·Basic Research·

The inhibitory effect of triamcinolone acetonide on the proliferation of monkey choroid –retinal endothelial cells in hypoxia condition

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Abstract

• AIM: To observe the inhibitory effect of triamcinolone acetonide (TA) on the proliferation of monkey choroid- retinal endothelial cells (RF/6A) in hypoxia or normal conditions.

• METHODS: TA was added into the cultured RF/6A of rhesus monkey. The effect of TA on the cellular activity was observed by MTT, the effect on cellular proliferation and apoptosis was detected by flow cytometry (FCM).

• RESULTS: The numbers of cells in s-phase of a cell cycle were reduced and the proportion of cells in G2-M phase was increased under the hypoxia condition. TA had a great effect on the cell cycle of RF/6A of rhesus monkey and it induced apoptosis of endothelial cells. It relatively increased the s-phase cells and reduced G2-M phase cells under both normal and hypoxia conditions, which indicates its role in blocking cell cycle from s-phase to G2-M phase and reducing mitosis.

• CONCLUSION: Hypoxia promotes the proliferation of RF/6A cells while TA has the opposite effect in both normal and hypoxia conditions. TA can also induce apoptosis of endothelial cells.

• KEYWORDS: triamcinolone acetonide; cell cycle; hypoxia; apoptosis

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INTRODUCTION

Recently, a kind of steroid, triamcinolone acetonide(TA) is used intravitreally in inhibiting the progression of proliferative vitreoretinopathy (PVR) and the angiogenesis of retina, choroid and iris. The clinical therapy is observed to be pretty good and the toxicity or side effect to retina is not obvious. But, the mechanism of inhibiting angiogenesis is not clear. This study is to observe the effect of TA on choroid-retinal endothelial cells (RF/6A)(*in vitro* cultured) under normal condition and in hypoxia by using MTT and flow cytometry. This study also reveals the total course of TA in the eye to explore the clinical therapeutic effect of TA.

MATERIALS AND METHODS

Materials Main: MTT solution, MSO, RNA enzymase. Equipments: flow cytometry (BD corporation, U.S.A),CO_2 incubator, enzyme-mark instrument，ultra-clean workbench, inverted phase contrast microscope(OlympusIX70, Japan).

Methods

Cell culture The choroid-retinal EC line RF/6A was obtained from cell bank of Chinese Acadamy of Science (CAS) and cultured according to the direction and guidance.

Drug concentration and groups ① normal control group② normal low concentration group ③ normal median concentration group ④ normal high concentration group ⑤ hypoxia control group ⑥ hypoxia low concentration group ⑦ hypoxia median concentration group ⑧ hypoxia high concentration group. In normal control group, the RF/6A cells were cultured under normal condition, without drugs. In hypoxia control group, the RF/6A cells were cultured under hypoxic condition and without drugs. In normal or hypoxia low/median/high concentration group, the concentration of TA was 0.05,0.1,1g/L.

Evaluation of effect of TA to RF/6A cell by MTT colorimetric method The cells were seeded onto 96-well tissue culture plates with the density of 7×10^3 per well, 200μL 1 640 culture medium which contained 100mL/L fetal bovine serum was added into each well. The

concentration of TA was diluted to 5, 2.5, 1, 0.25, 0.025g/L. Every 8 parallel wells was a group. After 24 hours, the drugs were removed and the seeded cells were washed gently with phosphate-buffered saline (PBS) for 3 times. Adding 200μL 1 640 culture medium that contained 100mL/L fetal bovine serum into each well again, after 24 hours culture, the upper fluid was removed. Adding 110μL MTT solution into each well, the culture plates were put into the incubator for 4 hours. Then, 80μL MTT solution was removed and 100μL DMSO was added, shaking for 10 minutes until the crystal were totally dissolved. The quantity was determined colorimetrically by using a spectrophotometer at λ = 570nm. The results were recorded and the survival rate of cell was calculated (%)= A of each well / mean A of normal groups×100%).

Hypoxia model According to the method introduced by kuwabara[1], we also made some improvement. Get a dry pot and remove the glass stopper by using a rubber stopper with two holes (one for gas inlet, the other for gas outlet). The 6-well tissue culture plates were put in the pot and obturated by Vaseline. The pot was perfused with 950mL/L N_2; 50mL/L CO_2 through the inlet at the velocity of 2-3L/min at the temperature of 37℃ .The concentration of oxygen is below 10mL/L by using the oxygen analyzer (made by Lida equipment factory, Jiande, Zhejiang, China). The inlet and outlet was closed by using hemostat and the pot was cultured in the 37℃ incubator for 4 hours.

