ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8): 759 - 764
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Regulation of telomerase activity in camptothecin-induced apoptosis of human leukemia HL-60 cells[1]

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KEY WORDS camptothecin; apoptosis; telomerase; *bcl*-2 genes

ABSTRACT

AIM: To explore the regulation of telomerase activity in camptothecin induced apoptosis of human leukemia HL-60 cells. **METHODS**: Apoptosis of HL-60 cells was induced by camptothecin (1 mg/L) for 2, 4, and 6 h. Apoptosis was determined by agarose electrophoresis and flow cytometry analysis. To assess telomerase activity, a PCR-based telomeric repeat amplification protocol assay (TRAP) was used. RT-PCR was performed to examine the mRNA levels of hTR (human telomerase RNA component), hEST2/hTERT (human telomerase reverse transcriptase), TLP1/TP1 (telomerase association protein 1), and *bcl*-2 (B cell leukemia/lymphoma 2 gene) in HL-60 cells before and after camptothecin treatment. **RESULTS**: Telomerase activity was decreased in a time-dependent manner during the camptothecin induced apoptosis of HL-60 cells. However, no difference in expression of each telomerase subunit was detected, while expression of *bcl*-2 was progressively down-regulated. **CONCLUSION**: These lines of evidences indicated that down-regulation of the telomerase activity in HL-60 cells was closely related to camptothecin-induced apoptosis, and the telomerase activity was not blocked at the transcriptional levels of the genes of the known ribonucleoprotein (RNP) complex components. We also found that *bcl*-2 did not regulate the mRNA expressions of telomerase subunits directly.

[1] Project supported by the National Natural Science Foundation of China (№ 39670840), Science Foundation of Chinese Academy of Sciences (№ KY-951-A1-301-01-02), and Shanghai Research Center of Life Sciences.
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Received 1999-12-21 Accepted 2000-03-30

INTRODUCTION

Most chemotherapeutic drugs exert their anti-tumor effects by inducing apoptosis. Apoptosis, the physiological mode of cell death, is representative of an endogenous mechanism which can be selectively triggered by cells in response to largely unknown stimuli. It has been described as programmed, as opposed to accidental, cell death. Deregulation of apoptosis has been shown to contribute to the pathogenesis of a number of human diseases including cancer[1].

In addition to deregulation of apoptosis, the process of neoplasia is characterized by reactivation of telomerase (which synthesizes TTAGGG telomeric DNA onto chromosomal end *de novo*). Telomeres consist of tandem repeats of the hexanucleotide 5'-TTAGGG-3' at the end of chromosomes. Putative functions of telomeres include protecting the ends of the chromosomes against exonucleases and ligases, and preventing the activation of the DNA damage checkpoint. In normal diploid somatic cells, telomeres are progressively shortened with each division (150 - 200 nucleotides/cell division) due to the inability of DNA polymerase to completely replicate the end parts of linear chromosomes, finally to critical length and lead to cell senescence and death. In contrast, most immortal cell lines can maintain their telomeres at a constant length due to the activity of telomerase. Telomerase activation has been reported in 98 % of established immortal cell lines and in about 90 % of tumor tissues tested, but not in most somatic cells[2-4], suggesting that activation of telomerase may play an important role in carcinogenesis and immortalization.

Although both activation of telomerase activity and deregulation of apoptosis have been widely reported in human tumor cells, little is known about specific links between regulation of telomerase activity and drug-induced apoptosis. Recently, accumulating evidences indicated that telomerase activity was mechanistically involved in the regulation of apoptosis[5-7]. In this communication, camptothecin (CPT), a topoisomerase I in-

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8)
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hibitor, was used to induce apoptosis of human leukemia HL-60 cells according to previous report[8]. The telomerase activity during the apoptosis was measured by an advanced TRAP (telomeric repeat amplification protocol) assay, and the mRNA levels of telomerase ribonucleoprotein (RNP) components and B cell leukemia/lymphoma 2 gene (*bcl*-2) were also analyzed to determine whether their expressions correlated with telomerase activity.

MATERIALS AND METHODS

Agents Camptothecin (CPT) was obtained from Feiyun Pharmaceutical Factory (Hubei, China); 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI) and RNase were purchased from Sigma Chemical Co; 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propane-sulphonate (CHAPS), TRIzol and RPMI-1640 medium were the products of GIBCO. SYBR green I was from FMC Bioproducts. All other reagents were of analytical reagent.

