gamma (IFN-γ), TNF-α and monoclonal antibody against the CD3 surface antigen (mAb CD3) were all purchased from Gibco Co. The F1t-3 ligand, mouse anti-human FITC-conjugated CD3 and CD56 monoclonal antibodies were obtained from BD-Pharmingen and Fetal calf serum (FCS) from Hyclone. BEL-7402 is human HCC cell line kept in liquid nitrogen in our laboratory.

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Cell separation
The patients with primary HCC and healthy donors were submitted to cytopenia after their writing contents were signed. An enriched peripheral blood mononuclear cells (PBMC) product was collected using the specific program of the Cobe Spectra blood separator. Cells were resuspended in phosphate-buffered saline (PBS) without calcium and magnesium. The separation of PBMC was performed as previously described[2,19-21]. The concentrated PBMC cells were used immediately for CIK cell culture.

Generation of cytokine-induced killer (CIK) cells
CIK cells were generated as described previously[2,3,22,23]. Briefly, non-adherent Ficoll-separated human peripheral blood mononuclear cells were prepared and incubated in RPMI1640 medium containing 100mL−1 FCS and various types of cytokines added according to the reported protocol with minor modifications[23]. The final concentrations of the cytokines and antibody added were as follows: IL-2, 1000×103U·L−1; IL-1, 100×103U·L−1; IFN-γ, 100×103U·L−1; mAb CD3, 50µg·L−1. Cells were incubated at 37°C in a humidified atmosphere of 50mL·L−1 CO2 and fed every 3 days in fresh complete medium with 100mL·L−1 FCS and various types of cytokines at 0.5×109cells·L−1.

Immunofluorescent staining of effector cells
Starting PBMC, LAK or CIK effector cells were stained with various types of FITC- or PE-conjugated mouse mAb’s against human surface antigens. Antibodies used included anti-CD3, anti-CD4, anti-CD16, anti-CD56 and anti-TCR-α/β, anti-TCR-γ/δ (all from Becton Dickinson, Beijing, China). Five ×104 of cells were incubated with a optimal amount of antibody at 4°C for 30min. To remove excessive antibody, the incubation mixture was centrifuged at 250×g for 10min. The pellet was resuspended in PBS containing 2mL·L−1 of human AB serum and treated as described before[21]. Stained cells were washed and analyzed with FACScaliber (Becton Dickinson) in our laboratory.

Isolation of primary HCC cells from patients
Fresh liver cancer tissues were obtained from the HCC patients at the department of Surgery in our hospital and immediately immersed in sterilized PBS (pH 7.0) solution containing penicillin, 100×103U·L−1 streptomycin, 100×103U·L−1 and gentamycin, 500×103U·L−1 for 1h at room temperature, then washed with PBS for three times. The liver cancer tissues were cut into small pieces (1-2mm3 in size), and digested with 37°C-prewarm serum-free medium with 1.25g·L−1 of collagenase-V for 2h. In order to separate them into single cell suspension, all the digested pieces of tissues were filtered through the 200 mesh of sterilized copper wire bag. The single cell suspension was further isolated by Percoll gradient centrifugation. Finally, the resuspended cells in DMEM medium with 200mL·L−1 FCS were the primary HCC cells.

Cytotoxic effects of CIK cells by 51Cr release assay
51Cr release cytotoxic assays were performed as reported previously[24]. Briefly, one ×106 of human primary HCC cells were incubated with tumor cells at various ratios of effector cells to target cells as 6.25 : 1, 12.5 : 1, 25 : 1 and 50 : 1. After 4h incubation, all cells were collected by centrifugation and an aliquot of supernant was counted in a gamma counter. The percentage of specific release was calculated as described[20].

Inhibitory Effects of CIK cells on HCC in tumor-bearing nude mice models
In pilot experiments, 1.5×106 of BEL-7402 cells injected subcutaneously could produce solid tumor in Balb/c nude mice at the tenth day with 100% of incidence rate. The Balb/c nude mice used in the study were randomly divided into four groups treated with physical solution, PBMC, LAK and CIK cells, respectively. The nude mice received 3.0Gy of whole body irradiation and injected subcutaneously with 5×106 BEL-7402 cells, which were at the stage of exponential growth (designated as day 0). On day 1, 0.25mL of 1×106 effector cells such as CIK, PBMC or LAK cells were injected locally where tumor cells were inoculated for 6 consecutive days. In controls, the nude mice received 0.25mL physical solution. The size of tumor was recorded every other day. The solid tumors were peeled off after the nude mice were dislocated by killing them on the 35th day.

