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Down-regulation of telomerase activity via protein phosphatase 2A activation in salvicine-induced human leukemia HL-60 cell apoptosis

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Abstract

Salvicine is a novel topoisomerase II inhibitor possessing significant antitumor activity, both *in vitro* and *in vivo*. The antitumor effect of salvicine is associated with its ability to induce tumor cell apoptosis. Telomerase plays an important role in the apoptotic pathway. However, little is known about the mechanisms of telomerase regulation during apoptosis induced by anticancer drugs. This study investigated the regulation of telomerase activity in salvicine-induced human leukemia HL-60 cell apoptosis. Salvicine treatment resulted in HL-60 cell apoptosis and down-regulation of telomerase activity in a time- and concentration-dependent manner. Repression of telomerase activity preceded a decrease in expression of the telomerase catalytic subunit (hTERT) and telomerase-associated protein (TP1) at the mRNA level, suggesting that the salvicine-induced decrease in telomerase activity may be additionally regulated by mechanisms other than telomerase subunit transcription. We observed that okadaic acid (OA), a protein phosphatase inhibitor, prevented the induction of apoptosis and the down-regulation of telomerase activity by salvicine. The significant increase in protein phosphatase 2A (PP2A) activity induced by salvicine treatment was blocked completely by OA. Moreover, although salvicine induced HL-60 cell apoptosis in a caspase-3-dependent manner, a specific caspase-3 inhibitor, Z-DEVD-FMK, did not prevent a decrease in telomerase activity or an increase in PP2A activity in apoptotic HL-60 cells, ruling out a role for caspase-3 in PP2A activation by salvicine. The results collectively suggest that the salvicine-induced decline in telomerase activity is not a consequence of HL-60 cell apoptosis and that it may be caused principally by the dephosphorylation of telomerase components mediated by PP2A activation.

Keywords: Telomerase; Apoptosis; Salvicine; Protein dephosphorylation

1. Introduction

Apoptosis represents an evolutionarily conserved form of cell suicide. Deregulation of apoptosis contributes to the pathogenesis of a number of human diseases, including cancer, in which apoptosis is suppressed. In addition to apoptosis deregulation, neoplasia development is characterized by the reactivation of telomerase, which synthesizes TTAGGG telomeric DNA onto the chromosomal end *de novo*. Telomerase activation has been reported in 98% of established immortal cell lines and in 90% of malignant tumor tissues, but not in most somatic cells [1,2], indicating its important role in carcinogenesis and immortalization [3].

Recent studies revealed a close relationship between telomerase activity and apoptosis. First, both telomerase activity and apoptosis are modulated by a common gene, *bcl-2*. It has been reported that telomerase activity is increased by Bcl-2 overexpression in human cancer cells with low expression of this gene [4]. Moreover, Bcl-2 overexpression in pheochromocytoma cells protects them from apoptosis in the presence of telomerase inhibitors [5]. Second, treatment of pheochromocytoma cells with telomerase inhibitors significantly enhanced apoptosis induced by staurosporine, Fe^{2+}, and β-peptide, suggesting that telomerase plays an important role in suppressing apoptotic

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DAPI, 4′,6-diamidino-2-phenylindole; hTR, human telomerase RNA subunit; hTERT, human telomerase catalytic subunit; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide; OA, okadaic acid; PMSF, phenylmethylsulfonyl fluoride; PP2A, protein phosphatase 2A; RT–PCR, reverse transcriptase–polymerase chain reaction; TP1, telomerase-associated protein 1; TRAP, telomeric repeat amplification protocol.

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signaling cascades [5]. Third, direct inhibition of telomerase using antisense telomerase RNA [6] or peptide nucleic acid and 2′-*O*-MeRNA oligomers [7] resulted in progressive telomere shortening and apoptosis. However, it is noteworthy that the telomerase inhibition sometimes resulted in cell growth arrest rather than apoptosis. Apoptosis was detected only in some transfectants of antisense against telomerase RNA over a long exposure time, although all transfectants expressed a high level of the pro-apoptosis protein interleukin-1-converting enzyme [8]. Therefore, it is difficult to conclude that telomerase inhibition is a requirement for apoptosis. Most anticancer drugs can induce tumor cell apoptosis. However, little is known at present about how telomerase is regulated during this process. At the same time, the changes in telomerase activity associated with anticancer drug-induced apoptosis are very complex according to the published data [9–12]. Hence, studying the mechanisms of telomerase regulation during apoptosis induced by anticancer drugs will be helpful for exploring the possible links between telomerase and apoptosis. A better understanding of the regulation of telomerase activity will also provide a basis for further investigation and manipulation of telomerase activity as a potential therapeutic modality.

