

Original Article

Bioactivity of umbilical cord blood dendritic cells and anti-leukemia effect

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Abstract: *Objective:* We investigated the effect of umbilical cord blood dendritic cells (DCs) on in vitro proliferation, immunophenotypes and levels of homologous cytokine-induced killer cells (CIK) and the toxicity on leukemia cells. *Method:* Mononuclear cell-induced DC-CIK cells derived from umbilical cord blood were collected and co-cultured in the proportion of 1:5. Cord blood CIK cells or peripheral blood DC-CIK cells were used as control. Phenotypes were analyzed by flow cytometry; vial cell counting was performed using trypan blue, and the killing activity of effector cells against leukemia cells was measured by MTT assay. The levels of interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α) and interleukin-12 (IL-12) were determined by ELISA. *Results:* The proliferative capacity of DC-CIK cells was obviously improved compared with cord blood CIK cells and peripheral blood DC-CIK cells ($P < 0.05$, $P < 0.05$). During the co-culture of cord blood DC-CIK cells, the ratios of $CD_3^+CD_8^+$ and $CD_3^+CD_{56}^+$ cells were obviously higher than that of CIK cells under the same conditions ($P < 0.05$). On day 3 of co-culture, the levels of IL-12, IFN- γ and TNF- α in cultured supernatant of cord blood DC-CIK cells were all higher than those secreted by CIK cells cultured alone ($P < 0.01$, $P < 0.05$, $P < 0.05$). When the effector to target ratio was 2.5-20:1, the killing effect of cord blood DC-CIK cells against each subtype of acute leukemia cells was obviously higher than that of CIK cells ($P < 0.05$). No significant differences in killing effect were observed for different subtypes. This finding was consistent with the killing effect of peripheral blood DC-CIK cells against leukemia cells. *Conclusion:* Cord blood DCs can enhance the proliferative capacity of homologous CIK cells and its anti-leukemia effect. Though cord blood DC-CIK cells showed a higher proliferative capacity than peripheral blood DC-CIK cells, the two types of DC-CIK cells did not differ significantly in terms of cytotoxicity. With a high availability and the low probability of graft rejection reaction, cord blood DC-CIK cells have a brighter prospect for application in immunotherapy.

Keywords: Umbilical cord blood, dendritic cells (DCs), cytokine-induced killer cells (CIK), co-culture, leukemia, cytotoxicity

Introduction

Dendritic cells (DCs) are the most powerful and the only professional antigen-presenting cells that can activate naïve T cells. As the initiator of immune response, DCs play an important role in mediating the anti-tumor activity of immunocytes [1]. Cytokine-induced killer cells (CIKs) represent a group of heterogeneous cells induced by anti- CD_3 monoclonal antibody and several cytokines. Combining the anti-tumor activity of T cells and non-MHC-restricted killing similar to the function of NK cells, CIK cells offer a great prospect in immunotherapy for hematologic malignancies [2]. The combined use of DC-CIK cells against malignancies is expected to have a synergistic effect. After co-culture of DC-CIK cells, the proliferative capac-

ity of DC-CIK cells and the anti-leukemia activity were both higher than those of CIK cells cultured alone [3]. We analyzed the effect of cord blood DCs on the proliferative activity, immunophenotypes, secreted cytokines and cytotoxicity of homologous CIK cells. A comparison was made with peripheral blood DC-CIK cells. The experimental findings provide valuable information for the clinical application of cord blood DC-CIK cells and the choice of DC-CIK precursors.

Materials and methods

Equipments

Model 311 CO_2 incubator (Thermo Electron, USA), IM inverted microscope (Olympus, Japan),