Cell culture and cytotoxicity assay *in vitro* Human leukemia HL-60 cell line kept in the cell collection of Shanghai Institute of Materia Medica, Chinese Academy of Sciences was used. Cells were cultured in RPMI-1640 medium supplemented with 10 % heat-inactivated calf serum, streptomycin 100 mg·L^{-1}, benzylpenicillin 100 kU·L^{-1} in a 5 % CO_2 humidified incubator at 37 ℃.

The cytotoxicity of CPT on HL-60 cells was tested by MTT assay with minor modification[9]. Briefly, exponentially growing HL-60 cells were seeded in 96-well, flat-bottomed plates at 5×10^8 cells·L^{-1} (90 μL). CPT (10 μL per well) was added immediately to achieve desired concentration of 1 mg·L^{-1}. The plates were incubated for 2, 4, and 6 h respectively and each period was tested in triplicate wells. At the end of each exposure, MTT (5 g·L^{-1}) 20 μL was put into each well and the plates was incubated at 37 ℃ for 4 h. After that 50 μL "triplex solution (10 % SDS – 5 % isobutanol-HCl 12 mmol·L^{-1})" was added and the cells were incubated at 37 ℃ for an additional 12 h. The absorbance A_{570} was read with a plate reader. The cell viability (percentage of growth) was calculated for each well: % viability = $A_{570\ \text{treated cells}} / A_{570\ \text{control cells}} \times 100$ %.

Apoptosis assay DNA fragmentation assay in agarose gel was performed as follows. The HL-60 cells were lyzed in a 0.5 mL buffer consisting of Tris-HCl 10 mmol·L^{-1} (pH 8.0), edetic acid 25 mmol·L^{-1} (pH 8.0), NaCl 100 mmol·L^{-1}, 0.5 % SDS, and proteinase K 200 mg·L^{-1}. After incubation at 37 ℃ over 12 h, the lysate was extracted with phenol, phenol:chloroform (1:1), and chloroform respectively. The aqueous phase was made up to NaCl 300 mmol·L^{-1}; nucleic acids were precipitated with 2 vol of ethanol and then dissolved in 50 μL water. After digestion of RNA with RNase A (50 mg·L^{-1}) at 37 ℃ for 15 min, the sample was electrophoresed in a 1.8 % agarose gel with 0.5 × TBE (Tris-borate edetic acid). DNA was visualized with ethidium bromide staining.

Cellular DNA content was analyzed by flow cytometry using PI staining. HL-60 cell sample was collected and washed by phosphate-buffered saline (PBS) once, then it was fixed by 70 % ethanol at −20 ℃ over 12 h and followed by PBS washing. The pellet was dissolved in PI solution (PI 100 mg·L^{-1}, RNase 20 mg·L^{-1} in PBS) in the dark at room temperature for 30 min. For each sample, at least 1×10^4 cells were analyzed by flow cytometry (FACsalibur, Becton Dickinson, USA). Results were analyzed by software of CELLQUEST and ModFIT LT for macV1.01 (Becton Dickinson, USA).

Telomeric repeat amplification protocol (TRAP) assay Preparation of telomerase extracts and the TRAP assay were performed as described previously[2,10] with minor modification. To prepare telomerase extract, about 5×10^5 HL-60 cells were washed once in ice cold PBS, pelleted at 5000 × *g* for 5 min at 4 ℃, then re-suspended in 100 μL CHAPS based telomerase lysis buffer [Tris-HCl 10 mmol·L^{-1} (pH 7.5), $MgCl_2$ 1 mmol·L^{-1}, egtazic acid 1 mmol·L^{-1}, PMSF 0.1 mmol·L^{-1}, β-ME 5 mmol·L^{-1}, 0.5 % CHAPS, 10 % glycerol]. The suspension was kept on ice for 30 min, then centrifuged at 15 000 × *g* for 30 min at 4 ℃. The supernatant was removed carefully and snap frozen in liquid nitrogen, then stored at −80 ℃. Protein extracts were diluted appropriately, and an aliquot of 0.5 μg protein was assayed in 46 μL of reaction mixture composed of [Tris-HCl 20 mmol·L^{-1} (pH 8.3), $MgCl_2$ 1.5 mmol·L^{-1}, KCl 63 mmol·L^{-1}, 0.005 % Tween-20, egtazic acid 1 mmol·L^{-1}, 10 mmol·L^{-1} of each deoxynucleotide triphosphate, 0.1 μg TS primer (5'-AAT CCG TCG AGC AGA GTT-3'), 2 μL telomerase extract]. Each TRAP reaction mixture was placed in a preheated thermocycler block and incubated at 30 ℃ for 30 min. After heating at 85 ℃ for 5 min to inactivate telomerase, 0.1 μg ACX return primer [5'-GCG CGG (CTT ACC)3 CTA ACC-3'], 0.01 amol internal control template

