Study of Multi –directional Derivation of Cord Blood Mononuclear Cells and Its Killing Activity Effect to SHG44 Glioblastoma Cell Strain in Vitro

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ABSTRACT **Objective** To study multi−direction derivation from cord blood mononuclear cells to CD_3AK, LAK and CIK cells as well as variations of killing activity to SHG44 Glioblastoma cells strain in vitro. **Methods** CD_3mAb and IL−2 were used to induce CD_3AK cells;IL−2 was used to induce LAK cells; IFN−r was used in the beginning, then IL−1, CD_3mAb and IL−2 were used to induce CIK cells after 24h for observing amplification status and analysis of the relationship; cultivated CIK cells were taken to analyze phenotype by flow cytometer, subsequently, the SHG44 Glioblastoma cell strain was taken as target cells applied to evaluating killing activity respectively of CD_3AK, LAK and CIK cells by MTT method. **Results** The amplification activity of CD_3AK and CIK cells were all far higher than LAK cells. Compared with CD_3AK cells, the amplification activity of CIK cells had no obviously difference at prophase, but was far higher than CD_3AK cells at about 20d. The analytic results of flow cytometer disclosed that the amount of CD_3^+ CD_{56}^+ cells which were major effector cells after CIK cells being cultivated was far higher than before, moreover, the amount of CD_8^+ cells was far higher than before as well. The killing activities of CD_3AK and CIK cells to the SHG44 Glioblastoma cell strain were all far higher than LAK cells, besides, killing activity of CIK cells was far higher than CD_3AK cells. **Conclusion** CIK cells have higher amplification activity and higher killing activity, which can be taken as more effective killing cells applied to the tumor adoptive immunotherapy.

Key words CD_3AK cells; LAK cells; CIK cells; Amplification activity; Killing activity

Natural Killer Cells (NK cells) have the activity of non−specificity killing tumor cells [1.2]. Lymphokine Activated Killer Cells (LAK cells) are NK cells activated by IL−2, which have the capability of broad−spectrum, high performance dissolving and killing many kinds of tumor cells, and have surely curative effect in the adoptive immunotherapy [3]. Anti−CD_3 monoclonal antibody activated killer cells (CD_3AK cells) are cytotoxic cells induced by CD_3McAb and rIL−2, which have some characteristics such as more speedy amplification, longer survival time, higher anti−tumor activity as well as lower toxin and by−effects [4.5.6]. Cytokine Induced Killer Cells (CIK cells) are immune competent cells (ICC) with broad−spectrum killing activity, which can be induced by many kinds of cytokines such as IFN−r, IL−1, IL−2 and CD_3McAb,etc[7. 8. 9].

The adoptive immunotherapy is an important method of adjunctive therapy to treat malignant tumors. Compared with other anti−tumor drugs, it can directly kill tumor cells without injuring organic immune system and function, furthermore, accommodates and enhances the immune function of organism. At present, some immunocytes have been commonly used such as CD_3AK cells, LAK cells, CIK cells and dendritic cells (DC), which must have higher cytotoxicity and amplification activity. This experiment intended to seek high performance ICC applied to the adoptive immunotherapy and to find out the relationship among them by adding various kinds of factors for multi−direction derivation in vitro. We had compared the amplification activity and the killing activity to MGC−803 among LAK, CD_3AK and CIK cells, as following:

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MATERIALS AND METHODS

General materials

Cord blood was collected from delivery room of The First Hospital of Xi'an Jiaotong University. The

selected indexes consisted of healthy puerpera, 18－42 years old, without urgent or chronic diseases and infection diseases, normal hepatic function, serologic examination of hepatitis virus being negative, HIV and CMV examination being negative, routine examination of blood and urine being normal, fetus being ripe term delivery and not dead foetus or abnormity, without apnoea neonatorum, without dropsy and choloplania, and the amount of cord blood being collected about 75－122 ml.

Main agentias

IL－2, IL－1, CD_3AK, IFN－r (Bangding Biology Company, Beijing,china); CD_3－FITC, CD_4－PE, CD_8－PE, CD_3－FITC, CD_{56}－PE and caprine－anti－mice IgG fluorescent antibody (Immunotech Company, France). MTT (Huamei Company, China); SHG44 Glioblastoma cell strain (subcultivated by our lab)

Abstraction of cord blood mononuclear cells

We collected some fresh cord blood anticoagulated by natrium citricum. After erythrocytes were settled, we explanted upper layer fluid which contained the karyocytes. Then we could get the cells of middle layer which contained the mononuclear cells by FISCO abstraction method. Subsequently, they were conserved after being washed by PBS 3 times.