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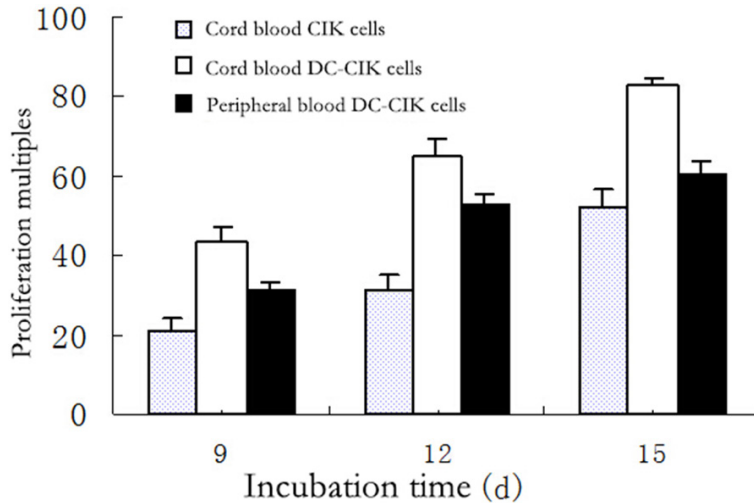


Figure 1. Proliferation multiples and culture time.

MK-3 microplate reader (Labsystems, Finland), KR-702 centrifuge (KUBOTA, Japan), JJJT-2 ultra-clean workbench (Beijing Semiconductor Device Factory, China), flow cytometer (Becton Di Ckison, USA), 96-well culture plate (Costar, USA).

Reagents

rhIL-2, RPMI1640 medium and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma Corporation (USA); CD₃McAb, Neomarkers Inc. (USA); rhIL-1, rhIFN-r and rhTNF-a, Shanghai Institute of Biological Products; rhGM-CSF, Schering-Plough Corporation; rhIL-4, Gibco BRL Co., Ltd. (USA); ELISA detection kits for labeled antibodies in flow cytometry CD3, CD1a, CD14, CD83, CD80, CD86, CD40, HLA-DR, CD3CD4, CD3CD8, CD3CD56 and cytokines (IL-12, IFN-r, TNF-a), Jingmei Biological Engineering Co., Ltd (Shenzhen).

Sources of target cells

Leukemia cells were collected from patients preliminarily diagnosed as acute leukemia at Department of Hematology of our hospital or brother institutes. With the addition of heparin as anticoagulant, 3-5 ml of peripheral blood (WBC>10×10⁹/L) or 1-1.5 ml of bone marrow was sampled and centrifuged by Ficoll-Hypaque density gradient centrifugation. The leukemia cells obtained were cryopreserved at -196°C in liquid nitrogen with ratio of leukemia cells >95% and that of viable cells >90%.

In vitro induction of CIK cells

Umbilical cord blood was sampled after severing of umbilical cord in full-term babies born vaginally. With heparin anticoagulation, mononuclear cells (MNCs) were harvested by Ficoll-Hypaque density centrifugation and washed with RPMI1640 medium three times. The cell concentration was adjusted to 4×10⁶/ml. Adherent culture was performed for 2 h, and the suspension cells were harvested and adjusted to 1×10⁶/ml using RPMI1640 medium containing 10% fetal calf serum (FCS). At 0 d, rhIFN-r 10-

00 U/ml was added and the cells were cultured at 37°C in a 5% CO₂ incubator for 24 h. After that, the cells were further incubated by adding rhIL-2 300 U/ml, rhIL-1 100 U/ml and CD₃McAb 50 ng/ml. The culture medium was replaced every 3 days and supplemented with rhIL-2.

In vitro culture of DCs

The method described in literature [4] was adopted. Adherent MNCs in the cord blood were cultured in RPMI1640 medium containing 10% FCS and supplemented with rhGM-CSF 550 U/ml and rhIL-4 500 U/ml. Half of the culture medium was replaced every two days with the supplementation of cytokines. At 72 h before harvest, rhTNF-α 50 U/ml was added to induce maturation of DCs.

Co-culture of DC-CIK cells

At 9 d of incubation, the viable DCs and CIK cells were counted and mixed in the proportion of 1:5 for 3 and 6 days, respectively. Then the cells were collected and detected for biological activity.

