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ORIGINAL ARTICLE

Effectiveness of allogeneic CD3AK cells on transplanted human renal cell cancer in mice with severe combined immune deficiency

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Summary

Purpose: *To assess the activity of allogeneic anti-CD3 antibody induced activated killer (CD3AK) cells on transplanted human renal cell cancer (RCC) in mice with severe combined immune deficiency (SCID), thus to provide theoretical and experimental support for clinical application of allogeneic CD3AK cells in the treatment of RCC.*

Methods: *A culture system which can massively increase allogeneic CD3AK cells was constructed. CCK-8 method was used to detect lethal effect of allogeneic CD3AK cells on human OS-RC-2 renal cancer cell line. Then, tumor-bearing mice models were constructed. SCID mice were randomly divided into four groups: group A (caudal vein was injected with allogeneic CD3AK cells before tumor bearing), group B (the control group of group A: caudal vein was injected with PBS before tumor bearing), group C (caudal vein was injected with allogeneic CD3AK cells after tumor bearing) and group D (the control group of group C: caudal vein was injected with PBS after tumor bearing), and spleen parameters were calculated to observe any inhibitory effect of allogeneic CD3AK cells on the growth of renal cancer cells, as well as their effect on the immune system of mice.*

Results: *Compared with the control groups B and D, spleen parameters of groups A and C increased significantly ($p<0.05$); compared with group C, spleen parameters of group A showed no obvious difference ($p>0.05$); compared with the control groups B and D, tumor weight of groups A and C decreased significantly and tumors grew slowly ($p<0.05$); the weight of mice in all groups increased while the weight of mice in the experimental groups increased less than the weight of mice in the control groups ($p<0.05$); compared with group C, tumor weight of group A reduced and grew slowly ($p<0.05$). Immunohistochemistry revealed that CD3+ cells showed positive expression in tumor tissues of mice in group C, while no positive expression was found in tumor tissues of mice in other groups.*

Conclusion: *Allogeneic CD3AK cells exerted lethal effect on OS-RC-2 cell line and significant inhibitory activity on the growth of transplanted cancer in mice with SCID. Also CD3AK cells expressed certain preventive effect on the development of implanted cancer in SCID mice; allogeneic CD3AK cells possessed antitumor activity and could enhance the immunologic functions of SCID mice with human renal cell-bearing cancer.*

Key words: *allogeneic CD3AK cells, combined immune deficiency, implanted cancer, OS-RC-2 cells, renal cancer*

Introduction

RCC is one of the most common malignancies of the urinary system, accounting for 85% of kidney cancers in the adults (2-3% in adult cancers, 4% in newly diagnosed cancer in males and 3% in newly diagnosed cancer in females). The incidence rate of RCC ranks second in tumors of the urinary system [1,2]. Global cancer statistical data shows that in the recent several decades, the incidence and mortality rates of RCC are increasing year by year [3,4]. Due to lack of obvious symp-

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toms and signs in early-stage RCC, about 30-40% of RCC patients are first diagnosed with locally advanced tumors or with distant metastasis. Median survival of such patients is 6-12 months only and 2-year survival is 10-20% [5]. At present, the main treatment method of RCC remains radical nephrectomy and the postoperative recurrence rate is 20%. RCC is a notorious multidrug resistant tumor and advanced metastatic RCC is insensitive to traditional therapies like radiotherapy and chemotherapy [6]. On the other hand, RCC is an immune-related malignancy which provokes strong immunogenicity and it is verified that various types of immuno-biological therapies have shown certain therapeutic effect in the treatment of RCC [7]. In the past 10 years, with the development of immunology and molecular biology, adoptive cellular immunotherapy of RCC has become a focus of research.