Preparation of LAK cells originated by cord blood

We added IL－2 500 U/ ml into the conservative cells 1×10^6/ml to be cultivated in the incubator(37 ℃, 5% CO_2) and exchanged fluids per 3d as well as supplied IL－2. Then we collected cells 1×10^6/ ml for being evaluated respectively the killing activity at 0, 10, 20 and 30d.

Preparation of CD3AK cells originated by cord blood

We added CD_3McAb 50ng/ml and IL－2 500 U/ ml into the conservative cells 1×10^6/ml to be cultivated in the incubator(37℃, 5 % CO_2) and exchanged fluids per 3d as well as supplied CD_3McAb and IL－2. Then we collected cells 1×10^6/ ml for being evaluated respectively the killing activity at 0, 10, 20 and 30d.

Preparation of CIK cells originated by cord blood

We added IFN－r 1000U/ ml into the conservative cells 1×10^6/ml to be cultivated in the incubator(37 ℃, 5 % CO_2) , and added IL－1 100 U/ ml, CD_3McAb 50ng/ ml and IL－2 500 U/ml after 24h, then exchanged fluids per 3d as well as supplied CD_3McAb and IL－2. Subsequently, we collected cells 1×10^6/ ml for being evaluated respectively the killing activity at 0, 10, 20 and 30d.

The phaenotype detection of CIK cells

CIK cells were washed by PBS 2 times after being cultivated, and then were marked respectively by fluorescent monoclonal antibody (CD_3－FITC, CD_4－PE, CD_8－PE, CD_3－FITC, CD_{56}－PE). The comparisons used IgGI－FITC, IgGI－PE. Then they were incubated in dark place at ordinary temperature for 15 min. Subsequently, we added PBS buffer 0.5ml into them and detected surface antigen by flow cytometer (Coulter Company, US).

The evaluation of killing activity

Fig.1 Effective relationship of amplified cells

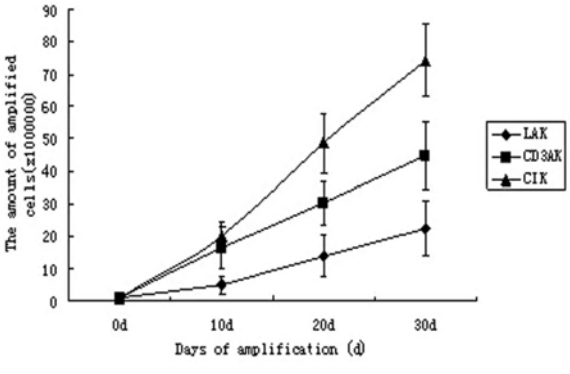
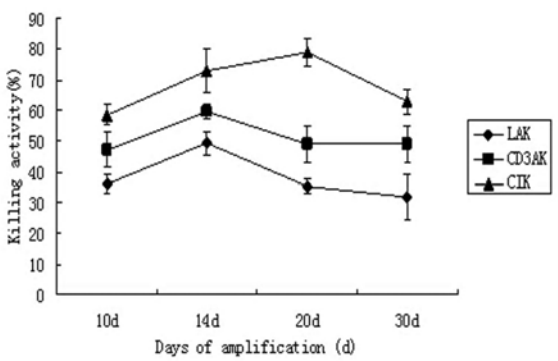


Fig.2 The relationship of killing activity of effector cells



We evaluated the anti-dyeing ratio of effector cells and target cells by trypan blue dyeing method. The results respectively were >95.4% and >96%. And then we put them (target cell: effector cell = 10:1) into the 96 orifice board. 3 teams were set up simultaneously such as an effector cell team, a target cell team as well as a blank team, and every team included 3 repeating holes. Subsequently, the 96 orifice board was taken into incubator (37 ℃, 5%CO_2) to be cultivated for 24h, after that we evaluated respectively the killing activity by MTT method. (Every hole was taken out of supernatant liquid 100ul and added MTT 20ul 5mg/ml to continue incubating for 4h, then was added HCL acidifying avantin 100ul0.04N. Subsequently, the OD values were measured by 570nm wave length. The results were a series of average values calculated by OD values of 3 repeating holes.)

Killing activity (%)=[1-(experiment team OD value - effector cell team OD value)/target cell team OD value]×100%

Statistic analysis

All data were performed by $x\pm s$, and analyzed by t or t′ analysis. When $P<0.05$, the result was seen as significant difference, which were calculated by SPSS10.0.