Salvicine [4,5-seco-5,10-friedo-abieta-3,4-dihydroxy-5(10),6,8,13-tetraene-11,12-dione], a novel diterpenoid quinone compound, is a structurally modified derivative of a natural product lead from a traditional Chinese herb, *Salvia prionitis* Hance (Labiatae) [13]. It possesses significant antitumor activity, both *in vivo* and *in vitro*, especially against various solid malignant human tumors, such as lung and gastric cancers [13,14]. Moreover, salvicine has a prominent cytotoxic effect on three multidrug-resistant (MDR) cell lines with an average resistance factor of 1.2, which is accompanied by a decrease in the expression of MDR-1 mRNA and P-glycoprotein in MDR tumor cells (Miao ZH, Tang T, Ding J, unpublished data). Mechanistic studies demonstrate that salvicine is a new non-intercalative topoisomerase II inhibitor, which exerts its effects by trapping enzyme–DNA cleavage complexes, and its mode of action differs from that of other topoisomerase II inhibitors [15]. Recent research on a yeast genetic system shows that DNA topoisomerase II is the primary cellular target of salvicine in *Saccharomyces cerevisiae* [16]. The antitumor effect of salvicine is associated with its ability to induce tumor cell apoptosis [17]. Preferential damage of the P2 promoter of the c-*myc* gene was observed, accompanied by a down-regulation of c-*myc* and an up-regulation of c-*fos* and c-*jun* expression in the early stages of salvicine-induced tumor cell apoptosis [18]. These observations suggest that salvicine is a very promising anticancer drug candidate. Salvicine has been promoted recently to clinical trials in China. The present study investigated whether telomerase activity changes in salvicine-induced HL-60 cell apoptosis, and the possible mechanisms of telomerase regulation in this process.

2. Materials and methods

2.1. Materials

Salvicine, a tangerine-colored crystalloid, was provided by Professor Jin-Sheng Zhang (Department of Phytochemistry, Shanghai Institute of Materia Medica, Chinese Academy of Sciences). It was dissolved in DMSO at 0.1 M as a stock solution and was kept at −20° in the dark. OA was purchased from GIBCO BRL. The caspase inhibitors Ac-YVAD-CHO, Z-VAD-FMK, and Z-DEVD-FMK were purchased from Calbiochem. The kits for PP2A and caspase activity assay were obtained from Promega.

2.2. Cell culture

The HL-60 cell line was obtained from the cell collection of the Shanghai Institute of Materia Medica. Cells were cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated calf serum, 100 μg/mL of streptomycin, and 100 units/mL of penicillin in humidified 5% CO_2/95% air at 37°.

2.3. Experimental treatments

Exponentially growing HL-60 cells at 5×10^5/mL were treated with salvicine at various concentrations alone or with OA (200 nM) for the desired period. The concentration of the DMSO vehicle in the growth medium was less than 0.02% (v/v). In an experiment involving caspase inhibitors, the cells were pretreated for 1 hr with the caspase inhibitor (200 μM) prior to exposure to salvicine. All experiments were repeated at least two times.

2.4. Cytotoxicity assay

The cytotoxic effect of salvicine on HL-60 cells was quantified by an MTT assay with minor modifications [19]. Briefly, exponentially growing HL-60 cells were seeded at 5×10^5/mL in 96-well flat-bottom plates. Then salvicine was added to achieve the desired concentration of 10 μM. After incubation for 2, 4, or 6 hr, 10 μL (5 mg/mL in PBS) of MTT (Sigma) was added to each well. The cells were then incubated for an additional 4 hr, after which 50 μL of "triplex solution" (10% SDS–5% isobutanol-HCl, 12 mmol/L) was added, and the cells were incubated overnight at 37°. Next, the plates were read at 570 nm on a scanning multiwell spectrophotometer (VERSAmax, Molecular Device). The percentage of control absorbance, which represented the surviving cell fraction, was calculated.