Biotherapy, a treatment method which develops rapidly in recent years, has become the 4th treatment modality along with the other 3 traditional therapies (surgery, radiotherapy and chemotherapy). At present, biotherapy of tumors mainly includes cytokine therapy, adoptive cellular immunotherapy (ACI), molecular targeting treatments, tumor vaccines, gene therapy, etc. [8]. With the development of immunological techniques and people's awareness of the complexity of immune system, immuno-biological therapies have gradually become the main treatment methods of RCC patients. However, in different kinds of ACI, application of tumor infiltrating lymphocytes and natural killer cells activated by lymphokines is limited due to problems like difficult cell source, slow speed of cell's expansion *in vitro*, poor cytotoxic activity, high dependence on exogenous interleukin-2 (IL-2), strong toxic side effects etc. Anti-CD3 antibody-induced activated killer cells, known as CD3AK cells, are alloplasm cell populations with CD3+, CD8+ and CD4+ cells as main effector cells, whose proliferation *in vitro* and immunological competence can last for about 2 weeks. Culture and separation of CD3AK cells are simple, and CD3AK cells can proliferate massively in culture *in vitro*. Moreover, CD3AK cells have strong antitumor activity [9,10].

The purpose of this study was to discover a new effective therapeutic schedule for RCC clinical adoptive immunotherapy as well as to provide a theoretical basis and accumulate experimental data for later clinical trials. In addition, this study also aimed to provide ACI of RCC patients with new choices and options.

Methods

Experimental materials

The main materials used in this study were: human RCC OS-RS-2 cell line (Cell Resource Center of Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences, China); mice with SCID (Beijing Huafukang Bioscience Co., Ltd., China); fetal calf serum and trypsin (Nanjing SenBeiJia Biological Technology Co., Ltd., China); penicillin (Shanghai Yu Duo Biological Technology Co., Ltd., China); streptomycin (Shanghai Yong Ye Biological Technology Co., Ltd., China); anti-human CD3 monoclonal antibody (T&L Biotechnology Co., Ltd.; Beijing, China). Main experimental instruments used included bechtop (Shanghai Ba Jiu Industrial Co, Ltd., China); 1X70-141 inverted fluorescent microscope (Olympus Co., Japan); K5600 ultra-micro ultraviolet spectrometry photometer (Beijing Kaiao Science and Technology Development Co., Ltd., China); FSX100 non-eyepiece microscope (Olympus Co., Japan); M5 multi-function microplate reader (Beijing An Maige Trading Co, Ltd., China); low-speed table centrifuge (Shanghai Seyfer Biological Technology Co., Ltd., China); vortex mixer (Beijing Ai Shang Yang Guang Science and Technology Co., Ltd.; China) and cryogenic refrigerator (Shanghai Ba Jiu Industrial Co, Ltd, China).

This study was approved by the Medical Ethics Committee and verified by pathologists. All patients signed written informed consent.

A) Experiment of cytotoxicity in vitro of co-culture of allogeneic CD3AK cells and OS-RC-2 cells

Culture and passage of human RCC OS-RC-2 cell line

Roswell Park Memorial Institute (RPMI) 1640 sterile culture medium was used for culture of human cancer cell lines, supplemented with 10% fetal calf serum + 100 U/ml penicillin and 100 μg/ml streptomycin. Human RCC OS-RC-2 cell line (Cell repository, Shanghai) was cultured in sterilized culture flask and put in an incubator with humidified atmosphere, 37°C, and 5% CO_2 for 2-3 hrs. Then the culture flask was taken out and was put in the biohazard safety equipment for passage.

Passage method: firstly, the culture flask was shook gently and fluid was removed; phosphate buffered saline (PBS) solution was drawn by Pasteur pipette to wash cells twice. Trypsin 0.25% was added into the culture flask and should cover all anchorage-dependent cells on the bottom. After 5 min digestion under 37 °C, cells were observed using microscope: intercellular spaces became wider and cells' edges rolled up. After that, trypsin was removed and complete medium was added to end the digestion. Cells were then dispersed using Pasteur pipette and cells that were not adherent were observed under the microscope. Next, cell suspension was drawn out and added into a centrifuge tube for centrifugation. Then, the supernatant was removed and certain

amount of complete medium was added into the cell pellet. Resuspended cells were dispersed using sterile Pasteur pipette to obtain single-cell suspension. Last, cell suspension was added into 2-4 culture flasks and was cultured in an incubator with humidified atmosphere, 37°C, and 5% CO_2.

