

Expression on *MDR 1* gene in cord blood nuclear cells.

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Abstract

Chemotherapy is at present one of the most commonly used methods to treat malignant tumors, but along with the killing of the tumor, normal tissue cells and bone marrow cells are also damaged, which would ruin the effectiveness of chemotherapy. Currently focuses on chemotherapy research are overwhelming the side effects and ensure the smooth conduct and efficacy of chemotherapy. In this paper human umbilical cord blood mononuclear cells were used as target cells. *MDR 1* was inserted into the adenovirus vector. Recombinant adenovirus plasmid pHVaMDR 1 was constructed. And exogenous *MDR 1* could be effectively transferred into the umbilical cord blood nuclear cells by the method of concentrated virus supernatant. The result shows that umbilical cord blood nuclear cells which can express P-gp. P-gp expressed is used to transfer exogenous *MDR 1* gene into umbilical cord blood cells by concentrated virus supernatant transfection method.

Keywords: *MDR 1* gene, Cord blood nuclear cells, Concentration-recombined virus, P-gp.

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Introduction

Malignant tumor is a systemic and heterogeneous disease. Systemic chemotherapy has a pivotal status in the comprehensive treatment of malignant tumors. But in clinical therapy, the effects of chemotherapy are often unsatisfactory. Research found *MDR 1* genes are expressed excessively in tumour cells, resulting in large amounts of P-gp which leads to the resistance. This is the main mechanism of drug resistance. Located on the cell membrane, P-gp can pump out drugs from the cell and reduce drug concentration within the cell. Taking advantage of the knowledge of this mechanism, people use P-gp monoclonal antibodies and some novel technologies and therapy to reverse the tumor cell expression of P-gp. However, due to the side effects, it is difficult to use clinically. Experiment shows that the bone marrow cells' P-gp expression is extremely low, and due to the sensitivity to chemotherapy drugs, it is easy to lead to bone marrow restraint. Therefore, if *MDR 1* gene is induced into bone marrow hematopoietic cells and P-gp is highly expressed, this can strengthen bone marrow hematopoietic cells' chemotherapy toleration. The patients then can tolerate higher and more doses during the whole course of chemotherapy. The above study confirmed that the human *MDR 1* gene can be transfected into various types of cells with stable P-gp expression.

Methods

The supernatant preparation of concentration

According to adenovirus kit instructions, the supernatant preparation of concentration was recombined with virus and the determination of titer of recombined virus.

The separation of cord blood nuclear cells (CBNC)

Two-step separation of umbilical cord blood components: Separate whole blood and Process white film which is rich of nucleated cells.

CPD (S-200) anticoagulation umbilical blood and centrifuge 2000 rpm 20~30 min were used to absorb plasma slowly by plasma exhaust tube or sucker. It was then put it into a non-bacterial bottle. Multiple umbilical plasma (including A, B, O and AB blood group of two or more) samples were collected after mixing and cryopreservation spare. Absorb white film; put it into a tube for further process. Cryopreserve the remaining erythrocyte.

5 ml of umbilical cord blood 5 ml was taken and 4°C precooled erythrocyte lysis buffer was added to a volume of 50 ml. It was left for 10 min after mixing, centrifugated and the liquid supernatant was discarded. It was then washed twice and the resulting suspension of CBNC was stored.

Transfected CBNC by supernate of concentrated viral

The heparinized CBNC layer into 1×10^7 /ml was separated and prepared. The CBNC mentioned above was taken. Transfection system was used; it had 24 holes 1ml in size. In

each hole, 1×10^7 cells, 3% L-Glu for 10 μ l. 2-ME 5 μ l (10^{-4} mol), polyberene 30 μ l (200 μ g/ml), 10% BSA 0.1 ml, EPO 40 μ l (50 U/ml), IL-3 50 μ l (2000 U/ml), IL-6 50 μ l (100 ng/ml), SCF 50 μ l (2000 U/ml) was added. Then wet incubate with 20 percent FBS's IMDM complement was added to 1 ml at 37°C 5% CO₂.