TSNT(5'-AAT CCG TCG AGC AGA GTT AAA AGG CCG AGA AGC GAT-3'), 0.1 μg internal control return primer NT(5'-ATC GCT TCT CGG CCT TTT-3'), and 3 unit Taq DNA polymerase were added in the mixture, and telomerase products were amplified for 29 PCR cycles at 94 ℃ for 30 s, 60 ℃ for 30 s. The PCR products were examined by electrophoresis on 10 % non-denatured acrylamide gel in 0.5 × TBE, and visualized by SYBR green I Nucleic Acid Gel Stain for 30 min. Relative telomerase activities were quantitated by Gel Document System GDS8000 (UVP, USA).

RNA isolation and RT-PCR Total RNA was prepared from HL-60 cells by TRIzol. PCR primers were as follows: hEST2/hTERT (human telomerase catalytic subunit), 5'-CGG AAG AGT GTC TGG AGC AA-3' (sense) and 5'-GGA TGA AGC GGA GTC TGG A '3'(antisense), amplicon 145 bp; TLP1/TP1 (human telomerase associated protein), 5'-TCA AGC CAA ACC TGA ATC TGA G-3' (sense), 5'-CCC GAG TGA AAA TCT TTC TAC GC-3' (antisense), amplicon 264 bp; hTR (human telomerase RNA component), 5'-TCT AAC CCT AAC TGA GAA GGG CGT AG-3' (sense), 5'-GTT TGC TCT AGA ATG AAC GGT GGA AG-3' (antisense), amplicon 136 bp; bcl-2, 5'-TGC ACC TGA CGC CCT TCA C -3' (sense), 5'-AGA CAG CCA GGA GAA ATC AAA CAG-3' (antisense), amplicon 293 bp. GAPDH (glyceral-dehyde-3-phosphate dehydrogenase), 5'-CCA TGG AGA AGG CTG GGG-3' (sense), 5'-CAA AGT TGT CAT GGA TGA CC-3' (antisense), amplicon 208 bp. The cDNA was synthesized using random hexamers from 1 μg of RNA. To amplify the cDNA, 2 μL aliquot of the reverse-transcribed cDNA was subjected to 29 cycles of PCR in 50 μL of 1 × buffer [Tris-HCl 10 mmol·L^{-1} (pH 8.3), $MgCl_2$ 1.5 mmol·L^{-1}, KCl 50 mmol·L^{-1}, dNTPs 50 μmol·L^{-1}, 3 units of Taq DNA polymerase, and 0.2 μmol·L^{-1} of specific primers]. Each cycle consisted of denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 40 s, and extension at 72 ℃ for 40 s. The amplified products were separated by electrophoresis on 3 % agarose gel and visualized by staining the gel with ethidium bromide. Each RT-PCR was repeated thrice using different preparations of RNA. Internal control GAPHD RT-PCR was done on all of the samples simultaneously.

RESULTS

Cytotoxic effect of CPT on HL-60 cells CPT at the concentration of 1 mg·L^{-1} demonstrated a potent cytotoxic activity against HL-60 cells even in short exposure periods. Following the addition of CPT to incubation medium for 2, 4, and 6 h, HL-60 viability progressively decreased by 82.6 %, 48.7 %, and 38.9 %, respectively (Fig 1).