Statistical analysis
The Wilcoxon matched-pairs test was used to analyze for statistical significance. The P value less than 0.05 was considered as significant difference.

RESULTS
Proliferation and phenotype of CIK cells
Figure 1A and B show the proliferation of CIK cells from healthy donors and patients with primary HCC at different incubation days. During cell generation, there was a steady increase in both the absolute number and the percentage of CD3+/CD56+ cells, e.g. the percentage of CD3+/CD56+ cells was 7.5% on 14d and 51.3% on 56d of in vitro incubation, respectively. After 14d in vitro incubation, the number of total incubated CIK cells increased significantly (500 fold from 8.07×1010 to 1.02×1010). The majority (as high as 82±6.4%) of these cells were positive for TCR-α/β. Cells expressing TCR-γ/δ were relatively rare (4.5%±2.6%). The proliferation capability of PBMC obtained from normal donors was slightly higher than that of PBMC obtained from the HCC patients. The percentage of double CD3+/CD56+ positive cells varied during CIK cell generation. The percentage of CD3+/CD56+ positive cells in total CIK cells from healthy donors and HCC patients significantly increased from 0.1-0.13% at day 0 to 18.95-20.5% at day 21, which suggested that the CD3+ CD56+ positive cells proliferated faster than other populations of CIK cells in the protocol used in this study.

In peripheral blood, 24.2% of CD56+ cells coexpressed CD3+ as compared to 36.2% in LAK cell cultures and 76% in CIK cell cultures (Figure 2). Conversely, only 17.8% and 42.1% of CD3+ cells in peripheral blood and LAK cells coexpressed CD56+, whereas 76.6% of CIK CD3+ cells coexpressed CD56+. At d 28 of CIK cell generation, the percentage of CD3+ CD56+ cells expressing CD56+ increased to 82.4%.

Table 1 Phenotype of CIK cells and cytotoxic activity of CIK subsets

| Subset | Percentage positively of CIK | LU/10⁵ cell stained cells | Cell number ×10⁶ | Total LU per culture |
|--------|-----------------------------|--------------------------|-----------------|---------------------|
| CIK    | 43.6±4.8                    | 712±24.3                 | 29,074          |                     |
| TCR-α/β| 82.0±6.4                    | 20.6±3.8                 | 583±41.6        | 12,088              |
| CD56   | 30.4±5.6                    | 78.4±6.9                 | 214±70.1        | 13,948              |
| CD16   | 15.8±5.4                    | 60.4±6.0                 | 89±40.9         | 4106                |

Note: Total LU per culture was calculated by multiplying the number of LU per million cells by the total number of cells.

Effect of different sera on CIK proliferation
When CIK cells were incubated in RPMI1640 medium containing 100 mL·L−1 of fetal calf serum or 100mL·L−1 human AB serum or free-

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serum medium, respectively, they exhibited different growth curves (Figure 3). CIK cells had the highest proliferation rate in human AB serum-containing medium, the lowest in serum-free medium. The proliferated numbers of CIK cells increased by 500 fold in human AB serum-containing medium, 450 fold increase in fetal calf serum-containing medium, and 50-60 fold in free-serum medium, respectively.

**Figure 1A** Proliferation of CIK cells *in vitro* culture system

**Figure 1B** The percentages of double CD3+ CD56+ positive cells at various incubation time *in vitro* culture system

**Figure 2** Subsets of CIK cells from patients with primary HCC. CIK cells were counterstained for CD3 and CD56 at day 12 of CIK generation and the percentage of positively stained cells was determined by flow cytometry. PBMC and LAK were stained like CIK cells. LAK cells were used at day 6 of generation. Upper: CD56 subsets of CIK cells, LAK, PBMC. Subsets of CD56+ cells were compared to the total number of CD56+ cells. The figure represents data from three different experiments. Results are presented as mean value ±SEM. Lower: CD3+ subsets of CIK cells, LAK, PBMC. Subsets of CD3+ cells were compared to the total number of CD3+ cells. The figure represents data from three different experiments. Results are presented as mean value ± SEM.