MTT assay for assessing the killing effect of target cells

Using acute leukemia cells as target cells and CIK cells and DC-CIK cells as effector cells, different effector to target ratios were selected, namely, 2.5:1, 5:1, 10:1 and 20:1. The effector cells and target cells were co-inoculated to

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Table 1. Phenotypic variations of cord blood CIK cells and DC-CIK cells (% , $\bar{x} \pm s$)

Group	n	Incubation time (d)	CD ₃ ⁺	CD ₃ ⁺ CD ₄ ⁺	CD ₃ ⁺ CD ₈ ⁺	CD ₃ ⁺ CD ₅₆ ⁺
Contrast	8	0	54.73±4.12	33.69±5.01	22.75±3.41	5.11±2.15
CIK	7	3	66.41±3.83	29.10±1.87	41.64±5.10	20.98±5.70
DC-CIK	7	3	72.57±4.13	24.91±3.09	55.43±6.11*	32.83±3.96*
CIK	7	6	75.98±5.12	25.02±5.53	60.88±7.10	34.21±5.11
DC-CIK	7	6	77.78±3.10	20.91±7.89	72.13±2.77*	48.22±2.70*

Cord blood MNCs before induction were used as control; *P<0.05 compared with CIK cells at the same culture time.

Table 2. Comparison of levels of cytokines secreted by cord blood CIK cells and DC-CIK cells (pg/ml, $\bar{x} \pm s$)

Group	n	IFN-r	TNF-a	IL-12
CIK	12	265.12±98.33	197.63±45.49	25.67±8.76
DC-CIK	10	532.76±189.42*	390.75±80.12*	85.22±11.95**

*P<0.05, **P<0.01 compared with CIK cells.

96-well plates, and separate cultures of effector cells and target cells were also set up, 3 replicates for each well. After treatment at 37°C for 48 h in a 5% CO₂ incubator, 20 ul of MTT solution was added into each well for further culture for 4 h. Then 100 ul of DMSO was added with proper mixing. The absorbance value (A) was measured with a microplate reader at 570 nm to assess the killing rate:

Killing rate (%) = [1 - (A of effector-target cell well/A of effect cell well/A of target cell well)] ×100%

Phenotypic analysis

Immunophenotypes of DCs, CIK cells and DC-CIK cells were identified by using flow cytometry.

Determination of cytokine expressions

The levels of IL-12, IFN-r and TNF-α in the cultured supernatant were measured according to the ELISA kit user manual.

Statistical analysis

The data were expressed as $\bar{x} \pm s$, and t test was used for intergroup comparisons. P<0.05 was considered as statistically significant.

Results

Proliferative capacity of CIK cells and DC-CIK cells

Cord blood CIK cells increased significantly on day 3. Some cells showed an enlargement of

volume and a trend towards irregular shape. Most cells underwent morphological changes on day 6 of culture, proliferating massively in clusters or colonies. Co-culture with DCs could improve the proliferative speed of CIK cells. Viable cells were counted using trypan blue on day

15. Results showed that cord blood CIK cells had proliferated by 52.28±8.03 folds, while DC-CIK cells proliferated by 82.20±6.35 folds, thus a significant difference was noted (P<0.05). Under the same conditions, peripheral blood DC-CIK cells proliferated by 60.75±4.89 folds [3], showing a much lower proliferative capacity compared with cord blood DC-CIK cells (P<0.05) (**Figure 1**).

Phenotypic analysis

Morphological features and phenotypic analysis of DCs: Cord blood DCs observed under the inverted microscope on day 9 of culture presented with typical dendritic shape or burr-like protuberances. Flow cytometry revealed the ratios of specific markers as follows: CD_{1a}⁺ (82.47±2.10)%, CD₈₃⁺ (80.12±1.68)%, CD₈₀⁺ (94.89±2.11)%, CD₈₆⁺ (93.77±3.10)%, CD₄₀⁺ (88.45±2.77)%, HLA-DR⁺ (95.48±3.00)% and CD₁₄⁺ (7.88±1.79)%. These findings indicated the presence of high-purity, adherent mature DCs derived from cord blood.

Phenotypic variations of CIK cells and DC-CIK cells: As the separate culture of cord blood CIK cells proceeded, the ratios of CD₃⁺CD₈⁺ and CD₃⁺CD₅₆⁺ cells increased gradually. Cord blood DC-CIK cells in co-culture showed much higher ratios of CD₃⁺CD₈⁺ and CD₃⁺CD₅₆⁺ cells than CIK cells cultured alone (P<0.05) (**Table 1**).

