The Effect of Overexpression of hTERT on Etoposide (VP-16)-Induced Apoptosis in Raji Cells

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OBJECTIVE To explore whether overexpression of human telomerase reverse transcriptase (hTERT) in Raji cells can protect against etoposide (VP-16)-induced apoptosis.

METHODS A lipofectin-mediated gene transfection method was used to transfer the hTERT gene into Raji cells. The polymerase chain reaction enzyme-linked immunoassay was employed to determine telomerase activity. The expression levels of hTERT protein were assayed by immunofluorescence using a fluoresce isothiocyanate label. Cell viability was determined using the trypan blue dye exclusion assay. Apoptosis was assessed by morphological observation and flow cytometry analysis.

RESULTS The results showed that there was a marked increase in both mean fluorescence intensity of hTERT-protein-positive cells and telomerase activity in hTERT- transfected Raji cells ($P<0.05$), but there was no difference in hTERT protein and telomerase activity levels between Raji cells and vector-transfected Raji cells ($P>0.05$). There were more viable cells at 48 h and 72 h after treatment of hTERT-transfected Raji cells with 10 μmol/L VP-16 compared to either vector-transfected Raji cells and Raji cells ($P<0.05$). Apoptosis rates at 72 h after treatment with 10 μmol/L VP-16 were 4.34 ±1.03% in hTERT-transfected Raji cells, 33.21± 3.12% in vector-transfected Raji cells, and 31.63 ± 3.06% in Raji cells. There was a significant difference in the percentage of apoptotic cells between hTERT-transfected Raji cells and either vector-transfected Raji cells or Raji cells ($P<0.05$).

CONCLUSION Overexpression of telomerase by transfection of hTERT gene can protect against etoposide-induced apoptosis in Raji cells.

KEYWORDS: telomerase, Htert, transfection, Raji cells, etoposide, apoptosis.

Human telomerase reverse transcriptase (hTERT),which is a limiting component for telomerase activity,has significant correlation with telomerase activity. [1,2] Telomerase is specifically activated in most malignant tumors but is usually inactive in normal somatic cells. The activation of telomerase results in the addition of telomeric DNA onto the ends of chromosomes ends. Many human cancers have high levels of telomerase activity, whereas most normal somatic cells have little or no activity. In tumor cells, inhibition of telomerase usually leads to telomere shortening and apoptosis while maintenance of telomerase activity is associated with an increased resistance to apoptosis. [3-6] However, there have been no reports

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regarding the effect of forced overexpression of telomerase on apoptosis in Raji cells. To study this effect, we transfected Raji cells with the hTERT gene to obtain a Raji cell line overexpressing telomerase. In this report, we explored whether overexpression of hTERT in Raji cells can protect against etoposide (VP-16)-induced apoptosis. An answer to this question will be helpful for telomerase-targeted therapy.

MATERIALS AND METHODS

Reagents and cell culture

Etoposide was purchased from Sigma. Polyclonal antibodies against hTERT were purchased from Santa Cruz, and RPMI-1640 and newborn calf serum were obtained from Gibco BRL. Telomerase-PCR-ELISA was purchased from Boehringer-Mannheim (Mannheim, Germany). The Raji cell line was cultured in 5% CO_2 at 37℃ in RPMI-1640 supplemented with 10% newborn calf serum and 100 units/ml of penicillin plus 100 μg/ml of streptomycin. Sub-culture was performed 3~4 days later and the cells in a logarithm growth phrase were used in all experiments.

Transfection of the hTERT gene

The plasmid (pcDNA3.1-hTERT) containing the full-length cDNA of hTERT was a kind gift from Dr. Jun-ichi Nakayama (Department of Life science, Tokyo Institute of Technology, Japan). The pcDNA3.1 vector was a kind gift from Dr.Sun Fenyong (Bio-Engineering Institute of Jinan University). The plasmid was amplified as described previously. [7] Aliquots (2 μg) of the expression vector or empty vector were transfected by Lipofectamine, and transfected cells were screened in 800 mg/ml G418 (Gibco) screening medium. Finally a single colony was isolated from the semisolid medium. The clonal cells were then maintained in RPMI-1640 medium, and the expression telomerase was examined periodically.

Examination of cell morphology

Apoptosis was evaluated by cellular morphology using Giemsa stain. The cells were harvested after treatment, and fixed with methanol after which the morphology of the cells was examined under a light microscope.

Determination of hTERT protein by flow cytometry

Cells (5×10^5) were collected and fixed with 70% formaldehyde at 4℃ for 15 min. Then the cell suspension was washed and resuspended two times in a specific PBS (s-PBS) (containing 1% human AB serum, 1% Tween-20, pH7.2), which causes perforation of the surface of the cells so as to permit easy access of the antibodies into the cells. The cells were incubated with polyclonal antibodies against hTERT for 1 h at 4℃ in the dark. After washing the cells twice with s-PBS, they were incubated with a fluoresce isothiocyanate-conjugated secondary antibody (Sigma Co. USA) for 30 min at 4℃. The cells were washed twice with PBS, examined by flow cytometry and the mean fluorescence intensity for the hTERT protein was determined.