B) Culture of allogeneic CD3AK cells

First, anti CD3 monoclonal antibody was coated in a 50 cm^2 culture flask (20 μg/ml density) and the flask was conserved in the dark at 4 °C overnight. The next day, 20 ml peripheral blood was drawn from a healthy adult and heparin was used for anticoagulation. Then, CD3AK complete medium was made, which contained 80% RPMI 1640 culture medium + 20% fetal calf serum + double antibody + 100 U/ml penicillin + 100 μg/ml streptomycin + 800 U/ml rIL-2. After 11-day culture, the culture flask was taken out and cellular morphology was observed under inverted microscope. Then, trypan blue staining was used to detect the viability of cells.

C) Detection of immunophenotype CD3, CD4 and CD8 of CD3AK cells using flow cytometry

On the 11th day, CD3AK cells were collected and centrifuged and the supernatant was removed. PBS was added to cell sediment to resuspend into single-cell suspension. Cell concentration was adjusted to 5×10^5/ml, and the solution was added into pre-marked flow tubes, 1 ml for each tube. All flow tubes were centrifuged and the supernatant was removed. Cells were blended using vortex apparatus and were added with fluorescein isothiocyanate (FITC) marked anti-CD3 antibody, phycoerythrin (PE) marked anti-CD8 antibody and PE-Cy5 marked anti-CD4 antibody, 5 μl for each. Cells were then kept in the dark for 30 min reaction at room temperature. After that, each tube was added with 1 ml PBS solution, it was centrifuged and the supernatant was removed. Then, 400 μl PBS were added and cells were blended for standby application.

D) Detection of lethal effect of allogeneic CD3AK cells on OS-RC-2 cells using CCK-8 method

First step: Human renal cancer OS-RC-2 cells were digested by 0.25% trypsin; then, the cells were dispersed and blended to obtain single-cell suspension. Cell viability was over 95% by using trypan blue dye exclusion method. The cells were washed with sterile PBS, were centrifuged and were made into single-cell suspension using 1640 complete medium; their concentration was adjusted to 5×10^5/ml cells. The cell suspension was added into a 96-well culture plate, 100 μl for each well; meanwhile, peripheral wells were added with 100 μl 1640 culture medium. Next, the 96-well culture plate was put in an incubator with humidified atmosphere, 37°C, and 5% CO_2 for 4-h culture.

Second step: On the 11th day of CD3AK cell culture, cells were collected and made into single-cell suspension; cell viability was over 95% by using trypan blue dye exclusion method. Then, cells were washed with sterile PBS, were centrifuged and were made into single-cell suspension using 1640 complete medium.

Third step: The 96-well culture plate was taken out of the incubator and 100 μl of the supernatant fluid from each well were removed. Then, 100 μl newly made 1640 complete medium was added in each well. Besides, wells of target cells and effector cells which were in the same volume and same number were prepared. Next, the culture plate was put in an incubator with humidified atmosphere, 37°C, and 5% CO_2 for 12-h culture; then, 10 μl CCK-8 reagent were added in each well and the culture was continued for another 4 hrs. The optical density (OD) of each well was measured using microplate reader at 450 nm to calculate the inhibiting activities of the cells.

Kill rate = [1–(OD value of experimental well –OD value of effector cell well)]/OD value of target cell well × 100%

E) Inhibiting effect of allogeneic CD3AK cells on transplanted human renal cell tumor model in SCID mice

1: Cell inoculation and construction of subcutaneously transplanted tumor models of SCID mice

Human renal cancer OS-RC-2 cells in logarithmic growth phase were selected and washed once using sterile PBS solution; after trypsinization, cells were made into single-cell suspension. Trypan blue exclusion test showed over 95% cell viability. After that, cells were centrifuged and the supernatant fluid was removed; then PBS was used to resuspend cells and cell concentration was adjusted to 1×10^7 cells/ml. Cells were placed into 15 ml centrifuge tubes and were conserved in an ice box for later use.

