

Research for the influence of telomerase inhibitors on myeloma cell and therapy

Zhao Zhihua¹, Sun Miaomiao², Yang Jianping¹ and Chen Kuisheng^{1*}

¹Department of Pathology, the First Affiliated Hospital, Zhengzhou University, Zhengzhou, China

²Department of Pathology, Henan Tumor Hospital, Zhengzhou, China

Abstract: This paper aims to study the effect of telomerase inhibitors zidovudine (AZT) on cell morphology, survival rate and telomerase activity of in-vitro culture myeloma cell line (U266). It has provided experiment basis for applying telomerase inhibitors in multiple myeloma treatment. The myeloma cell line in logarithmic phase (U266) could be divided into AZT group and negative control group, which are added with 100 μL AZT respectively and diluted into different concentrations with culture solution (1,10,100 and 1000 $\mu\text{mol}\cdot\text{L}^{-1}$) of AZT, as well as culture solution of the same volume. After cultivating for 24, 48, 72 h, morphological changes of U266 cell is observed under optical microscope. Survival rate of cell is detected with MTT method, the change of telomerase activity is detected with TRAP-PCR-ELISA method. There is significant change in U266 cell morphology after AZT effect, their volume has been smaller and shrunken. Its shape has changed from short spindle to polygon or irregular shape, and broken away from the neighboring cells. Through comparing with control group, the survival rate of U266 cell in AZT group has decreased significantly ($P<0.05$), it represents time and concentration dependence. Median lethal concentration of AZT on U266 cell (IC_{50}) is 1000 $\mu\text{mol}\cdot\text{L}^{-1}$. The drop in telomerase activity has decreased by 55.74% ($P<0.05$) through applying 1000 $\mu\text{mol}\cdot\text{L}^{-1}$ AZT on U266 cell for 72 h. AZT has significant inhibiting effect on the proliferation of myeloma cell line, and could reduce the telomerase activity of myeloma cell.

Keywords: Telomerase inhibitors, Multiple myeloma, Cell proliferation.

INTRODUCTION

Telomerase is a special kind of RT (reverse transcriptase), and could compound telomere DNA sequence through taking its own RNA as template. Its structure and behavior abnormalities are thought to have close relationship with cell aging and cancer development. There are some current researches indicating that (Guangbin and Kailai, 2000): 85%~95% malignant tumors have telomerase activity expression, while most of the somatic cell has no telomerase activity. Thus telomerase will possibly become the new targets for treating tumor. There are scholars putting forward the tumor gene therapy, which takes the inhibition of telomerase activity as the objective (Jin *et al.*, 2011). The inhibitor, which could inhibit telomerase activity and genetic expression is hoped to be developed, thus the purpose of curing cancer would be achieved. Osteogenic sarcoma is the malignant tumor originating from mesenchymal tissue, which has the highest morbidity in primary bone tumors and accounts about 35% (Guangyin, 2009). It is easy to attack among teenagers, such kind of bone tumors have the highest fatality and lethality rate among teenagers. Zidovudine (AZT) is a kind of nucleoside analogues, which has the effect of fighting AIDS, as well as acting as a kind of telomerase inhibitors. There are domestic and foreign literature (Che *et al.*, 2011, Wen *et al.*, 2000) reporting that: AZT is able to

inhibit the telomerase activity of liver cancer cells, human brain glioma cells, lung and colon cancer cells, thus the proliferation of tumor cells could be inhibited, and irreversible shortening would occur in the telomere of HeLa cell, while there are rare reports about applying AZT in osteogenic sarcoma. This experiment has applied AZT into osteosarcoma cell lines (HOS), by which to observe its effect on tumor cell growth and the activity of telomerase. Its aim is to provide experiment basis for better applying telomerase inhibitors into clinic treatment of osteosarcoma and other tumors.

