Brief Report

Application of Serum-Free Culture Medium for Preparation of A-NK Cells

Zhihua Wang[1, 2], Zhibin Zhang[1], Hui Zhang[1] and Yan Zhang[1]

To compare the differences between proliferation and cytotoxicity of adherent natural killer (A-NK) cells cultured with serum-free medium AIMV and standard serum-containing medium *in vitro*, and also observe the assisting effect of IL-12 on the activation and the morphology character of IL-2-treated A-NK cells, cellular proliferation was evaluated by MTT method *in vitro*. The morphology of the target cells killed by A-NK cells was observed through electroscope. All of the A-NK cells cultured in serum-free medium AIMV could rapidly proliferate and keep high cytotoxicity compared with that in standard serum-containing medium. A-NK cells activated by both moderate-dose IL-2 and IL-12 were superior to the high-dose IL-2-treated A-NK cells. These data indicated that serum-free medium AIMV could replace standard serum-containing medium for culturing A-NK cells, and moderate-dose IL-2 and IL-12 could reduce side effects caused by high-dose IL-2. The study provided a new experimental basis for experimental and clinical preparation of A-NK cells. *Cellular & Molecular Immunology*. 2006;3(5):391-395.

Key Words: A-NK cell, serum-free medium AIMV, IL-12

Introduction

Recently, tumor adoptive immunotherapy has focused on adherent natural killer (A-NK) cells since it has strong ability to proliferate *in vitro* and to kill tumor cells. Now complete medium (CM) is widely used for culture of A-NK cells. However, because of human blood group AB serum in CM, there is a risk in transmission some of infectious diseases. Therefore, application of serum-free medium for culture of A-NK will be an alternative strategy to avoid these defects, such as bad security, discrimination among batches, hard quality control and expensive price. Serum-free culture medium can also meet the requirements of non-organism and has definite ingredients and stable quality, which is easy to control the quality and overcome the defects of serum containing culture medium. IL-12, a stimulatory factor of NK cell, can vigorously trigger NK cell proliferation and high level of anti-tumor response. In this study, we try to improve a new way to the preparation of A-NK cells for the experimental and clinical trial in the absence of human serum.

[1]Institute of Oncology, Harbin Medical University, China;

[2]Corresponding to: Dr. Zhihua Wang, Institute of Oncology, Harbin Medical University, China. Tel & Fax: +86-451-666-5003, E-mail: hljwzh000 @163.com.

Received Mar 11, 2006. Accepted Oct 23, 2006.

Materials and Methods

Reagents and materials

Recombinant human IL-2 (rhIL-2) was bought from Nanjing Military Medicine Science Institute. rhIL-12 was gifted by Dr. Changyou Wu in National Institute of Health. Ficoll, lymphocyte separation medium, phenylalanine methylester (PME) and MTT were purchased from Sigma. RPMI 1640 medium was purchased from Gibco. Human AB type sera were obtained from Red Cross Center of Harbin for culturing human A-NK cells. Fetal calf serum was obtained from Hematology Institute in Chinese Academy of Medical Sciences for culturing tumor cells. Serum-free culture medium (AIMV) was the product of Gibco. Human lung adenocarcinoma cells (Anip973) and human erythroleukemia cells (K562) were kept in our lab. All the plastic culture flasks and culture plates were purchased from Costar Company in America.

Preparation of human peripheral blood mononuclear cells

Human peripheral blood was collected from healthy volunteer. Peripheral blood mononuclear cells (PBMC) were routinely separated on Ficoll-isopaque gradient, adequately rinsed and then conducted with PME in plastic centrifuge tube for 40 min at room temperature to deplete adherent cell mainly consisting of monocytes and B lymphocytes. Then the non-adherent lymphocytes were obtained for preparation of A-NK cells.

Table 1. The proliferation of A-NK cells at different culture time (OD)

Day	Group A	Group B	Group C	Group D
1	0.197 ± 0.014	0.199 ± 0.015	0.202 ± 0.015	0.197 ± 0.025
7	1.031 ± 0.073	1.037 ± 0.068	1.112 ± 0.084	1.104 ± 0.063
10	0.756 ± 0.046	0.721 ± 0.055	0.948 ± 0.030*	0.960 ± 0.025*

* $p < 0.05$.