Five-week old male SCID mice which were raised in specific pathogen free (SPF) animal feeding department in the medical research center of our hospital were selected for this study. The left inguinal skin of SCID mice was disinfected using 75% ethyl alcohol. One-off 1 ml sterile empty needles were used to draw human renal cancer single-cell suspension from the ice box. Each mouse was subcutaneously inoculated with 0.2 ml human renal cancer single-cell suspension in the left groin, thus each mouse was inoculated with about 2×10^6 cells. Occurrence of subcutaneous skin rashes in the left groin of mice suggested successful construction of subcutaneously transplanted tumor models of SCID mice.

2: Grouping and drug injection of SCID mice

SCID mice were randomly divided into 4 groups (A-D) with 8 SCID mice in each group. Mice were injected with 0.2 ml allogeneic CD3AK cells in the caudal vein. The day of mice bearing cancer was recorded as day 0 (d 0). Intervention methods in the experiment were as follows: 3 days before bearing cancer, SCID

mice in group A were injected with allogeneic CD3AK cells resuspended in sterile PBS solution in the caudal vein and the total number of cells was 2×10^7; 3 days before bearing cancer, SCID mice in group B were injected with sterile PBS solution in the caudal vein; on D 6, D 11, D 16, D 21 and D 26 of bearing cancer, SCID mice in group C were injected with allogeneic CD3AK cells resuspended in sterile PBS solution in the caudal vein and the total number of cells each time was 4×10^6; on D 6, D 11, D 16, D 21 and D 26 of bearing cancer, SCID mice in group D were injected with sterile PBS solution in the caudal vein.

3: Detection of CD3+ expression in tumor tissues of all SCID mice using immunohistochemistry (streptavidin–peroxidase method)

After being fixed by neutral formic acid, tumor tissues of mice were processed with dehydration, transparentizing, waxing and embedding. Then, tumor tissues were cut in 3-4 μm; CD3+ expression in tumor tissues was detected using streptavidin–peroxidase (SP) method. Yellowish brown staining of the cell membrane and cytoplasm was taken as positive expression.

Statistics

PASW Statistics 19.0 software was used for t-test of all data; normal distribution of data was expressed by mean±SD and skewness data and logarithmic transformation were expressed by median; repeated measurement deviation analysis was used for comparison among groups, and chi-square test was used for comparison of enumeration data. A p value <0.05 was considered as statistically significant.

Results

Experimental results of cytotoxicity in vitro

A) Morphology of CD3AK cells and detection results of immunophenotyping

The growth of CD3AK cells was observed under inverted microscope. In the culture flask where CD3 monoclonal antibody (CD3mAB) was previously coated, non-adhering peripheral blood mononuclear cells (PBMC) were found to be in adhering growth and were round, transparent and bright. On the fourth and fifth day of culture, the number of cells increased significantly and cell clusters and colonies began to form; cell shapes were irregular (Figure 1).

Flow cytometry analysis of CD3AK cells suggested that CD3+ cells were the main part in CD3AK cells; positive expression rates of CD3+, CD4+ and CD8+ cells were 81.22, 20.91 and 60.12%, respectively; CD3AK cells were heterogeneous cell populations, mainly containing CD3+, CD4+ and CD8+ cells.

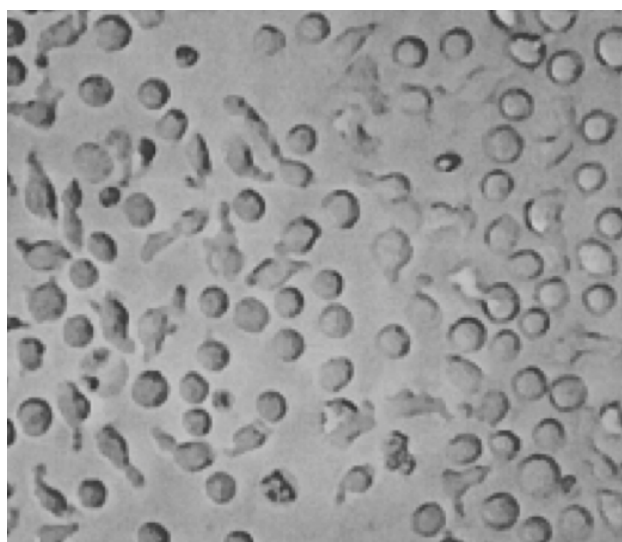

Figure 1. This figure shows the morphology of CD3AK cells after one-week culture, in which cells were continually increasing and formed into colonies, the volume of cells increased and cell shapes became irregular.