2.5. Apoptosis assay by morphology, DNA fragmentation, and flow cytometric analysis

Cells were harvested to determine apoptotic features using the following methods. Morphological changes

characteristic of apoptosis were determined by staining cell nuclei with DAPI. The nuclear morphology was observed with an Olympus IX70 UV light fluorescence microscope. For the DNA fragmentation assay, total DNA was extracted according to a procedure reported previously [18]. DNA samples were subjected to electrophoresis in a 1.8% agarose gel, visualized under UV light, and photographed. Quantification of the apoptotic HL-60 cells was carried out by determining the fluorescence intensity of propidium iodide (PI) using flow cytometry (FACSCalibur, Becton Dickinson). At least 1×10^4 cells were analyzed for each sample, using the software of CELLQUEST and ModFIT for macV1.01 (Becton Dickinson).

2.6. Telomerase activity assay

Telomerase activity was determined by the telomeric repeat amplification protocol (TRAP), using a method described previously with minor modifications [1,20]. In brief, telomerase was extracted by a CHAPS-based telomerase lysis buffer [10 mM Tris–HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol]. Protein extracts were diluted appropriately, and an aliquot of 0.5 μg protein was assayed in 46 μL of reaction mixture [20 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween-20, 1 mM EGTA, 46 μM of each deoxynucleotide triphosphate, 0.1 μg TS primer (5′-AAT CCG TCG AGC AGA GTT-3′), 2 μL telomerase extract]. To determine the direct effect of salvicine on telomerase activity, 2 μL of salvicine at a final concentration of 10 μM was added to the TRAP reaction buffer containing telomerase crude extracts from cells not treated with salvicine. 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 units *Taq* DNA polymerase were added to the mixture, and telomerase products were amplified at 29 PCR cycles at 94° for 30 sec, 60° for 30 sec. The PCR products were examined by electrophoresis on a 10% non-denatured acrylamide gel in 1× TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA), and visualized by SYBR green I Nucleic Acid Gel Stain (FMC Bioproducts) for 30 min. The experiment was repeated three times using different telomerase extracts from independent treatments. Relative telomerase activities were quantitated by the Gel Document System GDS8000 (UVP).

2.7. RNA isolation and RT–PCR

Cellular total RNA was isolated from HL-60 cells by TRIzol (GIBCO BRL). PCR primers were as follows: human telomerase catalytic subunit (hTERT), 5′-CGG AAG AGT GTC TGG AGC AA-3′ (sense) and 5′-GGA TGA AGC GGA GTC TGG A-3′(antisense); amplicon, 145 bp; telomerase-associated protein 1 (TP1), 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; human telomerase RNA subunit (hTR), 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; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 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 aliquots of the reverse-transcribed cDNA were subjected to 29 cycles of PCR in 50 μL of 1× buffer [10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl], 50 μM dNTPs, 3 units of *Taq* DNA polymerase, and 0.1 μM of specific primers. Each cycle consisted of denaturation at 94° for 30 sec annealing at 60° for 40 sec, and extension at 72° for 40 sec. The amplified products were separated by electrophoresis on a 3% agarose gel and visualized by staining the gel with ethidium bromide. Each RT–PCR was repeated three times using different preparations of RNA. The individual bands were quantitated, and the intensities of the subunits were expressed as intensities relative to those of the GAPDH, respectively. A value of 100% was given to the relative intensity of untreated cells (control).

2.8. PP2A activity assay

The PP2A activity of the total cellular lysate was determined by measuring the generation of free PO_4 from the phosphopeptide RRA(pT)VA, using the molybdate-malachite green-phosphate complex assay. Cell lysates were prepared in a low detergent lysis buffer [0.25% Nonidet P-40, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM PMSF, 10 μg/mL of aprotinin, 10 μg/mL of leupeptin]. The phosphatase assay was performed in a PP2A-specific reaction buffer [final concentration 50 mM imidazole (pH 7.2), 0.2 mM EGTA, 0.02% 2-mercaptoethanol, 0.1 mg/mL of bovine serum albumin] using 100 μM phosphopeptide substrate and 5 μg of protein isolated from HL-60 cell lysate. After a 60-min incubation at room temperature, molybdate dye was added, and the amount of free phosphate generated was determined from the optical density at 630 nm (VERSAmax, Molecular Device), using a standard curve for free phosphate. Phosphatase activity was defined as picomoles of free PO_4 generated per minute per microgram of protein.