To detect the insertion of gene MDR 1 in CBNC by the method of PCR

Transfected and untransfected culture cells were taken on the 2nd, 4th and 6th day; around 6×10^6 /ml was taken and the conventional method was used to extract DNA. Phenol-chloroform extraction DNA of cells was assayed. 25 μ l amplification system (including distilled water 17 μ l, primer P1 P2 each 0.5 μ l, buffer 2.5 μ l, Taq enzyme 0.5 μ l, DNA touched plate 2 μ l, dNTPs 0.5 μ l, MgCl₂ 1.5 μ l) was used. Cycling conditions: 94°C 5 min; 94°C 45 s, 58°C 45 s, 72°C 60 s, a total of 35 cycles, 72°C 10 min; Primer sequences: justice chain "5'-CCCATCATTGCAATAGCAGG", antisense chain "5'-GTTCAAACCTTCTGCTCCTCA". A rubber board was used to carry 10% ethidium bromide to show color 10min. it was observed and photographed by ultraviolet methods.

To determine the transfection rate of exogenous genes MDR 1 transfected CBNC by the method of immunohistochemistry

Transfected cells and untransfected cells were collected on the 2nd, 4th and 6th day, and washed with PBS with a centrifugal. Cell concentration was adjusted to about 1×10^7 /ml with PBS. Smears were prepared with centrifugal smear machine; 20°C acetones were used to dry them; they were washed three times with PBS and then soaked in 75% ethanol. Finally, they were stored at -20°C for immunohistochemistry. The first antibodies are P-gp monoclonal antibody; the second antibodies are biotin mark antibodies. Cell membrane and the cytoplasm which are both not responders are designated (-). The cytoplasm and cell membrane which are shallow brown coloured are designated (+). The cytoplasm and cell membrane which are light brown coloured are designated (++) . The cytoplasm and cell membrane which are dark brown coloured are designated (+++). The cell membrane which contained dark brown particles and the cytoplasm which was dark brown coloured was designated (++++).

Detection on P-gp positive rate of Exogenous MDR 1 gene transfected CBNC

Cultured cells to be tested were separated into 2×10^6 /ml suspensions. Adding Daunorubicin (DNR) into each tube, the final concentration is 7.5 μ g/ml. Then incubating them at 37°C, 5% CO₂ for 10 min. After the function of termination of the pump by verapamil ingot, the concentration is 10 μ g/ml. They were then washed twice with PBS before observing and counting them under a fluorescence microscope.

Determination on P-gp expression of the CBNC by indirect ELISA

Our experiment collected cell culture of 293 transfected cells on the 2nd, 4th and 6th day under the condition of -20°C; they were thawed before using. At same time, a comparison was made between the collected transfected cells and 293 normal cells. This was done by centrifuging them overnight with 5000 r for 10 min to coated microtiter plates and then adding to each 50 μ l 18% of formaldehyde at 37°C for 30 min. Then, remove the coating buffer; it was closed with 3% gelatin at 37°C for 1 h and washed 3 times with lotion which contains 0.5 ml/L Tween-20 PBS (Ph7.2) for 3 min each time. After this, MdrC McAb (1:200 ascites) for 2 h was added, it was rewashed three times, goat anti-mouse secondary antibody (1:4000) with the HPR label was added, a sense was made for 2 h and it washed three times. Finally, chromogenic substrate solution was dropped and OD490 values were measured.

Detection of biological characteristics of the cord blood cell cycle of transfected MDR 1 gene

The cultured cells to be tested were made into 2×10^5 /ml single cell suspensions, and washed two times with PBS. Collected cells were centrifuged for 5 min under 1500 rpm. The 0.1 μ g/ml RNase solution was left in a 37°C water bath for 30 min, propidium iodide working solution dark stain at 4°C was added at 30 min, and then detection with flow cytometry was conducted. This produced 293 normal human lymphocyte cells as controls.

Result

Virus titer was determined. Recombinant adenoviral vector was diluted by dilution method; 293 cells were infected. TCID₅₀ was calculated by using the Karber formula. The titer is about 4×10^9 PFU/ml.