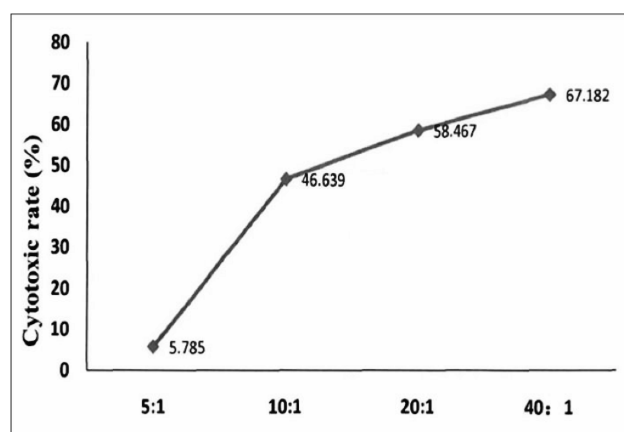

Figure 2. This figure shows the curve of *in vitro* cytotoxicity of allogeneic CD3AK cells on OS-RC-2 cells, and the effector target ratios were in direct proportion to *in vitro* cytotoxicity of allogeneic CD3AK cells on OS-RC-2 cells.

B) Detection of in vitro cytotoxicity of allogeneic CD3AK cells on OS-RC-2 cells

Allogeneic CD3AK cells and OS-RC-2 cells were co-incubated for 12 hrs; CCK-8 method was used to detect the killing activity of CD3AK cells on OS-RC-2 cells. When effector target ratios were 5:1, 10:1, 20:1 and 40:1, the killing rate of the cor-

responding CD3AK cells were 5.79, 46.64, 58.47 and 67.18%. Therefore, with the increase of ratio between effector cells and target cells, the killing activity of allogeneic CD3AK cells on OS-RC-2 cells also increased (Figure 2).

Experiment of internal inhibition

A) Inoculation of OS-RC-2 cells

Human renal cancer OS-RC-2 cells were cultured in an incubator with humidified atmosphere, 37°C, and 5% CO_2, using 1640 complete medium. When cells covered 85% of the flack bottom, cell medium was removed. The next day, cells were digested by 0.25% trypsin for 3 min; then trypsin was removed and 1640 complete medium was added to end cell digestion. After that, cells were dispersed and resuspended; then, after centrifugation, cells were dispersed and washed using PBS solution. Next, the number of cells was counted and cell concentration was adjusted to 1×10^7/ml. Details are shown in Figure 3.

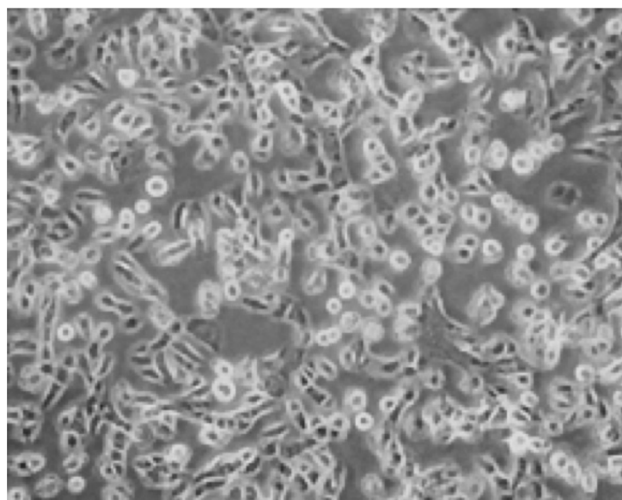

Figure 3. This figure shows the shapes of OS-RC-2 cells before inoculation. Cells were aggregated and in high density.