**Figure 3** Effects of different culture system on growth of CIK cells

**Cytotoxic activity of CIK cells on primary HCC cells**

The CIK cell subpopulations were tested for cytotoxicity against the HCC cells as measured by $^{51}$Cr release. The primary HCC cells were used as the target cells and the CIK cells from the same patients with HCC as the effector cells. Both of them at various ratios of effector cells to target cells were mixed together for evaluation of the cytotoxic activity of CIK cells. CD3+/CD56+ T cells were identified to be the major cytolytic effectors, as previously reported, the CD3+/CD56+ subpopulation of CIK cells exhibited maximum $^{51}$Cr release cytotoxicity toward target cells (Figure 4) with the increased ratio of effectors and target cells, the cytotoxic effects of CIK cells correspondingly became stronger. In controls, PBMC cells only showed a weaker cytotoxic effect on primary HCC cells compared with CIK cells, and maintained at a lower platform level even if the ratio of effector and target cells increased.

**Figure 4** Cytotoxic activity of CIK, PBMC cells against primary HCC cells

**Inhibitory effects of CIK cells on HCC in tumor-bearing nude mice**

Inhibitory effects of CIK cells on HCC cells in tumor-bearing nude mice are summarized in Table 2. In CIK-treated group, 15 nude mice were inoculated with tumor cells and received treatment of CIK cells for six day consecutively. Tumorgenesis was found in ten of fifteen (66.7%) nude mice. However, tumorgenesis was formed in all the mice in PBMC and LAK treated groups. Furthermore, the tumor size was smaller and limited in the mice of CIK-treated group as compared with those in the mice of PBMC- and LAK-treated groups. There was an obvious difference between the CIK group and LAK group.
(P<0.05), and between CIK group and PBMC group (P<0.001), which suggest that CIK cells have a great priority of inhibitory effects on tumorgenesis in tumor-bearing nude mice as compared with LAK cells.

Table 2 Inhibitory effects of CIK, PBMC and LAK cells on HCC cells in tumor-bearing nude mice

| Group | n  | Tumor /g | Tumorgenesis (%) | Inhibitory rate (%) |
|-------|----|----------|------------------|--------------------|
| Control | 9  | 2.10±0.41 | 100              | 0                  |
| PBMC   | 11 | 1.32±0.27 | 100              | 37.1               |
| LAK    | 11 | 0.99±0.33 | 100              | 52.8               |
| CIK    | 15 | 0.32±0.31 | 66.7             | 84.26*             |

*P<0.05 vs LAK group, *P<0.01 vs PBMC group.

**DISCUSSION**

In this study, we found that aotologous CIK cells had a higher proliferation rate and enhanced cytotoxic activity compared with lymphokine-activated killer cells[26,27]. The CIK cells from the primary HCC patients had a lower proliferation rate and enhanced cytotoxic activity than LAK cells[26,30,31]. The CIK cells of CIK cells from healthy donors were proved to be stronger than those from the patients with primary HCC. This result might reflect the functions of CIK cells in HCC patients is probably inhibited to some extent by itself, which is consistent with the fact that there is a decreased function of peripheral blood dendritic cells in patients with primary HCC[28,29]. Whether the dysfunctional dendritic cells led to the decreased proliferation and function of CIK cells? Martin et al reported that CIK cells significantly increased the cytotoxic activity after co-cultured with aotologous dendritic cells, and they considered this phenomenon to be induced by IL-12 cytokine secretion from dendritic cells and cell-cell interactions between the two population[29].

Though LAK cells recognize and kill target cells by a non-MHC restricted mechanism in the same way like CIK cells, the CIK cells were found to have the greatest killing activity[31]. Our results suggested that the CIK effector cells are the subpopulation coexpressing CD3+/CD56+ phenotypes, which supported the facts that CIK cells possessed a higher proliferation rate and higher antitumor cytotoxic activity in vitro than LAK cells[26,29,31].

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