The cells growth conditions after the *MDR 1* gene transfected CBNC. CBNC were transfected by *MDR 1 in vitro* and half of the medium was changed every other day. Observation by an inverted microscope revealed that the group of cells that had been transected for 2 to 4 days was growing well. The cells were homogeneous and transparent. The group of cells that had been transected for 4 days produced a large number of cell colonies. The cell number in every hole increased to $1.0 \times 10^6 \sim 1.5 \times 10^6$ from $2.0 \times 10^5 \sim 5.0 \times 10^5$. However, the group of cells that had been transected for 6 days did not grow well. The contrasts of the cell contour were enhanced. Grains and vacuoles were appearing in the cytoplasm.

Integration and expression results of exogenous *MDR 1* gene in CBNC. Application of *MDR 1* plasmid transfected CBNC using specific primers for PCR amplification of the *MDR 1* yielded the result shown in Figure 1. From the results it can be seen that specific amplified product bands appeared on 157 bp in cells, but not in negative control and untransformed cells. This proves successful integration of exogenous genes into the DNA of target cells.

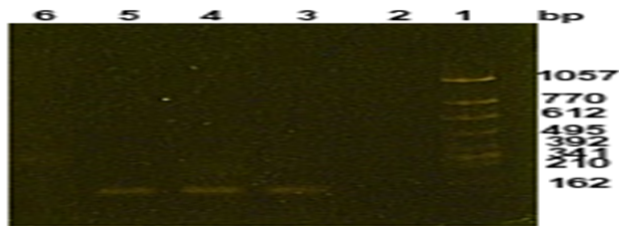


Figure 1. PCR result of MDR 1 transferred and untransferred on CBNC.

Transfection ratio on exogenous MDR 1 transfection CBNC by immunohistochemical

CBNC were cultured for two, four and six days respectively. Transfected MDR 1 group and untransfected group showed immunohistochemical results; P-gp expression is shown in Table 1, (293 cells as the positive control). As seen from Table 1, the transferred group and untransfected were compared on the same day with $P < 0.01$; transfection ratio increased in transfected group with time, $P < 0.01$.

Table 1. Transfection ratio of MDR 1 transferred and untransfected in CBNC on different time.

Transferred time and division groups		P-gp level				Transfected rate (%)
		+	++	+++	++++	
2 d	Transfected group	9	6	2	0	17*
	Untransfected group	1	0	1	0	2
4 d	Transferred group	10	12	5	3	30*
	Untransfected group	0	1	1	0	2
6 d	Transferred group	12	13	8	5	38*
	Untransfected group	0	1	0	0	1
293 cells as positive control		20	40	30	10	100

Positive rate determination of P-gp on exogenous MDR 1 transfected CBNC and comparison between groups

CBNC were cultured on 2nd, 4th and 6th days. Transfected MDR 1 group and untransfected group showed that P-gp was

expressed (293 cells as the positive control). The results are shown in Table 2. Seen from the Table 2, through statistical analysis, difference in P-gp expression was statistically significant between transfected MDR 1 group and untransfected group ($P < 0.05$). It is clearly seen that the more days the group is transfected, the higher the transfection ratio.

Table 2. Positive rate determination of P-gp on exogenous MDR 1 transfected CBNC and comparison between groups.

Transferred time and division groups		P-gp level				The positive ratio of cellular DNR
		+	++	+++	++++	
2 d	Transferred group	9	6	2	0	68**
	Untransferred group	1	0	1	0	94
4 d	Transferred group	10	12	5	3	55**
	Untransferred group	0	1	1	0	95
6 d	Transferred group	12	13	8	5	24**
	Untransferred group	0	1	0	0	95
293 cells as positive control		20	40	30	10	0

Test results of biological characteristics of the MDR 1 gene transfected cord blood cells

In the transfection group, umbilical cord blood cells in the proliferative phase (G_2 -M+S) cells accounted for 6.13%, quiescent (G_0 - G_1) cells accounted for 93.87%. The untransfected cord blood cells in the proliferative phase (G_2 -M

+S) cells accounted for 5.38%, quiescent (G_0 - G_1) cells accounted for 94.62%; there was no aneuploidy, debris or accumulation in the body. The difference in biological characteristics was not statistically significant between the two groups of cell cycle by χ^2 . ($P > 0.05$), the results are shown.