Evaluation of interference of TA to RF/6A on cell cycle and apoptosis RF/6A cells were seeded at a density of 5.0×10^8 cells/L onto 6 well tissue-culture plates and two 50mL culture flasks for 24 hours by using the 1 640 culture medium which contains 100mL/L fetal bovine serum. For each of the two 6-well plants, every two wells were add with TA of 1,0.1, 0.05g/L separately. After 24 hours, put a set of 6-well plant with culture flask into hypoxia equipment for 4 hours. The drugs were removed and the plates were washed by PBS, routine digestion centrifugation and fixed by 700mL/L ethanol through the night. Then, the plates underwent centrifugation again and washed by PBS for 3 times. The upper fluid was removed and 100μL RNAase was added at 4℃ for 10 minutes , and stained by 300μL PI. The plates were put into the refrigerator at 4℃ for 30 minutes, filtered by 300 nylon filters. 10 000 cells were counted every sample, and DNA dosage of each cell cycle was detected by flow cytometer to calculate the ratio of apoptosis cells and analyse cell cycle.

Table 1 Effect of TA to RF/6A cell

Concentration (g/L)	*A* value	Survival rate of cell(%)	Ratio of inhibited cell(%)
Control group	0.87±0.08		
0.025	0.44±0.06	50.36%	49.64%
0.25	0.35±0.01	40.00%	60.00%
1	0.32±0.01	36.21%	63.79%
2.5	0.21±0.02	23.71%	76.29%
5	0.15±0.01	16.95%	83.05%

Table 2 Interference of TA to cell cycle of RF/6A (%)

Group	G0-G1	S	G2-M
Normal control	70.85±0.31	17.63±0.15	11.85±0.68
Hypoxia control	57.50±0.33	4.06±0.09	38.44±0.24
Hypoxia low concentration	68.13±0.02	17.42±0.20	14.46±0.22
Hypoxia median concentration	69.17±0.05	14.58±0.03	16.24±0.04
Hypoxia high concentration	67.31±0.04	15.74±0.04	16.95±0.02
Normal low concentration	65.10±0.02	26.89±0.02	8.01±0.03
Normal median concentration	64.28±0.03	27.73±0.01	7.99±0.03
Normal high concentration	58.04±0.01	40.42±0.02	1.53±0.03

Statistical Analysis Analysis followed by SPSS for Windows13.0. Results were expressed as the mean ±SD. Data was analyzed by one-way ANOVA. $P<0.05$ was considered significant.

RESULTS

Interference of TA to RF/6A Cell by MTT The inhibiting effect of TA to RF/6A was parallel to the concentration of TA. The difference between experimental group and control group was obvious.($P<0.01$) see Table 1.

Interference of TA to Cell cycle of RF/6A The ratio of cells in each stage of cell cycle marked changed after adding TA for 24 hours. The ratio of S stage cell increased in normal low, median, high group while that of G2-M stage decreased when compared with normal control group, the differences were obvious ($P<0.01$), meanwhile the effect enhanced when the concentration increased(Table 2). These indicated TA may block RF/6A cells from S stage entering G2-M stage. Compared with normal control group, the ratio of S stage cell sharply decreased in hypoxia control group, while that of G2-M cells largely increased. Obvious difference existed ($P<0.01$). As for the hypoxia low, median, high group, the ratio of S stage cells also increased compared with hypoxia control group and in contrast, that of G2-M stage declined.The difference was also obvious($P<0.01$). All the results above indicated hypoxia stimulated proliferation of retinal endothelials while TA inhibited the proliferation no matter under normal condition or in hypoxia.