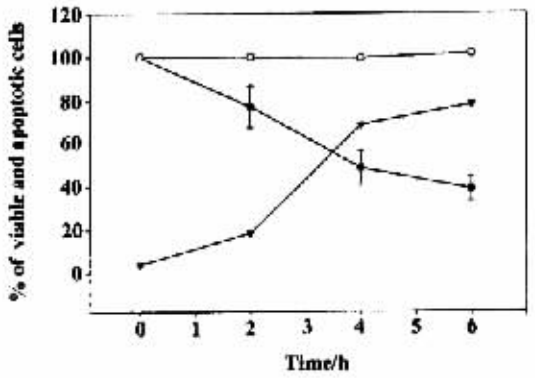

Fig 1. Flow cytometry analysis of CPT 1 mg·L^{-1} on viability and apoptosis of HL-60 cells. $n = 3$. $\bar{x} \pm s$. (○) Viability of control cells; (●) Viability of HL-60 cells treated with CPT; (▼) Apoptotic HL-60 cells treated with CPT.

CPT-induced apoptosis of HL-60 cells To determine whether CPT-treated HL-60 cells demonstrated the characteristic DNA ladder of apoptosis, we examined DNA prepared from HL-60 cells treated with CPT 1 mg·L^{-1} for different time periods by agarose gel electrophoresis. The typical nucleosome spacing ladder was detected in HL-60 cells treated with CPT for 4 h and the extent of ladder became more abundant after a 6-h exposure (Fig 2). The

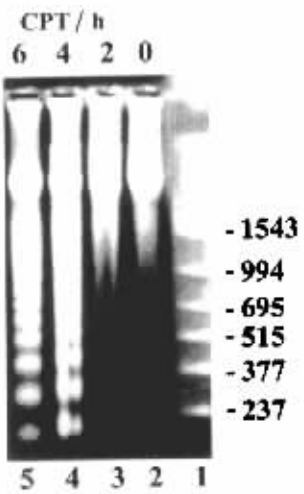

Fig 2. Agarose gel electrophoresis of DNA extract from HL-60 cells. Lane 1: DNA marker. Lane 2: untreated HL-60 cells. Lane 3 – 5: HL-60 cells treated by CPT 1 mg·L^{-1} for 2, 4, and 6 h, respectively.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8)
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number of apoptotic cells was quantitated simultaneously by flow cytometry analysis. The nuclear DNA of apoptotic cells was cleaved into regularly sized fragments by activated nucleases and some of these fragments might escape the cells before it lost membrane integrity. The hypodiploid population formed by cells having a reduced DNA content in DNA content frequency histograms obtained by flow cytometry analysis represented the presence of apoptotic cells, which allowed quantitative comparison of the degree of apoptosis induced by exposure to CPT 1 $mg \cdot L^{-1}$ for different time periods. As shown in Fig 1, the apoptotic population in the control was 3.8 %. It increased by 16.4 %, 68.4 %, and 77.8 %, respectively after co-incubated with CPT 1 $mg \cdot L^{-1}$ for 2, 4, and 6 h.

Down-regulation of mRNA expression level of bcl-2 To better understand the mechanism of CPT-induced apoptosis of HL-60 cells, we examined the mRNA expression of apoptosis-modulating gene *bcl-2*. The mRNA level of *bcl-2* in control cells was high, and it was pronouncedly down-regulated parallel with increased proportion of apoptotic cells (Fig 3).

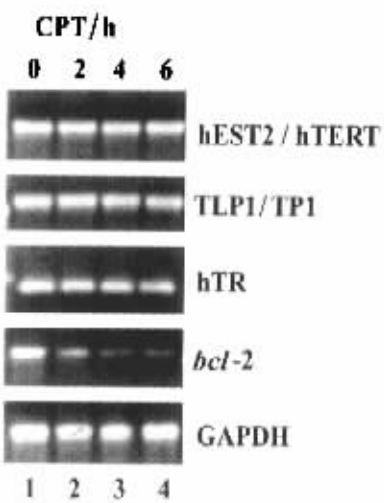

Fig 3. Analysis of mRNA expression in each of telomerase subunits and apoptosis-modulating gene bcl-2 in HL-60 cells undergoing CPT-induced apoptosis. Lane 1: untreated cells. Lane 2 – 4: HL-60 cells treated with CPT 1 $mg \cdot L^{-1}$ for 2, 4, and 6 h.

Down-regulation of telomerase activity during CPT-induced apoptosis We next examined whether level of telomerase activity changed in HL-60 cells undergoing CPT-induced apoptosis. Relative telomerase activities were quantitated by comparing the signal intensity of each lane with that of positive control. The telomerase activity value of positive (untreated HL-60 cells) was regarded as 100 %. Following exposure to CPT 1 $mg \cdot L^{-1}$, there was a progressive decrease in telomerase activity of HL-60 cells. Telomerase activity decreased to 82.3 %, 31.0 % and 28.2 % of the control levels respectively after being exposed to CPT for 2, 4, and 6 h (Fig 4).