B) Comparison of tumor growth between groups

On D 6 of inoculation, subcutaneous tumors of mice in groups B, C and D could already be seen, while subcutaneous tumors of mice in group A could be seen on D 11 of inoculation; the percent of successful inoculation of all SCID mice was 100%; tumors of mice in groups B and D grew fast while tumors of mice in groups A and C grew slowly. Mice were sacrificed on the 26th day; each group had one randomly chosen mouse dissected, and subcutaneous tumors in the left groin showed that tumor volumes of mice in groups A and C were significantly smaller compared with mice in groups B and D.

The weight of all mice showed an increasing trend and no cachexia was noticed. After the experiment, the average weight of mice in groups A, B, C and D was 30.00 ± 1.16 g, 34.62 ± 1.16 g, 30.98 ± 1.22 g and 34.93 ± 0.43 g, respectively; the weight of mice in groups A and C increased less significantly compared with mice in groups B and D ($p<0.05$).

Concerning the spleen parameters (splenic weight/weight), the average of these parameters of groups A, B, C and D were 0.0025 ± 0.0003, 0.0012 ± 0.0003, 0.0025 ± 0.0002 and 0.0011 ± 0.0002, respectively. The difference of spleen parameters between group A and group B was significant ($p<0.05$), while between group A and group C was not significant ($p>0.05$). Spleen parameters of group C were significantly higher compared with group D ($p<0.05$). Details are shown in Figure 4.

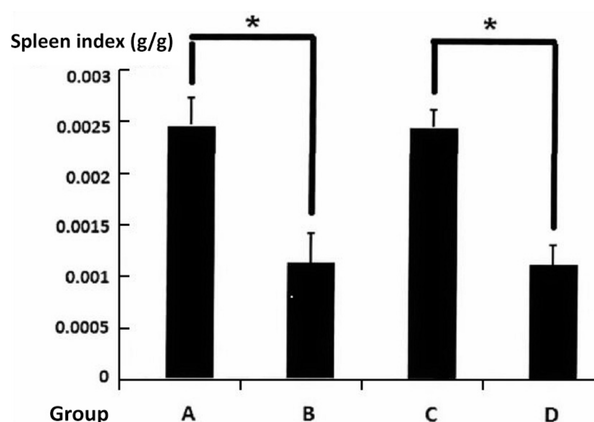

Figure 4. This figure shows the effect of allogeneic CD3AK cells on spleen parameters of SCID mice. *p<0.05.

The average tumor weight in mice of groups A, B, C and D after the experiment were 0.244 ± 0.034 g, 0.66 ± 0.071 g, 0.394 ± 0.047 g and 0.636 ± 0.084 g, respectively. The difference of tumor weight between groups A and B was significant ($p<0.05$) and the tumor inhibition rate was 63.03%. The difference of tumor weight between groups C and D was significant ($p<0.05$) and the tumor inhibition rate was 38.05%. The tumor weight of mice in group A was lower than that of mice in group C ($p<0.05$). The difference of tumor weight between groups B and D had no statistical significance ($p>0.05$). Details are as shown in Figure 5.

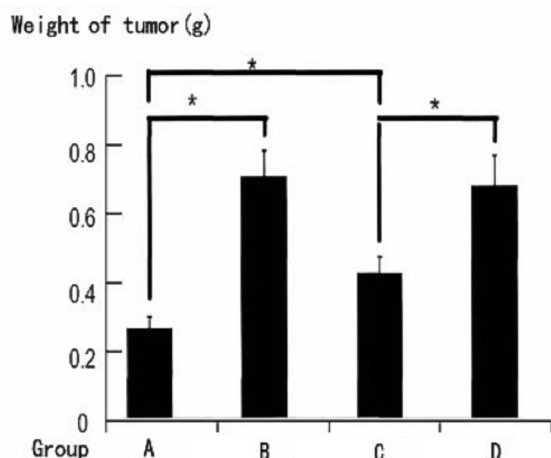

Figure 5. This figure shows the effect of allogeneic CD3AK cells on tumor weight of SCID mice. * p<0.05.