Zidovudine (AZT) is a kind of nucleoside analog and the first anti-AIDs drug approved by Food and Drug Administration (De Clercq E, 2009). ATZ inhibits the formation of virus double-stranded DNA by obstructing the reverse transcription of virus RNA gene to make virus lost replication template and thus lower replication rate (Kumar *et al.*, 2013). Therefore, it can be regarded as one telomerase inhibitor. Literatures (Che *et al.*, 2011; Wen *et al.*, 2000) at home and abroad reported that AZT can inhibit the telomerase activity of hepatoma carcinoma cells, human brain glioma cells, lung cancer cells and colon cancer cells, and thus inhibit the proliferation of cancer cells and make telomere of HeLa cells appear uninverted shrink. However, reports on the application of ATZ in osteosarcoma is few. This experiment applied ATZ to act on osteosarcoma cell strain, observed its function on the growth of tumor cells and telomerase activity. It aimed to provide experimental basis for the

*Corresponding author: e-mail: zzhsmmyjp@163.com

better application of telomerase inhibitor in osteosarcoma and other cancer.

MATERIALS AND METHODS

Cells, Reagents and main instrument

Multiple myeloma cell lines (U266) are purchased from Dingguo Biont Company in Beijing; MEM culture medium is purchased from Gibco Company; fetal bovine serum is purchased from Hyclone Company; AZT is the products of Sigma Company; methylthiazolyldiphenyl tetrazolium bromide (MTT) and BCA protein kits are the products of Beyotime Company; TRAP-PCR-ELISA kit is the product of Boehringer Mannheim GmbH.

Cell culture and grouping

U266 cell is cultivated in MEM culture medium containing 10% fetal calf serum, and placed in cell culture box with 5% CO₂ at 37° for culture. The U266 cell in logarithmic phase is divided into AZT group and negative control group, which are respectively added with 100 μL, then they are diluted to different concentrations with nutrient solution (1, 10, 100 and 1000 μmol·L⁻¹ AZT, as well as nutrient solution in the same volume, various indicators are observed after cultivating for 24, 48 and 72 h.

Morphological observation of cell

In MTT experiment, the change of cell morphology structure in each group of cultivation hole is observed with inverted difference microscope before and after dosing.

MTT colorimetric analysis method

Cells in logarithmic growth phase (preparation of cell suspension is 1x10⁶ mL⁻¹) is taken to inoculate on 96-well plates, each hole is 100 μL. The experimental group and control group are set up, 100 μL AZT of different concentration (1, 10, 100 and 1000 μmol·L⁻¹ diluted with culture solution and nutrient solution of the same volume are added with 100 μL AZT next day, each concentration is set up in triplicate, 20 μL MTT (5g·L⁻¹) is added into it after cultivating for 24, 48 and 72 h respectively, then 4 h culture is continued, nutrient solution is absorbed and discarded. Each hole is added with 150 μL dimethyl sulfoxide (DMSO), and then cultivation came to an end, room temperature oscillation is implemented on it about 10 min to sufficiently dissolve blue violet crystal. Mode-1 450 micro-plate reader (Bio-Bad) is applied to measure the absorbency (A) value at 490 nm wavelength. The cell survival rate (%) = (A value in experimental group/A value in control group) x100%, cell inhibition rate (%) = 1-cell survival rate. As inhibition rate reaches 50%, it is the best action time and concentration, which would be taken as the drug concentration and action time of measuring changed telomerase activity at the next step.