Telomerase activity assays

Telomerase activity was measured using a telomerase PCR ELISA assay kit. Briefly, a Raji cell extract was prepared at different times of treatment. Positive and negative control groups were established for each experiment; the cell extract was heated at 65℃ for 10 min as a negative control, and an extract of the 293 cell line having telomerase activity used as a positive control (from the kit). Telomeric repeat amplification protocol (TRAP) reaction: (1) primer elongation: transfer 25 μl of the reaction mixture into a tube suitable for PCR amplification , then add 2 μl of the cell extract and sterile water to a final volume of 50 μl; transfer the tubes to thermal cycler and perform one cycle at 25℃ for 30 min; (2) telomerase inactivation: perform one cycle at 94℃ for 5 min; (3) amplification: 50 μl reaction mixture contains dNTP, *Taq* polymerase, the biotin-labeled P1-TS primer and P2 primer. Perform 30 cycles, at 94℃ for 30 s for denaturation, at 50℃ for 30 s for annealing, at 72℃ for 90 s for polymerization, then at 72℃ for 10 min for the balance of synthesis. Hybridization and the ELISA reaction were carried out according to the manufacturer's instructions. We determined A (Absorbance) value at a wavelength of 450-655 nm,

based on the formula to calculate A=A450-A655.

Assay of the cell cycle

The cells were collected, washed two times, and then fixed with 70% cold alcohol at 4℃ for more than 24 h. Then the cells were centrifuged at 800 g for 15 min, and the supernatant was discarded to completely remove the ethanol. The pellets were resuspended in 0.5 ml PBS, treated with RNaseA for 30 min, and stained with a propidium iodide (PI) solution (Sigma, USA) for 30 min. At last the cell cycles were assayed by flow cytometry. The G_1-S phase and G_2-M phases were analyzed by Multicycle software (Phoenix Flow Systems, San Diego, CA).

Statistical analysis

Data were expressed as means ± standard deviations ($\bar{x} \pm s$). The significance was assessed using a one-way analysis of variance (ANOVA), using a significance level of $P<0.05$. All data represent at least three independent experiments performed in triplicate.

RESULTS

Effects of hTERT transfection on expression of the hTERT protein

The immunofluorescence assay by flow cytometry showed that hTERT-transfected Raji cells expressed high levels of hTERT protein. The mean fluorescence intensity and percentage of positive cells were 280.36 ± 18.91 and 99.85 ± 5.26, respectively (repeated three times) in hTERT-transfected Raji cells, whereas these values were 168.17 ± 10.12 and 95.62 ± 4.86, respectively in vector -transfected Raji cells, and were 172.68 ± 10.83 and 96.39 ± 5.17, respectively in parental Raji cells. There were no significant differences in the percentage of positive cells among hTERT-transfected Raji cells, vector-transfected Raji cells and parental Raji cells. However the mean fluorescence intensity of hTERT protein was significantly increased in hTERT-transfected Raji cells compared to either vector-transfected Raji cells or parental Raji cells.

Effects of hTERT expression on telomerase activity

Telomerase activities were 2.26 ± 0.27 A, 1.39 ± 0.15 A and 1.32 ± 0.12 A in hTERT-transfected Raji cells, vector -transfected Raji cells and parental Raji cells, respectively. Telomerase activity was significantly increased in hTERT-transfected Raji cells compared to either vector -transfected Raji cells or parental Raji cells. We found no significant differences in telomerase activity between the vector -transfected Raji cells and parental Raji cells.

Effect of VP-16 on growth of hTERT-transfected Raji cells

Cell viability was assayed by trypan blue exclusion. The proliferation of all cells was suppressed by 10 μmol/L VP-16 at 48 and 72 h. However, the number of viable cells was significantly increased in hTERT-transfected Raji cells compared to either vector-transfected Raji cells or parental Raji cells, $P<0.05$ (Fig.1). There was no significant difference between the vector-transfected Raji cells and parental Raji cells ($P>0.05$).

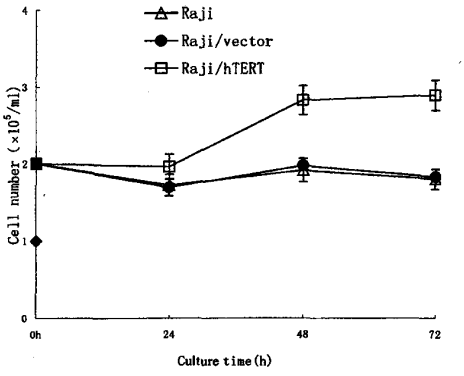

Fig.1. Effect of 10μmol/L VP-16 on cellular growth in hTERT-transfected Raji cells.

Effect of VP-16 on apoptosis of hTERT-transfected Raji cells

Morphological characteristics of apoptosis, such as cell shrinkage, chromatin condensation, nuclear segmentation and formation of apoptotic bodies, were observed at 48 and 72 h (Fig.2). The percentages of apoptotic cells at 72 h were 4.34 ± 1.03%, 33.21 ± 3.12% and 31.63 ± 3.06% in hTERT-transfected Raji

cells, vector-transfected Raji cells and parental Raji cells, respectively. Transfection of hTERT therefore significantly inhibited apoptosis induced by VP-16 at 48 and 72 h, $P<0.05$ (Fig.3). However, there was no significant difference in apoptosis between vector-transfected Raji cells and parental Raji cells ($P>0.05$).