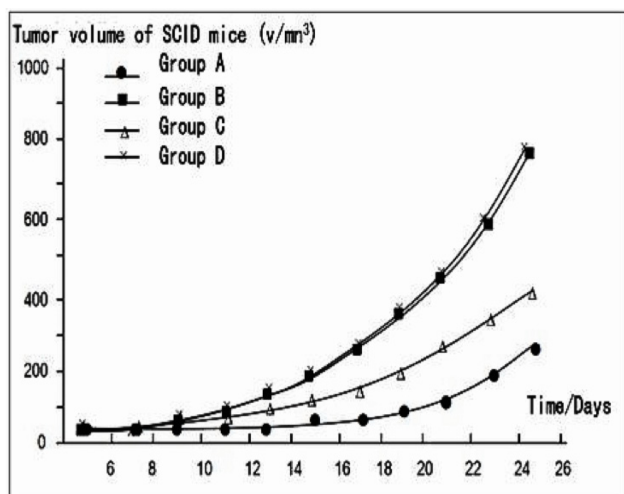

Figure 6. This figure shows the growth curves of tumor volumes of mice.

Regarding the tumor volume growth, the results showed that tumor growth of mice treated with allogeneic CD3AK cells in group A and C was slower compared with mice in the control groups B and D. Statistical analysis indicated that tumor volume of group A mice was 234.44 ± 40.24 mm^3, which was significantly smaller than that of mice in the control group B which was 774.86 ± 49.85 mm^3 ($p<0.05$); the tumor volume of group C mice was 393.42 ± 61.21 mm^3, which was significantly smaller than that of mice in the control group D which was 799.57 ± 85.67 mm^3 ($p<0.05$). Details are shown in Figure 6.

Immunohistochemical results of tumor tissues of mice

Immunohistochemistry (SP) was used to detect the expression of CD3+ cells in tumor tissues of mice. Experimental methods were processed in strict accordance with operating steps in specification. Staining results showed that positive expression of CD3+ cells was found in tumor tissues of group C mice, indicating that allogeneic CD3AK cells could reach tumor tissues and exert anti-tumor activity. No positive expression was found in tumor tissues of mice in groups A, B and D. Details are shown in Figure 7.

Discussion

RCC is a kind of relatively uncommon tumor, which accounts for 2-3% among malignant tumors in the adults and about 85% of idiopathic renal tumors are RCC [2]. On account of unconspicuous positions of the kidneys and unnoticeable symptoms of RCC in early stage, 30-40% of patients with RCC have already metastasis when first diagnosed. Prognosis of metastatic RCC is poor and 5-year survival rate is less than 10% and mean survival is only 10 months [1,11]. Among male populations in developed countries, RCC ranks sixth among the most common cancers. Active and passive smoking, hypertension and obesity are considered to be risk factors of RCC [12-14]. Besides, increased usage of medical imaging like computed tomography (CT) is also causes of increased cases of RCC [15].

Biotherapy develops fast in recent years and has become one of the four modern cancer treatment modalities along with surgical therapy, radiotherapy and chemotherapy [16]. Current biotherapies of tumors mainly include cytokine therapy, adoptive cellular immunotherapy, targeted therapy, tumor vaccines and gene therapy [17-21]. RCC is insensitive to traditional therapies like radiotherapy and chemotherapy, but it is the first kind of malignant tumor discovered to possess significant immunogenicity, thus creating adoptive immunotherapy of malignant tumors a hotspot in recent years [22]. Experiments of cytotoxicity *in vitro* showed that with increasing of ratio between effector cells and target cells, the killing activity of allogeneic CD3AK cells on OS-RC-2 cells also increased, which verifies that allogeneic CD3AK cells have cytotoxic effect on OS-RC-2 cells.