RESULTS

The comparison of amplification activity

We compared directly the amplification activity as well as the diversity of proliferative kinetics among LAK, CD_3AK and CIK cells within 30d for cultivating by counting living cell method. The results were as following table 1 and fig. 1. As we could see, the amplifi-

Table 1 Proliferation status of effector cells

Cells($x10^6$)	Cell amplification multiples			
	0d	10d	20d	30d
LAK cells	1	5.06±2.65	13.99±6.30	22.40±8.61
CD_3AK cells	1	16.43±6.26*	30.23±6.75*	44.69±10.41*
CIK cells	1	20.05±4.47*	48.67±9.02*#	74.33±11.27*#

Compared with LAK cells, $P<0.05$; # compared with CD_3AK cells, $P<0.05$

Table 2 Phenotype analysis of CIK cells(%, $x\pm s$)

Phenotype	Cell amplification multiples			
	0d	10d	20d	30d
CD_3	73.15±9.15	61.55±12.57	64.63±10.30	77.72±10.59
CD_4	45.65±8.05	30.10±8.69	24.15±12.37	27.17±10.80
CD_8	22.33±5.09	29.37±6.40	41.25±3.53*	54.23±7.18*
CD_3CD_{56}	0.79±0.34	3.63±1.17*	17.27±5.42*#	43.89±9.49*#
$CD_{16}CD_{56}$	18.26±6.93	35.64±10.41	36.80±12.46	40.04±14.65

* Compared with beginning, $P<0.05$; # Use t′test

Table 3 Killing activity of effector cells(%, $x\pm s$)

Cells	Cell amplification multiples			
	10d	14d	20d	30d
LAK cells	36.13±2.93	49.24±3.99	35.26±2.25	31.90±7.18
CD_3AK cells	47.26±5.67*	59.63±2.40*	48.89±6.01*	48.89±6.01*
CIK cells	58.63±3.28*#	73.00±7.28*#	79.01±4.50*#	62.86±3.92*#

*Compared with LAK cells, $P<0.05$; #compared with CD_3AK cells, $P<0.05$

cation multiples of CD_3AK cells and CIK cells were all far higher than LAK cells ($P<0.05$) because CD_3mAb had extremely apparent activation and amplification effect. Compared with CD_3AK cells, the amplification multiple of CIK cells had no obviously difference at protophase but was far higher than CD_3AK cells′ at 20d ($P<0.05$).

The phenotype analysis of CIK cells [10]

We took the cultivated cells to analyze phenotype via flow cytometer (Coulter Company, US), the results were as following table 2. As we could see, the amount of major effector cells ($CD3^+$ $CD56^+$ cell) after being cultivated increased far higher than before ($P< 0.05$), $CD8^+$ cells increased far higher than before as well ($P< 0.05$).

The killing activity comparison of LAK, CD3AK and CIK cells

We evaluated the killing activity to MGC−803 at 10,14,20,30d among LAK, CD_3AK and CIK by MTT method. The results were as following table 3 and fig. 2. As we could see, the killing activities of CD_3AK and CIK cells was all far higher than LAK cells ($P<0.05$). CIK cells had higher killing activity at 10d (58.63± 3.28)%, which were far higher than LAK cells′ (36.13± 2.93)% ($P<0.05$) and CD_3AK cells′ (47.26±5.67)% ($P< 0.05$), and could hit the highest value at 20d (79.01± 4.50)%. The killing activity of LAK cells team and CD_3AK cells team could hit the highest value together at 14d, which were respectively (49.24±3.99) % and (59.63±2.40) %.

DISCUSSION

Cord blood is also called placenta blood which is collected from interior of abdominal stalk and placenta blood vessel near the fetus. Cord blood contains not only haemopoietic stem cells (HSC)/ hemopoietic precursor cells (HPC) which has the capability of directional differentiation, but also the stem cells of multi−directional potentiality which are similar to the stem cells of marrow interstitium. Cord blood has been a sort of important method of stem cells transplantation, the relative foundational studies and clinical applications, which has received increased attention. The cord blood has lots of characteristics such as weaker lymphocyte antigen, immature function of lymphocyte, lower risk of graft versus host disease (GVHD), etc. As a sort of substitute of marrow, the stem cells of cord blood have been applied to therapy of the malignant tumor of hematological system, the aplastic anemia and a part of genetic diseases. At present, there have been established some pools for conserving the stem cells of cord bloods inside and outside of country. The stem cells of cord blood have tremendous potentiality of generally application to clinical tissue engineering due to various merits of theirs such as higher survival rate of transplantation and lower risk of GVHD, etc.