Table 2. The cytotoxicity of A-NK cells against Anip973 tumor cells at different culture time (%)

Day	Group A	Group B	Group C	Group D
1	42.75 ± 4.97	46.60 ± 4.05	45.85 ± 4.74	46.92 ± 4.45
7	82.67 ± 6.66	84.77 ± 6.54	80.92 ± 1.25	92.14 ± 4.63*
10	56.74 ± 7.82	59.62 ± 4.19	60.97 ± 7.74	70.36 ± 6.22

* $p < 0.05$.

Preparation of A-NK cells

The preparation of A-NK cells was described preciously (1, 2). In brief, to obtain enriched NK cells, peripheral blood lymphocytes were incubated with anti-CD3, anti-CD4, anti-CD14, anti-CD19 mAbs (30 min at 4°C) and purified by immunomagnetic depletion. Obtained cells were analyzed by flow cytometry. The purified human NK cells were divided into four treatment groups as follows. Group A: AIMV, IL-2 (6,000 IU/ml); Group B: AIMV, IL-2 (1,000 IU/ml), IL-12 (5 ng/ml); Group C: RPMI 1640 culture medium, 10% human AB type serum, IL-2 (6,000 IU/ml); Group D: RPMI 1640 culture medium, 10% human AB type serum, IL-2 (1,000 IU/ml), IL-12 (5 ng/ml).

Resting purified NK (R-NK) cells were cultured for 3-5 h in the cell density of 5×10^6/ml, and then removed the non-adherent NK (NA-NK) cells suspension and collected adherent NK (A-NK) cells. Then the A-NK cells were re-suspended respectively into the culture medium containing 50% self-containing medium, adjusted to cell density of 1-2 $\times 10^6$/ml, and continued to culture and changed with fresh medium every other day.

Determination of proliferation capability of A-NK cells

The modified MTT colorimetric method was used to determine cell proliferation as described previously (1). Briefly, the tumor cells (1×10^4) were washed twice with PBS, suspended in 180 μl medium, and inoculated to 96-wells plate, cultured at 37°C 5% CO^2 for 24 h. After added MTT 10 μl (5 mg/ml) continual culture for another 40 h, DMSO (200 μl) was added into each well, slightly shaked for 5 min, then OD values were determined by micro-plate reader at 570 nm.

Determination of cytotoxicity of A-NK cells

The tumor cell lines Anip973 and the K562 were used as target cells of A-NK cells treated with different culture conditions. A-NK cell cytotoxicity was evaluated with MTT assay as described above. Briefly, the A-NK cells were co-cultured with Anip973 and K562 target cells at effector (E) to target (T) ratio of 100:1 in 96-well plate. As control, the A-NK cells and target cells were respectively cultured in 96-well plate. Each sample was assayed in triplicate to determine OD value and get mean value of triplicate wells. Cytotoxicity of A-NK cells was calculated by the following: cytotoxicity (%) = $[1 - (OD_{E+T} - OD_E)]/OD_T \times 100$ (1).

Table 3. The cytotoxicity of A-NK cells against K562 tumor cells at different culture time (%)

Day	Group A	Group B	Group C	Group D
1	44.90 ± 5.64	44.85 ± 4.96	48.91 ± 4.16	47.19 ± 4.88
7	83.16 ± 6.67	84.78 ± 4.86	84.40 ± 5.26	90.96 ± 3.18
10	66.06 ± 8.50	66.73 ± 7.59	67.83 ± 10.10	71.59 ± 8.22

Preparation of specimen for scanning electroscope and transmission electroscope

The scanning electroscope specimen was prepared as follows. Effector cells and tumor cells were mixed and cultured overnight in the culture flask lay out sterilized cover glass. The next day, the cover glass was took out and fixed with 3% glutaraldehyde.

The cultured cells were collected with centrifuge tube, centrifuged (4,000× g for 5 min) and fixed with 3% glutaraldehyde. Then the fixed cells were observed by transmission electroscope.

Statistical analysis

Significance of differences in the proliferation capability of A-NK cells at different culture time was calculated using *t*-test. For analysis of data on cytotoxicity of A-NK cells, ANOVA was used. The significant level was set at p value < 0.05.