We also examined the effect of CPT on telomerase activity in a cell free system. CPT did not directly inhibit the telomerase activity at a concentration of 1 $mg \cdot L^{-1}$ (Fig 5). These results indicated that the down-regulation of telomerase activity in HL-60 cells was due to CPT-induced apoptosis.

Induction of apoptosis by CPT in HL-60 cells did not affect the mRNA expression of the RNP complex components To determine whether the inactivation of the telomerase occurred at the mRNA levels of the components of the RNP complex, RT-PCR was performed to examine the mRNA expression of each telomerase subunit in HL-60 cells before and after CPT treatment. The mRNA levels of hTR, hEST2/hTERT, and TLP1/TP1 were unchangeable during the apoptotic process (Fig 3).

DISCUSSION

Apoptosis, an orderly process that uses intracellular signal transduction pathways and digestive enzymes to degrade the cell in an organized way, is different from necrosis. The mechanism of necrosis is relatively well understood, whereas that of apoptosis is presently subject to intense investigation. Recent accumulating reports suggest that telomerase might be playing an important role in suppressing apoptotic signaling cascades. Treatment of pheochromocytoma cells with telomerase inhibitor (oligodeoxynucleotide TTAGGG or 3, 3'-diethyloxadicarbocyanine) has been shown to enhance mitochondrial dysfunction and apoptosis induced by staurosporine, Fe^{2+}, and amyloid β-peptide[5]. Other reports also revealed that an increase in apoptosis has been associated with a decrease in telomerase activity in quiescent and terminally differentiated cells[11,12]. Furthermore, glioblastoma cell lines with high levels of telomerase activity exhibited reduced sensitivity to cisplatin-induced apoptosis[6], and stable overexpression of bcl-2 has been reported to result in increased telomerase activity and decrease in apoptosis[7]. All these data suggested that telomerase maybe an important factor in modulating cell death. To determine whether induction of apoptosis down-regulates

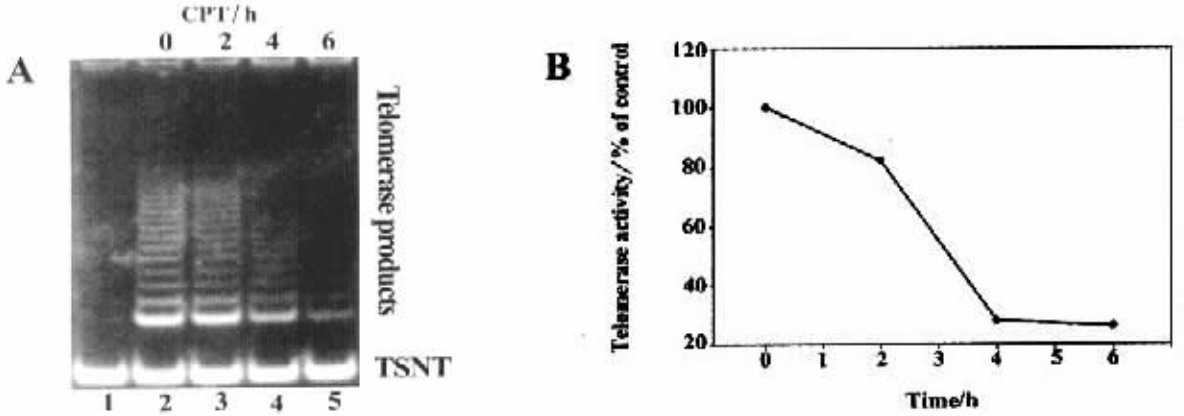

Fig 4. Telomerase activity in HL-60 cells undergoing CPT-induced apoptosis. (A) Lane 1: negative control, no telomerase added. Lane 2: positive control, extract from untreated HL-60 cells. Lane 3 – 5: telomerase activity in HL-60 cells treated with CPT for 2, 4, and 6 h. (B) Relative telomerase activities in CPT-treated HL-60 cells.