The stem cells of cord blood are sort of archaeocyte, which have the potential of differentiation, the capability of self−renewal and multiplication, and can be induced to become various kinds of cells and tissues by some definite factors. While the mechanisms of differentiation are not yet clear, researchers still can use various combinations of different cytokines to induce stem cells to differentiate along different directions. In this experiment, we used IL−2 to induce LAK cells, used CD_3mAb and IL−2 to induce CD_3AK cells, used IFN−r in the beginning and IL−1, CD_3mAb, IL−2 after 24h to induce CIK cells for observing of amplification and analysis of relationship. The results of amplification indicate: amplification ability of CD_3AK cells is far higher than LAK cells ($P<0.05$), moreover, the killing activity of CD3AK cells is far higher than LAK cells ($P<0.05$) in vitro as well. The results also indicate CD_3AK cells are sort of more effective anti−tumor ICC, which outweigh LAK cells. The previous work on cord blood has indicated that LAK cells were sort of killer cell activated by IL− 2, which had broad−spectrum activity of killing tumor cells and could be applied to clinical therapy of tumor immunity as an effective method.

The mechanism of killing tumor cells of LAK cell is similar to CTL, which can be divided into two ways: directly killing mode and indirectly killing mode. CD_3AK cells, after LAK cells, become another sort of ICC, which are heterogenicity cell populations that have the capabilities of highly effective activation and

amplification. Although therapeutic effects have been achieved in the therapy of tumor by CD_3AK and LAK cells, there still remain some defects: it has to use large doses of IL−2 to be inject into body when LAK cells are applied to clinical therapy. As a result, poison and by−effects can not be ignored. Furthermore, the amplification of LAK cells is relatively difficult. In 1991, Schmidtf et al. of Stanford University reported earliest that CIK cells were cells induced by various cytokines, moreover, this experiment further indicates that the amplification ability and the killing activity of CIK cells is far higher than LAK and CD_3AK cells ($P<0.05$).

In this experiment, cell population of CIK cells team could be amplified to 20.05±4.47 times at 10d, 74.33± 11.27 times at 30d. We discovered that $CD3^+CD56^+$ cells had been significantly amplified ($P<0.05$) based on detecting cell phaenotype via flow cytometer (Coulter Company, US). As reported by some prior experiment [11], $CD_3^+CD_{56}^+$ cells played a role as major effector cell, and it had been confirmed that the cells with $CD_{56}^+$of NK cells antigen simultaneously originated form CD_3^+ CD56− T cells and not form $CD_3^-CD_{56}^+$ T cells. The results disclosed that $CD_3^+CD_{56}^+$ cells were never amplified or never amplified obviously at protophase; but being amplified in high speed at 20d; and hit the highest value (43.89±9.49)% at 30d, after that the percentage of $CD_3^+CD_{56}^+$ began to decrease but still remained 29.11% at about 60d. In the same process, CD_3^+,CD_4^+ and NK cells performed increasing tendency and activated CD_4^+ cell also maintained higher level. It was reported by some prior experiments that the CD_4^+ CIK cells also had the effects of restraining and killing tumor, which were never confined by MHC. The cytotoxicity of CIK cells was relevant to $CD_3^+CD_{56}^+$ cells. As indicated by some prior studies [12], antigen CD_{56} might play an important role to reinforce cytotoxicity. Meanwhile, CIK cells themselves could excrete some cytokines such as IL−2,IL−6,TNF and GM−CSF, etc to enhance cytotoxicity or accommodate tumor cells sensitiveness to CIK cells.

Subsequently, we had discovered that the killing activity of CIK cells hit the highest value ((79.01±4.50)%) at about 20d, LAK and CD_3AK cells also hit the highest values ((49.24±3.99)% and (59.63±2.40)%)at about 14d by MTT method to evaluate cell killing activity. Compared with LAK cells, the highest values of killing activities of CD_3AK and CIK cells are significantly different($P<0.05$). Moreover, of them all, CIK cells are most powerful($P<0.05$).

To sum up, CIK cells have various aspects of merits such as: more powerful amplification ability in vitro; more powerful killing activity; inducing graft versus tumor (GVT) effect and restraining GVHD effect; it is different to immune effector cells induced by target cells that CIK cells can perform degranulation without influence of immunosuppressive drug (ISD) and can overcome medicine tolerance of chemotherapy without deleteriousness to human marrow stem cell and hemopoietic progenitor cells, which can be seen as highly effective anti−tumor cells of new generation. On account of time difference of killing activity hitting the highest value, it can be inferred that if both CIK cells and CD_3AK cells can be put together for application, the killing activity curve may become more stable and killing tumor effect can be more lasting, which is going to be a sort of foundation for study in the future, and to bring about some guidance effects in clinical therapy of SHG44 Glioblastoma. At the same time, we should understand that experiment is only the simple imitation to environment in vivo and there is still a long way to go.

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