Cord blood MNCs before induction were used as control; *P<0.05 compared with CIK cells at the same culture time.

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Table 3. Killing activity of DC-CIK cells against leukemia cells on day 3 of co-culture (% , $\bar{x} \pm s$)

Group	Target cell	n	Multiplicity of infection			
			2.5:1	5:1	10:1	20:1
CIK	AML-M _{1,2}	10	11.55±6.38	15.97±4.22	22.33±3.88	32.86±3.78
	AML-M ₃	12	13.32±2.12	18.27±1.98	24.11±4.01	35.17±5.10
	AML-M _{4,5}	17	16.55±2.00	20.32±3.65	27.90±3.77	37.18±6.10
	AML-M ₆	8	12.89±5.11	16.99±3.89	24.77±5.00	32.11±3.33
	ALL-L _{1,2}	16	12.34±6.00	19.22±4.77	25.88±5.11	35.98±2.77
	ALL-L ₃	6	13.28±2.86	21.12±2.75	24.47±2.97	33.63±4.89
DC-CIK	AML-M _{1,2}	10	18.97±4.34	25.66±3.57	33.22±1.98	45.23±2.95
	AML-M ₃	12	20.77±2.86	29.57±2.34	36.24±5.12	48.85±4.11
	AML-M _{4,5}	17	24.11±3.02	32.35±4.11	37.18±5.55	50.34±2.74
	AML-M ₆	8	19.24±5.77	27.13±3.24	36.90±1.73	43.28±3.11
	ALL-L _{1,2}	16	21.33±4.13	30.07±2.73	38.00±4.40	49.99±5.15
	ALL-L ₃	6	19.32±6.12	29.44±5.01	34.95±6.00	43.95±2.22

Determination of cytokine secretions by DC-CIK cells

After co-culture of cord blood DCs and CIK cells for 3 days, the supernatant was collected to determine the levels of cytokines. It was found that the levels of IFN- γ , TNF- α and IL-12 secreted by DC-CIK cells were significantly higher than those by CIK cells on average ($P < 0.05$, $P < 0.05$, $P < 0.01$) (Table 2).

Cytotoxicity

In vitro experiments indicated that when the effector to target ratio was 2.5:1-20:1, cord blood CIK cells and DC-CIK cells displayed a strong killing effect against each subtype of acute leukemia. Such killing effect was increased with effector to target ratio. Generally DC-CIK cells had a higher killing rate than CIK cells cultured alone under the same conditions for each subtype of acute leukemia ($P < 0.05$). The killing effect showed a certain variation from one subtype to another, but the difference was not significant ($P > 0.05$) (Table 3). As indicated by our preliminary research [3], the average killing rate of peripheral blood DC-CIK cells against acute leukemia cells was 43.31% when the effector to target ratio was 20:1. This result did not differ considerably from the killing rate of cord blood DC-CIK cells induced under the same conditions (48.43%) ($P > 0.05$).

Discussion

CIK cells are a group of immune effector cells derived from MNCs in peripheral blood, bone

marrow or cord blood. They can be induced in the presence of IL-2, IL-1, IFN- γ and CD3 monoclonal antibodies. CIK cells combine the strong anti-tumor activity of T lymphocytes and the non-MHC-killing typical in NK cells [2]. The use of CIK cells for adoptive immunotherapy of tumors effectively addresses the problems of limited proliferation of effector cells, a need for transfusion of IL-2, low efficacy and severe side effect. As the most powerful antigen-

presenting cell, DC can highly expression MHC-I and MHC-II molecules, co-stimulatory molecules and adhesion molecules while absorbing and processing antigens. DC triggers primary and secondary T cell-dependent immune response in vitro and secretes Th1 type cytokine IL-12; moreover, it induces the production of IFN- γ by T cells and NK cells and enhances the cytotoxicity of activated NK cells. Either directly or indirectly DC exerts an influence on the proliferation of B cells and activates humoral immune response [5]. Thus the combined use of CIK cells and DCs will produce a synergistic effect in treating malignancies.