A study has shown that culture of CD3AK cells *in vitro* revealed powerful anti-tumor activity of T lymphocytes and non-major histocompatibility complex (MHC) restricted tumor-cytotoxicity

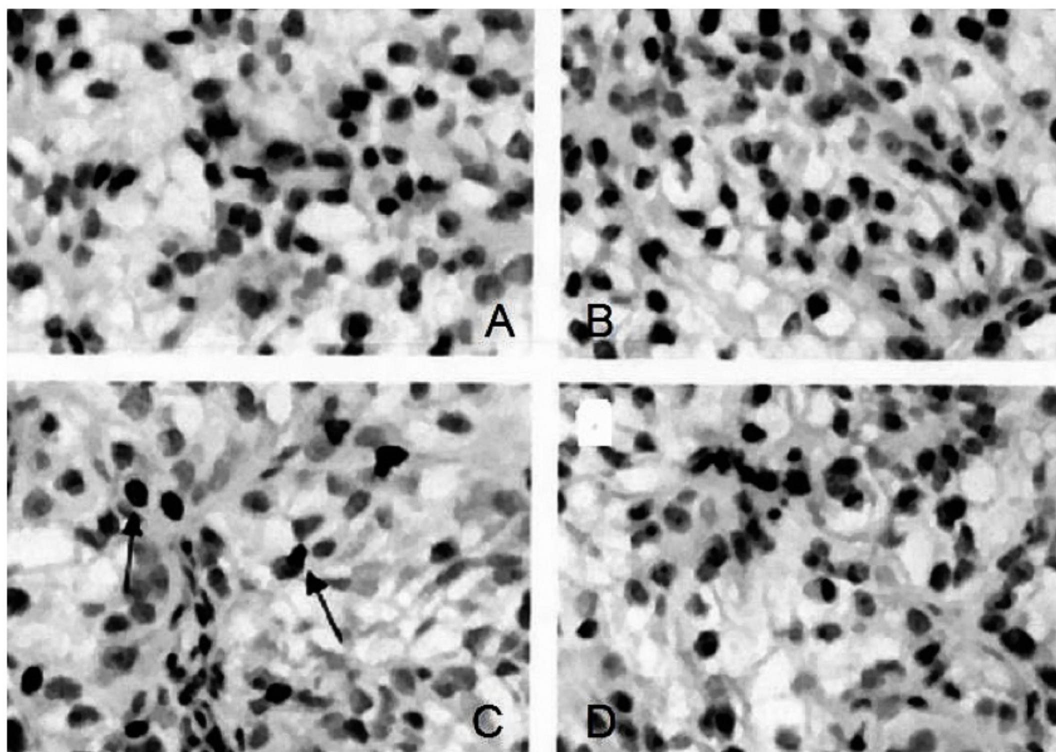

Figure 7. This figure shows the immunohistochemical results of tumor tissues of mice. No positive expression of CD3+ cells was found in tumor tissues of mice in groups A, B and D, while positive expression of CD3+ cells (arrows) was found in tumor tissues of group C mice.

[23]. In our study, allogeneic CD3AK cells were injected in the caudal vein of mice to treat human RCC. Comparison and statistical analysis of the four groups indicated that the net weight of tumor of mice in group A decreased significantly compared with mice in group B ($p<0.05$); the net weight of tumor of mice in group C decreased significantly compared with mice in group D ($p<0.05$); tumor growth of mice in groups A and C was slower than tumor growth of mice in groups B and D, indicating that allogeneic CD3AK cells exerted significant inhibiting effect on the growth of inoculated human RCC of SCID mice; the net weight of tumor of mice in group A decreased significantly compared with that of mice in group C ($p<0.05$); tumor growth of mice in group A was significantly slower compared with tumor growth of mice in group C. All the results of this study indicated that allogeneic CD3AK cells had certain preventive and inhibiting effects on the growth of transplanted tumors in SCID mice.

In conclusion, it can be considered that allogeneic CD3AK cells have considerable cytotoxicity in vitro on human OS-RC-2 cells as well as effective inhibiting effect on transplanted tumors in SCID mice. Besides, they exert certain preventive effect on the development of tumors in mice and they can stimulate body immune system against RCC cells.

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