Discussion

When cancer patients are receiving chemotherapy, the effects of chemotherapy can be influenced and it cannot be implemented on schedule for its damage to normal cells and bone marrow cells. Currently, the factors which affect hematopoietic growth are used to shorten the duration of neutropenia after chemotherapy in clinical treatments. Ingredient transfusion and other supportive therapy are used to reduce bone marrow suppression after chemotherapy. However, chemotherapy-induced myelosuppression cannot be prevented. Repetitive chemotherapies may also cause normal bone marrow hematopoietic cell reserves to decrease, even causing bone marrow failure. In this study, cord blood nucleated cells were transfected by adenovirus-mediated *MDR 1*, to enhance their resistance to chemotherapeutic drugs. They were expected to overcome the injury of bone marrow cells and leukocytes during cancer chemotherapy. This plays an important role in improving the efficacy of chemotherapy. The study also intends to explore a way which can resist the cytotoxic effects of anticancer drugs during treatment. It aims to learn to increase the dose of chemotherapy drugs without the damaging hematopoietic cells, thereby enhancing the effects of chemotherapy.

Target cells of gene transfection should have the functions of self-renewal, proliferation and differentiation. Cells with these functions are known to be none other than stem cells. Therefore, pluripotent stem cells are often used for transplantation and for gene therapy research as target cells [1]. CBNC which is rich in hematopoietic stem/progenitor cells was used as target cells for gene transfection. Reasons that $CD34^+$ stem/progenitor cells were not purified are as follows: (1) the great loss of stem/progenitor cell in purification process; (2) the decay of interaction between cells; (3) the damage of micro-environment in which cord blood stem/progenitor cells stay.

Some scholars believe that cord blood cell cytokines may be provided independently in the form of paracrines, which support stem/progenitor cell proliferation. Therefore, this experiment is about how *MDR 1* gene was transfected into CBNC of proliferating exuberant. The result of stem/progenitor cell proliferation was also shown at the end. After 6 days of culturing, cells counting per well in 24-well plate grew from 1×10^6 to $(1\sim 2) \times 10^7$. The *MDR 1* gene transfection rate was 30%.

The *MDR 1* gene was imported into cord blood hematopoietic cells after reinfusion to make colonies; also to exert its function after its reinfusion and colonization. The key is how to improve *MDR 1* gene transfection efficiency *in vitro* and colonization rate of transfected bone marrow hematopoietic cell. This makes a search for choosing *MDR 1* gene transfect cord blood nuclear cells, discussing feasibility, efficiency and stabilized condition of transfection *MDR 1* gene, while it is the base to further the establishment of *MDR 1* gene transfected

autologous cell transplantation model and chemotherapy experimental *in vivo*.

High efficiency and stable transfection system *in vitro* are the prerequisites for the successful application *in vivo*. There are three key factors in transfection system, namely, the carrier, transfection conditions (mainly refers to a combination of cytokines), and the target cells. *MDR 1* gene is not only the resistance gene of tumor cells, but also a protection gene for normal cells from foreign body violation [2]. But in the bone marrow, P-gp expression is lower than tumor tissue, making it prone to myelosuppression in chemotherapy. To increase protection of hematopoietic cells in chemotherapy, we chose human *MDR* genes to transfect into human umbilical cord blood hematopoietic cells. Research has shown that the addition of IL-3, IL-6 and SCF is the best for improving transfection efficiency. Our study chose the combination of IL-3, IL-6, SCF, EPO, GM-CSF to induce growth of umbilical cord blood nuclear cells from hematopoietic stem/progenitor cells which are rich of $CD34^+$ *in vitro*. The purpose is to facilitate the integration of the target gene while prompting stem/progenitor cells amplification *in vitro* [3].

Conclusion

The recombinant adenovirus is used to transfer exogenous *MDR 1* gene into umbilical cord blood cells by concentrated virus supernatant transfection method, and P-gp is expressed.

Acknowledgements

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