2.9. Caspase activity assay

Cells were harvested and washed with cold PBS. Pellets were resuspended in hypotonic lysis buffer [25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA (pH 8.0), 5 mM dithiothreitol, 1 mM PMSF, 10 μg/mL of pepstatin A,

10 μg/mL of leupeptin] and lysed by four cycles of freezing and thawing. Samples were then centrifuged at 16,000 *g* for 20 min at 4°, after which the supernatants were removed to new tubes and the protein concentration in each supernatant was determined by Coomassie brilliant blue assay. The same amount of protein was used to determine caspase activity using the CaspACETM Assay System (Promega Co.) according to the protocol of the manufacturer. Briefly, 10 μg of total protein was incubated with a 50 μM concentration of the substrate, acetyl-Asp-Glu-Val-Asp-methylcoumaryl-7-amide (Ac-DEVD-AMC) or acetyl-Tyr-Val-Ala-Asp-methylcoumaryl-7-amide (Ac-YVAD-AMC), at 37° for 90 min. The release of AMC was measured by excitation at 360 nm and emission at 460 nm using a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo Labsystems Co).

3. Results

3.1. Induction of apoptosis by salvicine in HL-60 cells

One of our previous reports showed that a brief exposure (30 min) of HL-60 cells to 10 μM salvicine induced early DNA breaks and triggered apoptosis [18]. In this study, the effect of salvicine on HL-60 cell proliferation was initially examined using an MTT assay. As shown in Fig. 1A, salvicine at 10 μM exhibited potent cytotoxic activity against HL-60 cells, even during short-exposure periods. Following incubation with the compound for 2, 4, and 6 hr, cell viability progressively decreased to 77.8, 41.2, and 35.9%, respectively, of untreated cells. The ability of salvicine to induce apoptosis was simultaneously determined by different methods, including fluorescence microscopy, DNA agarose gel electrophoresis, and flow cytometric analysis. After treatment with salvicine for 2 hr, only a few HL-60 cells exhibited the morphological features of apoptosis, such as chromatin condensation. After 4 hr, chromatin condensation became more abundant, and apoptotic bodies began to appear in DAPI-stained cell nuclei. After a 6-hr exposure, most of the observed cells were apoptotic (Fig. 1C). Flow cytometric analyses demonstrated that the apoptotic population was 3.1% in control cells, which increased to 33.8, 55.6, and 65.9% following co-incubation with 10 μM salvicine for 2, 4, and 6 hr, respectively (Fig. 1D). These findings suggest that salvicine induces HL-60 cell apoptosis in a time-dependent manner. Agarose gel electrophoresis of total DNA extracted from