DCs play an important role in the activation and proliferation of CIK cells. After co-culture of peripheral blood DCs and homologous CIK cells, CIK cells showed an obvious enhancement of proliferative activity. On day 14 of co-culture, CIK cells had a 2-fold increase in proliferation compared with that on day 7 [6]. We showed that after co-culture of cord blood DCs and CIK cells, the total number of DC-CIK cells was increased by 27 times compared with that of CIK cells cultured alone. Moreover, the proliferative capacity of cord blood DC-CIK cells was superior to that of peripheral blood DC-CIK cells. This indicated that co-culture of cord blood DCs and CIK cells could produce enough effector cells for immunotherapy. The differences in proliferative capacity of DC-CIKs derived from different sources may be explained in light of the number and differentiation degree

of immunocytes and cytokine regulatory network.

Literature [7] revealed that the interaction of CIK cells with DCs promoted the antitumor activity. We found that the killing rate of DC-CIK cells against each subtype of acute leukemia was considerably higher than that of CIK cells cultured alone under the same conditions. But the differences in killing effect against each subtype were not statistically significant. This conclusion was consistent with the anti-leukemia effect of human peripheral blood DC-CIK cells in our preliminary research [3]. DC-CIK cells derived from cord blood possess a stronger anti-leukemia effect than CIK cells.

CIK cells mainly refer to $CD_3^+CD_{56}^+$ and $CD_3^+CD_8^+$ T cells. The antitumor activity of CIK cells is related to the content of $CD_3^+CD_{56}^+$ and $CD_3^+CD_8^+$ T cells and the secreted cytokines such as IL-2, IFN- γ and TNF- α . Mature DCs can secrete IL-12, IFN- γ and TNF- α . The co-culture of DCs and CIK cells will result in a variation of cytokine secretions and expression of surface molecules. One report [6] demonstrated that after co-culture for 2 days, CD_{40} , CD_{80} , CD_{86} , *HLA-DR* in DCs were obviously upregulated, with a 1.5-fold increase compared with those under separate culture of DCs. In contrast $CD_4^+CD_{25}^+$ cells (regulatory T cells) and IL-10 level decreased, which was accompanied by an enhanced cytotoxicity against tumor cells [8]. Co-culture of peripheral blood DCs and CIKs significantly improved the specificity of antigen presentation by DCs and co-stimulatory molecules. Furthermore, IL-12 secretion by DC cells and cytotoxicity of CIK cells were increased [6]. Antigen presentation by DC cells helped prolong the time of IFN- γ secretion by CIK cells and also increase the secretion amount [9]. Our results also indicated that the levels of IL-12, IFN- γ and TNF- α in the cultured supernatant of DC-CIK cells were much higher than those of CIK cells cultured alone; the levels of $CD_3^+CD_{56}^+$ and $CD_3^+CD_8^+$ cells were also higher in co-culture. This indicated that the high killing activity of cord blood DC-CIK cells was associated with the massively proliferating $CD_3^+CD_{56}^+$ and $CD_3^+CD_8^+$ cells. The secreted cytokines IL-12, IFN- γ and TNF- α enhanced the tumor killing activity either directly or indirectly. Several mechanisms of target cell killing by DC-CIK cells are proposed: CIK cells release cytotoxic cytoplasmic particles to kill the target cells; CIK

cells produce a large number of inflammatory cytokines to enhance the cytotoxicity; CIK cells can express FasL to induce tumor cell apoptosis. DCs in co-culture may secrete co-stimulatory molecules and cytokines to promote the activation and differentiation of CIK cells; DC cells, by stimulating the proliferation of antigen-specific T cells, activate CIK cells indirectly [10, 11].

In vitro experiments indicated that cord blood DC enhanced the proliferative capacity of homologous CIK cells and its anti-leukemia effect. The proliferative capacity of cord blood DC-CIK cells was stronger than that of peripheral blood DC-CIK cells, but the DC-CIK cells of different origins did not differ significantly in cytotoxicity. Since cord blood DC-CIK cells are more abundant and induce less graft rejection reaction during transfusion, they have a wider scope of clinical application.

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Disclosure of conflict of interest

None.

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