Detecting telomerase activity with TRAP-PCR-ELISA method

The operation is performed according to kit instructions, specific steps are as follows: cell precipitation is collected and suspended on cold lotion, and then it is of ice-bath for 5 min, 1000 μmol·L⁻¹ centrifugal at 4° is implemented for 5 min, supernatant is discarded. The sediment will be of ice bath with cold pyrolysis buffer for 30 min, during which vortex oscillation is performed for 3~5 times, 13000r·min⁻¹ centrifugal is performed for 30 min at 4°, the supernatant is the required extracting solution, liquid supernatant is sucked up and placed in EP tube processed with DEPC, then they are packed in 20 μL/tube respectively. BCA method is adopted to measure one piece of protein concentration, the remaining is preserved for standby application at -70°. TRAP-PCR reaction: 25 μL reaction mixture and 3 μL cell lysates product are added into 0.2 ml amplification tube in turn, finally aquae sterilisata is added into it until its volume is 50 μL. PCR procedure: primer extension is performed for 30 min at 25°; telomerase inactivation is performed for 5 min at 94°; then they are looped for successive 36 cycles among 94° in 30 s, 50° in 30 s and 72° in 90 s. Further extension is performed for 10 min at 72°. PCR products are preserved at 4°. ELISA reaction: after the reaction of PCR product, denaturant and hybridization buffer, 100 μL solution is extracted to be added into the reaction hole with affinity element package, then it is of oscillation and incubation for 2 h at 37°. After washing it, anti digoxigenin-peroxidase is added into each hole, then it is of oscillation at room temperature for 30 min. After washing it, TMB and termination liquid are added into it successively, its A value at 450 nm is read from the enzyme mark instrument. That inactivated U266 cell extraction is heated through water bath for 10 min at 65° is taken as negative control, positive control is provided by the inside kit. Lowering rate of telomerase activity (%) = (average A value in experimental group-average A value in control group) / average A value in control group x 100%.

STATISTICAL ANALYSIS

SPSS 1310 statistical software is adopted for performing statistical analysis, the cell survival rate is denoted with $\bar{X} \pm s$, comparison among multiple sets is performed through adopting variance analysis; comparison between the two groups is implemented through applying SNK-q inspection.

RESULTS

Cell morphology change

Adherent cell growth in control group is observed under inverted microscope, it represents short spindle, the cells are arranged closely, its nucleus is larger, it represents

multilayer growth when the cells are intensive, and cell proliferation is in good condition. After applying $1\mu\text{mol}\cdot\text{L}^{-1}$ AZT into U266 cell for 48h, cell's volume become smaller, intercellular space will be widened, and we could see that a few cells fall off from bottle wall and suspend on nutrient solution, and a few cellular debris are also visible. Above change would become more significant with the increase of AZT concentration and the extension of time. After the interaction of U266 cell and $100\mu\text{mol}\cdot\text{L}^{-1}$ AZT for 24h, cell volume would become smaller; its form has changed from short spindle into polygonal or irregular shape. Cellular morphology would be of the most significant change after 48h, cell volume would shrink and break away from neighboring cells and the size of various oncocyte are not the same, most of them represent monolayer growth. The number of cell would be reduced further after 72h, the cell would get round and shrink, most of the cells would float, as it is shown in fig. 1.

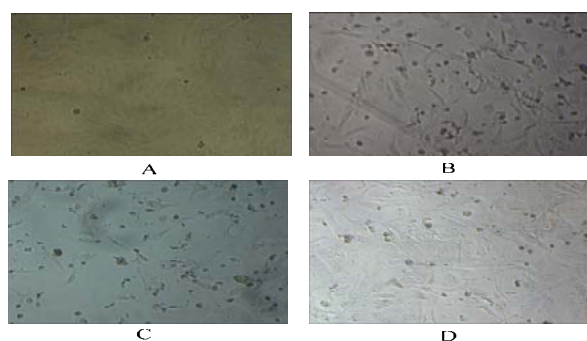


Fig. 1: Morphological change of U266 cell after effecting with $100\mu\text{mol}\cdot\text{L}^{-1}$ AZT $100\mu\text{mol}\cdot\text{L}^{-1}$ AZT.

Note: A: control group; B-D: AZT effect for 24,48,72h

The change of U266 cell' S survival rate after AZT effect

After exerting AZT of different concentration on U266 cell for respective 24, 48 and 72 h, cell growth has been significantly inhibited. After effecting 1, 10, 100 and $1000\mu\text{mol}\cdot\text{L}^{-1}$ AZT in processing U266 cell for 24, 48 and 72 h respectively, cell survival rate is shown as table 1. Proliferation rate in control group is $(100.000\pm 0.000\%)$, comparison for different time between concentration group and control group is made for representation: AZT could significantly inhibit the proliferation of U266 cell, and the inhibition effect would be reinforced with the increase of concentration and the extension of time ($P<0.05$), it represents time and the concentration dependence. The median lethal concentration (IC_{50}) of AZT on U266 cell is $100\mu\text{mol}\cdot\text{L}^{-1}$.