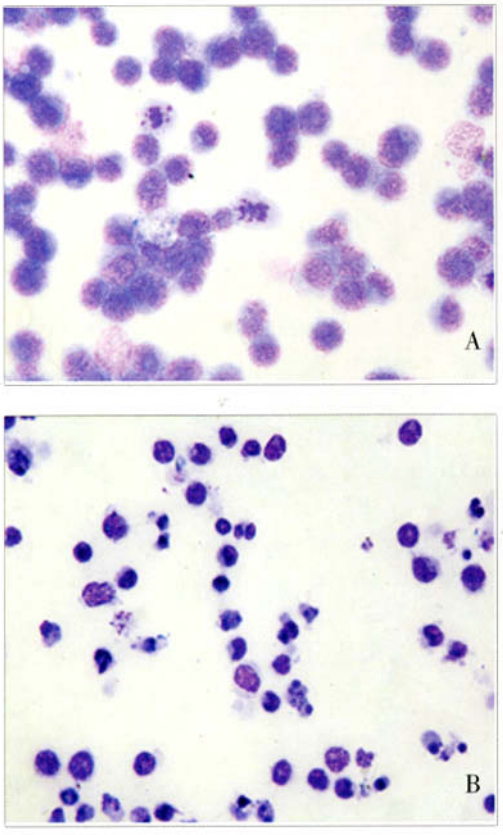

Fig.2. The morphological changes induced 72 h after 10 μmol/L VP-16 in hTERT-transfected Raji cells and Raji cells (Giemsa staining)× 400. A: hTERT-transfected Raji cells; B:Raji cells. Morphological characteristic of apoptosis, such as cell shrinkage, chromatin condensation, nuclear segmentation and formation of apoptotic bodies, were observed.

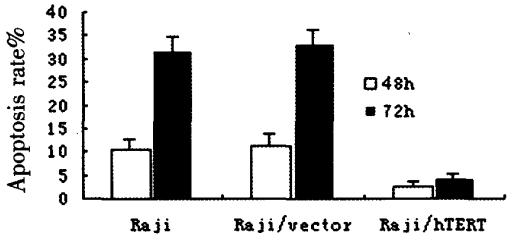

Fig.3. The percentages of apoptotic cells induced at 48 and 72 h after treatment with 10 μmol/L VP-16 in hTERT-transfected Raji cells, as well as in vector-transfected Raji cells and Raji cells.

DISCUSSION

Telomeres are specialized nucleoprotein complexes that serve as protective caps on linear eukaryotic chromosomes. Loss of telomere function is associated with genetic instability and loss of cellular viability. Telomerase is a ribonucleoprotein DNA polymerase that elongates the telomeres of chromosomes to compensate for losses that occur with each round of DNA replication. Unlimited proliferationin of tumor cells requires this enzyme to maintain chromosomal stability. Inhibition of telomerase could therefore provide a new strategy for anticancer therapy. We have previously reported that downregulation of hTERT mRNA and telomerase activity by hTERT antisense oligodeoxynucleotides could enhance cisplatin induced-apoptosis in leukemia cells.[6]

In this report, hTERT ectopic expression was able to increase hTERT protein and telomerase activity in hTERT-transfected Raji cells, whereas telomerase activity in vector -transfected Raji cells was not increased compared to parental Raji cells. These data suggest that the transfection of hTERT induced stable overexpression of hTERT. We next determined the effects of ectopic hTERT expression on apoptosis. Compared with the vector-transfected Raji cells and parental Raji cells, we found that the hTERT-transfected Raji cells were significantly more resistant to VP-16-induced apoptosis. However, there were no significant differences in the sensitivity to VP-16-induced apoptosis in vector-transfected Raji cells compared with parental Raji cells. Recently, we have reported that inhibition of telomerase enhances sensitivity of leukemia cells to cisplatin. [6] These results suggest that overexpression of telomerase by transfected hTERT is advantageous for stabilizing damaged chromosomes and double-stranded DNA break repair. Several recent reports have demonstrated that activation of telomerase by expression of an hTERT transgene protects the cells from stress-induced apoptosis, and decreases the sensitivity of human fibroblasts to different topoisomerase inhibitors. [5,8,9] Some investigetors also have reported that telomerase activation in originally

telomerase-negative human cells or overexpression in telomerase-positive human cancer cells markedly increased the resistance to hydroxyl radical-induced apoptosis.[10]

In conclusion, overexpression of telomerase by hTERT gene transfection can decrease the sensitivity of Raji cells to VP-16 and protect against apoptosis induced by VP-16.

Thus our study provides evidence for a role of telomerase in the regulation of apoptosis in cancer cells. Understanding the effects of telomerase on chemosensitivity may provide important insights for novel anticancer therapies.

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