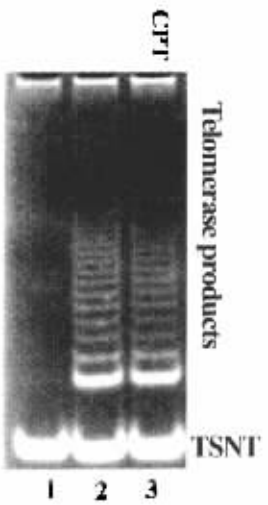

Fig 5. Effect of CPT on telomerase activity in cell free system. Lane 1: negative lysis buffer control. Lane 2: positive control (extract from untreated HL-60 cells). Lane 3: effect of CPT 1 mg·L^{-1} on untreated HL-60 cell extract.

the telomerase activity, we examined the telomerase activity in CPT-induced apoptosis in human leukemia HL-60 cells. We found that an increased apoptotic population caused by CPT treatment was accompanied by a pronounced down-regulation of telomerase activity; and CPT did not inhibit telomerase activity directly, demonstrating that the down-regulation of telomerase activity was due to CPT-induced apoptosis.

It is still unclear whether the inactivation of the telomerase occurs at the transcriptional levels of the components of the RNP complex. Some of the genes involved in the RNP complex have been cloned, include hTR, which contains the template for reverse transcription[13]; TLP1/TP1, the p80 homologue of the Tetrahymena telomerase gene[14,15]; and hEST2/hTERT, which is thought to be the enzyme's catalytic subunit[16]. Using the RT-PCR method we analyzed the mRNA level of each component of the RNP complex. All the mRNA levels of hTR, hEST2/hTERT, and TLP1/TP1 remained unchangeable after CPT-treatment. This indicates that telomerase activity in apoptotic HL-60 cells is not regulated at transcriptional levels of its subunits, and we propose that it might be getting inhibited by natural repressors in apoptotic HL-60 cells. Some recent studies have provided valuable evidences towards this notion. Since vertebrate telomerase is a ribonucleoprotein complex with two protein components containing multiple potential phosphorylation sites, protein phosphorylation was suggested to play a role in telomerase regulation. Protein phosphatase 2A (PP2A) has been proposed to be a telomerase inhibitory factor in the nucleus of human breast cancer cells[17], and its inhibitory action on telomerase activity was both concentration- and time-dependency and can be prevented by the protein phosphatase inhibitor okadaic acid. Additionally, Santoro *et al*[18] have reported an increase of protein phosphatase 2A activity by caspase-3 in anti-Fas antibody induced apoptosis in Jurkat cells. The above data establish a close relationship among PP2A, telomerase, and apoptosis. Whether the protein phosphatase has some influences on telomerase regulation in drug-induced apoptosis remains to be further explored, a more detailed study is underway in our lab.

The report that stable over-expression of *bcl*-2 results in up-regulation of both the telomerase activity and

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Aug; 21 (8)
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resistance to apoptosis, establishes a link between *bcl*-2 expression and telomerase activity[7]. This seems to be confirmed by our observation that decreased telomerase activity was accompanied by down-regulation of *bcl*-2. However, as *bcl*-2 had no effect on the expression of the telomerase RNP complex components, our results suggest that the telomerase activity may be regulated by *bcl*-2 in an indirect manner.

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喜树碱诱导人白血病 HL-60 细胞凋亡过程中端粒酶的调控[1]

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关键词 喜树碱；细胞凋亡；端粒酶；*bcl*-2 基因

目的：研究在喜树碱诱导人白血病 HL – 60 细胞凋亡过程中端粒酶的调节变化规律. **方法**：用 MTT 法测定药物对细胞存活率的影响；用琼脂糖电泳及流式细胞术检测和定量凋亡的发生；用以 PCR 为基础的 TRAP 法测定端粒酶活力；逆转录 PCR 检测凋亡过程中 *bcl*-2 及端粒酶亚基 hTR、hEST2/hTERT 和 TLP1/TP1 的基因表达水平的变化. **结果**：端粒酶活力伴随喜树碱诱导 HL-60 细胞凋亡的发生而逐渐降低，在此过程中端粒酶各亚基的 mRNA 水平无可见性变化，而 *bcl*-2 的基因表达水平则相应下调. **结论**：端粒酶活力的下调和喜树碱诱导的 HL-60 凋亡密切相关，端粒酶活力的阻断并非发生在其亚基基因转录水平，*bcl*-2 对端粒酶活力的调节也不是通过影响端粒酶亚基的转录水平来实现的.

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