The change of telomerase activity after AZT effect

A value in control group is 1.236 ± 0.22 . A value in AZT group is 0.547 ± 0.07 . A value in AZT group has significantly decreased when compared with control group ($P<0.05$), telomerase activity of U266 cell after

AZT effect has dropped by 55.74%, it indicates that AZT has significant inhibition effect on telomerase activity of U266 cell.

DISCUSSION

Telomerase is a kind of ribosome protein inside the cell, it is mainly composed of RNA ingredient (hTR), telomerase related proteins (hTEP) and catalytic subunit (hTERT) (Nakamura *et al.*, 1997). It is able to take its own RNA as template, telomere DNA is compounded at the end of chromosomes, thus telomere could be extended for making up for the shortening of chromosomes terminal during cell division and solving 'end replication problem', and then cells would be immortalized through stabilizing telomere length. Thus inhibiting telomerase activity has important effect in cancer treatment. There are researches reporting that (Jun *et al.*, 2012): the expression of telomerase activity could be detected in 84% myeloma and 60% soft myeloma, while it could not be detected in normal tissue beside tumor. This indicates that telomerase activity is a kind of signal for malignant tumor cells, it also represents that inhibiting telomerase activity maybe becomes a new target in curing myeloma. As telomerase inhibitors, AZT could compete with normal mononucleotide and combine with RNA template, inhibit the activity of RT (reverse transcriptase) inside the cell. They could also be mixed during DNA duplication until the process is stopped (Jinhai and Guohui, 2011). There are researches finding that (Lijun and Hongxue, 2010): AZT could inhibit the telomerase activity of malignant tumor, shorten the telomeres, thus the proliferation and growth of tumor cells would be inhibited.

CONCLUSION

The lowering rate of telomerase activity in this research is 55.74%, it is consistent with telomerase activity's lowering rate of AZT on liver cancer cell, lung cancer, colorectal cancer cells of culture in vitro (Xialiu *et al.*, 2004). Cell proliferation is inhibited after $1\mu\text{mol}\cdot\text{L}^{-1}$ AZT's effect on U266 cell for 24 h. The dose is far lower than the application dose of cells in tongue cancer, it indicates that myeloma cell is more sensitive to AZT. The inhibition effect would be strengthened with the extension of time and the increase of concentration. The inhibition ratio is up to 50 % when $100\mu\text{mol}\cdot\text{L}^{-1}$ AZT has been acted on U266 cell for 72 h. When $1000\mu\text{mol}\cdot\text{L}^{-1}$ AZT has been acted on U266 cell for 72 h, inhibition rate would reach the highest (90.92%). There is literature reporting that (Rihua *et al.*, 2013): AZT could block glioma cells within G1phase, interfere with the reverse transcription process, thus the growth of glioma cells is inhibited. The specific mechanism of AZT's proliferation inhibition effect on U266 cell is waiting for further research. The results in this experiment indicate that (1) zidovudine (AZT) has growth inhibition effect on the

Table 1: The survival rates of U266 cell lines after being treated with AZT (n=3, x±s, /%)

Group (t/h)	Survival rate		
	24	48	72
Control	100.000±0.000	100.000±0.000	100.000±0.000
1μmol·L ⁻¹ AZT	93.404±0.734	88.618±3.013	63.483±0.581
10μmol·L ⁻¹ AZT	89.593±1.818	60.781±8.218	51.184±4.179
100μmol·L ⁻¹ AZT	87.699±2.845	56.832±3.947	47.630±3.808
1000μmol·L ⁻¹ AZT	84.244±2.473	26.861±1.974	9.085±2.010

*P<0.05,**P<0.01 compared with control group.

human osteosarcoma cell line U266 in vitro culture, and the inhibition effect represents time and concentration dependence. Median lethal concentration of AZT on Cell lines U266 (IC50) is 100μmol L⁻¹. (2) zidovudine (AZT) could reduce the telomerase activity of human osteosarcoma cells, the lowering rate is 55.74%. Thus AZT has significant inhibition effect on U266 cell growth, it represents time and concentration dependence, AZT has the function of resisting myeloma, it is expected to become a new drug for curing myeloma.

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