Results

Proliferation of A-NK cells in vitro

All A-NK cells proliferated rapidly in different culture conditions. There was no significant difference between group A and group B and between group C and D ($p > 0.05$). It was shown that IL-12 could also stimulate A-NK cells to proliferate rapidly. Combined with IL-12 to activate A-NK cells, the dosage of IL-2 could be reduced. There was no significant difference between group C and group A and between group B and group D ($p > 0.05$) except for the tenth day ($p < 0.05$). It was showed that AIMV substituted for CM had no obvious influence on culture of A-NK cells. Only on the 10th day A-NK cells cultured with CM still had stronger proliferating potential (Table 1).

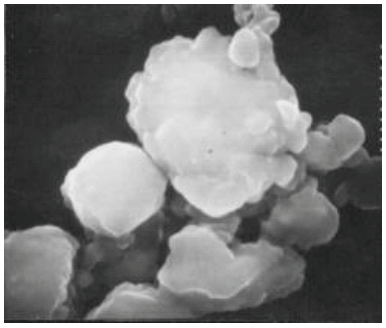

Figure 1. A-NK cells impinge on K562 tumor cell. The target cell swells and presents as vacuole.

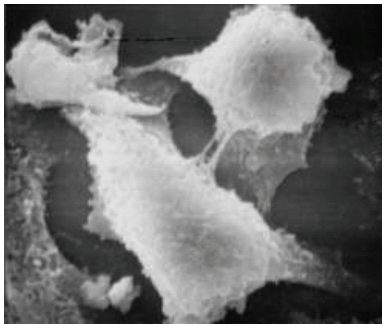

Figure 2. A-NK cell inserted to Anip973 tumor cell (as patrol) through protruding long pseudopods and prominencies, and tightly contacted with the tumor cell.

Determination of cytotoxicity of A-NK cells in vitro

There was no significant difference between group C and group A and between group D and group B in different culture conditions ($p > 0.05$). It was shown that AIMV substituted for CM had no obvious influence on the killing activity of A-NK cells. Except that, difference between group D and group C was significant at day 7 ($p < 0.05$). There were no significant difference between group A and group B and between group C and group D at other culture time ($p > 0.05$). It was shown that IL-12 can also improve the cytotoxicity of A-NK cells (Tables 2, 3).

The result of scanning and transmission electroscope

It can be seen under the scanning and transmission electroscope that A-NK cells congragated around the target cells and formed garland, part of which became deformed and protruded long prominences and microvilli that inserted to the target cells and contacted tightly with them in the form of point and spot (Figures 1, 2). It can be seen under the transmission electroscope that the tumor cells died in the form of necrosis and apoptosis (Figures 3). In a part of the target cells, the cellular endoplasmic reticulum swelled. The intima of the mitochondrial was apart from its adventitia. The cytoplasm vacuolated and the nuclear chromatin broke. The karyotheca and plasma lemma fragmented and the cell collapsed ultimately. These were typical symptom of necrosis. On the contrary, in the other tumor cells, the cellular nuclear chromatin condensed and aggregated at the nuclear membrane as hemilunar caps. The matrix occurred hydropic degeneration. The cell membrane swelled and became blebbing. But the karyotheca and plasmalemma remained intact. Typically withered body can be seen.

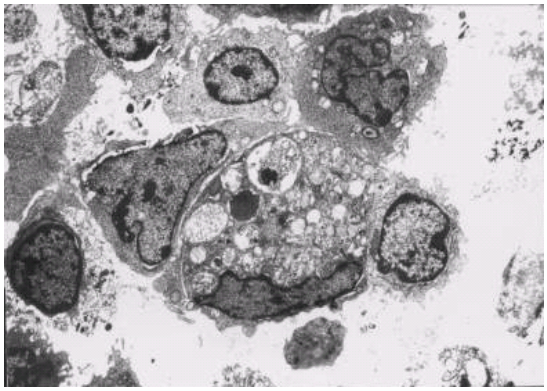

Figure 3. A-NK cells congregate, adherent to tumor cell and form the garland. The target cell appears necrosis symptom.