TA Induced Apoptosis of RF/6A Measured by Flow Cytometer There was an obvious "hypodiploid peak" of

Table 3 The induction of apoptosis by TA to RF/6A
(measured by Flow cytometer,%)

Group	Apoptosis
Normal control	0
Hypoxia control	2.6±0.03
Hypoxia low concentration	0.06±0.01
Hypoxia median concentration	1.01±0.01
Hypoxia high concentration	2.38±0.01
Normal low concentration	0.85±0.01
Normal median concentration	4.33±0.02
Normal high concentration	8.70±0.09

DNA prior to G stage of cells in hypoxia and treated by TA when measured by flow cytometer, this was the symbol of cells go apoptosis so it was also termed "apoptosis peak". As the concentration of drug went higher, the area of the peak became larger ,in another words, the destruction of DNA enhanced. From Table 3, it was found that the ratio of cells go apoptosis was positively parallel to the concentration of TA when the cells were cultured with TA for more than 24 hours. There was obvious difference between the experimental group and control group (P <0.01). All these indicated that TA induced apoptosis of RF/6A.

DISCUSSION

Retinal angiogenesis is the key point of kinds of fundus disease, the related factors are ischemia and hypoxia. Meininger *et al* [2] found that the endothelials of aorta and coronary artery may increase by 74%-202%. Smith[3]found that hypoxia might increase the activity of endothelials in the aorta of Rabbits.

There are many complex physical models of hypoxia, the equipments acquired are harsh. In this experiment, we triumphantly set up an economical and convenient model of hypoxia.

In this experiment, the ratio of G2-M RF/6A cells increased in hypoxia group while that of S stage decreased, which shows hypoxia stimulates the proliferation of RF/6A cell.

Nowadays, the treatments for angiogenesis-retinopathy include retinal laser photocoagulation and operations of vitreous body at late stage, but the outcome is not so ideal and with lots of complications. Thus , the drug of anti-angiogenesis becomes the trend. Recently, Wang *et al* [4] found that TA can inhibit the proliferation of choroidal endothelials; there are also some reports about the applications of TA on exudative age related macular degeneration, progression of diabetic retinopathy and retinal vein occlusion [5-8], but rarely about the direct inhibiting of proliferation of choroid-retinal endothelial cells.

We focused on the inhibiting of TA to the proliferation of cultured choroid-retinal endothelial cells of Rhesus monkey under normal and hypoxia conditions. Endothelial origin of these cells has been corroborated by morphology, growth patterns, and the presence of factor VIII-related antigen (von Willebrand factor). As we know, EC lines are evolutionarily close to those derived from humans and therefore are an attractive model for studies targeting neovascularization. Thus ,the result of this experiment provides more information for clinical treatment.

The method we used in this experiment is MTT method, which is a simple method with high sensitivity to detect the mitochondrion dehydrogenase of living cells. It reveals the activity of cells and indirectly, the proliferation of cells. The result of this experiment indicates the inhibiting of TA to the proliferation of cultured RF/6A cells, and this effect is enhanced with the increasing dosage of TA. As the concentration goes up to 2.5g/L and 5g/L, the toxicity gradually appearaces. Similar to the result of Yeung *et al*[9] and Spandau *et al* [10].

The most efficient dosage and safety dosage should be mentioned. Normally, The vitreous volume is about 4mL in humans, the equivalent vitreous concentrations used in various studies therefore correspond to 0.2g/L to 6.25g/L of vitreous [9]. Clinically, the most common dosage of TA is 4mg, which equivalent 1g/L in vitreous cavity. As the Rf/6A cell line is from Rhesus monky, As such, the concentration chosen in the present study was to be within 0.05g/L to 1g/L in detection by flow cytometer. Clinical experience indicates that efficiency and duration time depend on concentration, this is consistent to our result, which indicates high concentration of TA plays stronger effect on inhibiting the proliferation of choroid-retinal endothelial cells. What's more, our experiment also reveals the toxicity of ultrahigh dosage.

TA has significant effects on the endothelial cell cycle and induces apoptosis, which was confirmed in our experiment by flow cytometer. It can increase the cells of S stage and reduce that of G2-M stage, which means TA blocks endothelial cells at S stage from entering G2-M stage, thus, down-regulates the capacity of mitosis.

In conclusion, our study confirms hypoxia induces the

proliferation of choroid-retinal endothelial cells; TA inhibits the proliferation of choroid-retinal endothelial cells under normal condition and hypoxia condition. Meanwhile, it induces apoptosis. Its efficiency is enhanced as the dosage increases. It is conferred that TA inhibits the proliferation of RF/6A by impacting on the cell cycle, inhibiting the energy metabolism and inducing apoptosis.

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