Discussion

We try to find a rapid and simple approach to preparation of the A-NK cells. Taking advantage of the nature that NK cells become plastic-adherent within the first 3-5 hours of exposure to IL-12, the time of collecting A-NK cells can be shortened from 24-48 h to 3-5 h (2). Although nylon wool column used to be adopted as a filter, the procedure is too complex to be applied widely. Now we conduct human peripheral blood mononuclear cells with PME for 40 min at room temperature, which can also deplete some inhibitors in peripheral blood (e.g., B cells and mononuclear phagocytes) (3).

AIMV has been used more and more widely, such as in culture of hematogenous cells, expression of recombinant protein in eukaryote system, proliferation of virus parasite and so on. Most studies indicate that AIMV can replace CM since production in both culture mediums are equal quantitatively and functionally (4). It is also demonstrated in our research that while culturing in a little amount, proliferation speed of the cells cultured in AIMV medium is slightly lower than that of the cells cultured in CM, but it has no statistical significance though the cells cultured in CM still have strong proliferation capability on the 10th day.

IL-12 is an important cytokine, which can stimulate various immunocyte proliferation, and play a prominent role in the tumor immunotherapy and gene therapy (5-7). Our experiment suggests that IL-12 can also improve proliferation of A-NK cells and anti-tumor activity. As shown in Figures 2 and 3, A-NK cells can kill both NK-sensitive cell system (K562) and NK-insensitive cell system (Anip973) (p

< 0.05). That is to say, IL-2 or combined IL-2 and IL-12 activated NK (A-NK) cells have stronger capability of killing neoplasm and wider range of killing neoplasm. However, clinical study data about IL-12 from American Genetic Association shows that different tumor patients accepting IL-12 therapy have better safety and endurance, but to the kidney carcinoma patients, IL-12 can exert some digestive system toxicity (8). We use IL-12/IL-2 to activate A-NK cells, which can reduce the dosage of both IL-12 and IL-2 and their side effects.

Anti-tumor mechanism of A-NK cells is complex. It is supposed that A-NK cells could kill tumor cells through various mechanisms: 1) A-NK cells can release perforin and granzyme to initiate cell apoptosis; 2) A-NK cells contact with tumor cells directly, whose surface membrane TNF mediate tumor cells apoptosis, or which release secretory TNF and induce the apoptosis of the tumor cells indirectly; 3) A-NK cells may destroy or activate the microvasculature of tumor, which induces other effector cells and cytotoxic molecules to infiltrate into tumor tissue and as a result tumor cell necrosis (9, 10). We observed the morphology of tumor cells killed by A-NK cells through scanning and transmission electroscope, and found that they died in form of necrosis and apoptosis. The results showed that A-NK cells kill tumor cells through various mechanisms.

Recently, the receptors involved in triggering of NK cell mediated cytotoxicity have been unveiled (11, 12). These are represented by three NK-specific receptors, termed as NKp46, NKp44 and NKp30 (13-16), that belong to the immuno-globulin superfamily and represent the first members of a group of receptors collectively termed as NCRs. NKp46 and NKp30 are expressed by both resting and activated NK cells, whereas NKp44 is expressed only on activated NK cells. A direct correlation exists between the surface density of NCR and the ability of NK cells to kill certain tumors.

Another receptor involved in NK cell-mediated cytotoxicity is represented by NKG2D. Recent data indicate that in most instances NK cell triggering in the process of tumor cell lysis depends on the concerted action of NCR and NKG2D. A novel family of human GPI-linked MHC class I related molecules, termed as ULBPs could bind to NKG2D and activate NK cells (17-25). Whether or not A-NK cell cytotoxicity depending on these receptors is under the research in our laboratory.

Abroadly, A-NK cells have a great potential to be used for adoptive immunotherapy of advanced malignant solid neoplasm in clinical trial (26). Our study provided some theoretical basis for further study and wide application of A-NK cells.

Acknowledgements

This project has been funded by grants from the Ministry of Science and Technology of China (95 Project 96-960A-01-0-20) and Science and Technology Commission of Heilongjiang Province (Key Project G00C190401) and the Department of Education in Heilongjiang Province.

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