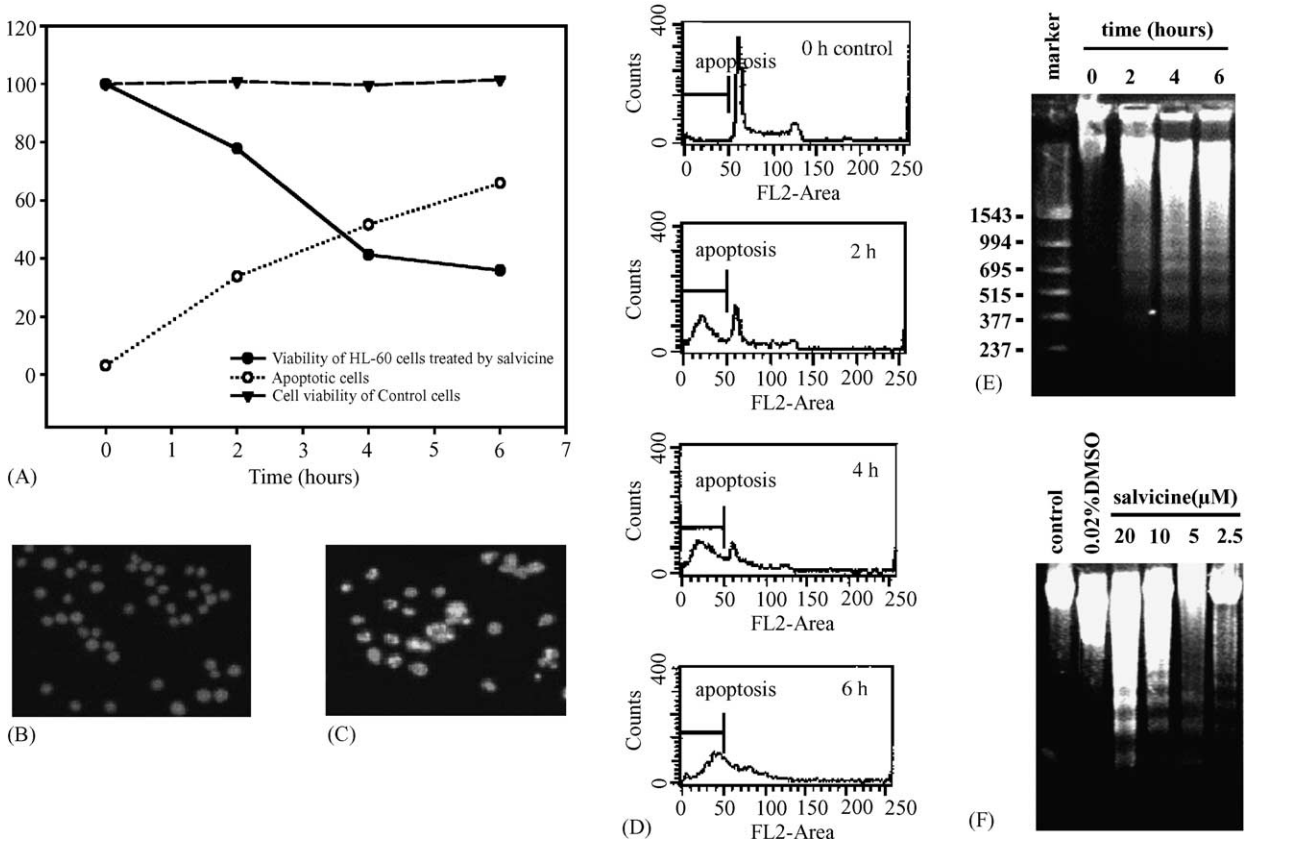

Fig. 1. Induction of apoptosis by salvicine in HL-60 cells in a time- and concentration-dependent manner. HL-60 cells were treated with either 10 μM salvicine for 0, 2, 4, and 6 hr or with various concentrations of salvicine for 4 hr. Cell viability was determined by the MTT assay (A), and apoptotic cells were quantitated by flow cytometry (D). Data points are representative of three independent experiments. Morphology of untreated (B) or salvicine-treated (10 μM) (C) HL-60 cells for 6 hr was visualized by DAPI staining (×200). DNA fragmentation in HL-60 cells treated with 10 μM salvicine for the indicated times (E) or with various concentrations of salvicine for 4 hr (F) was analyzed by agarose gel electrophoresis.

salvicine-treated HL-60 cells confirmed the time-course of DNA fragmentation. The typical nucleosome spacing ladder indicative of apoptosis was observed at a 2-hr exposure, and became more obvious after 4 and 6 hr (Fig. 1E). This cell death response was also concentration-dependent. Within 4 hr of treatment with 2.5 to 20 μM salvicine, digested genomic DNA was evident at 5 μM and became more obvious at 10–20 μM (Fig. 1F).

3.2. Down-regulation of telomerase activity during salvicine-induced apoptosis

We next determined, using a TRAP assay, whether telomerase activity was altered in salvicine-induced apoptotic HL-60 cells. The data presented in this study demonstrate that salvicine-induced telomerase activity decreased in a time- and concentration-dependent manner. As shown in Fig. 2, telomerase activity in untreated HL-60 cells was taken as 100%. Following exposure to 10 μM salvicine for 2, 4, and 6 hr, telomerase activities of HL-60 cells progressively declined to 77.9, 51.3, and 36.2%, respectively, compared to the control group (Fig. 2A and B). Within 4 hr of treatment with 2.5, 5, 10, and 20 μM salvicine, telomerase activity was 76.3, 60.1, 40.9, and 32.2% of control, respectively (Fig. 2C and D).

We also examined the effect of salvicine on telomerase activity in a cell-free system. Salvicine was added directly to the TRAP reaction buffer containing telomerase crude extracts. As shown in Fig. 2E, 10 μM salvicine (a concentration considerably higher than intracellular concentrations) did not inhibit telomerase activity directly. The results suggest that down-regulation of telomerase activity in HL-60 cells is associated with other cellular events that are linked to salvicine-induced apoptosis.

3.3. Expression of telomerase subunits in salvicine-induced HL-60 cell apoptosis

Human telomerase consists of two core subunits: an RNA component acting as a template for replication (hTR) [21], and a protein component responsible for enzymatic activity (hTERT) [22]. Telomerase-associated protein (TP1) has also been cloned as a component of telomerase, but its function remains unclear [23,24]. To investigate the

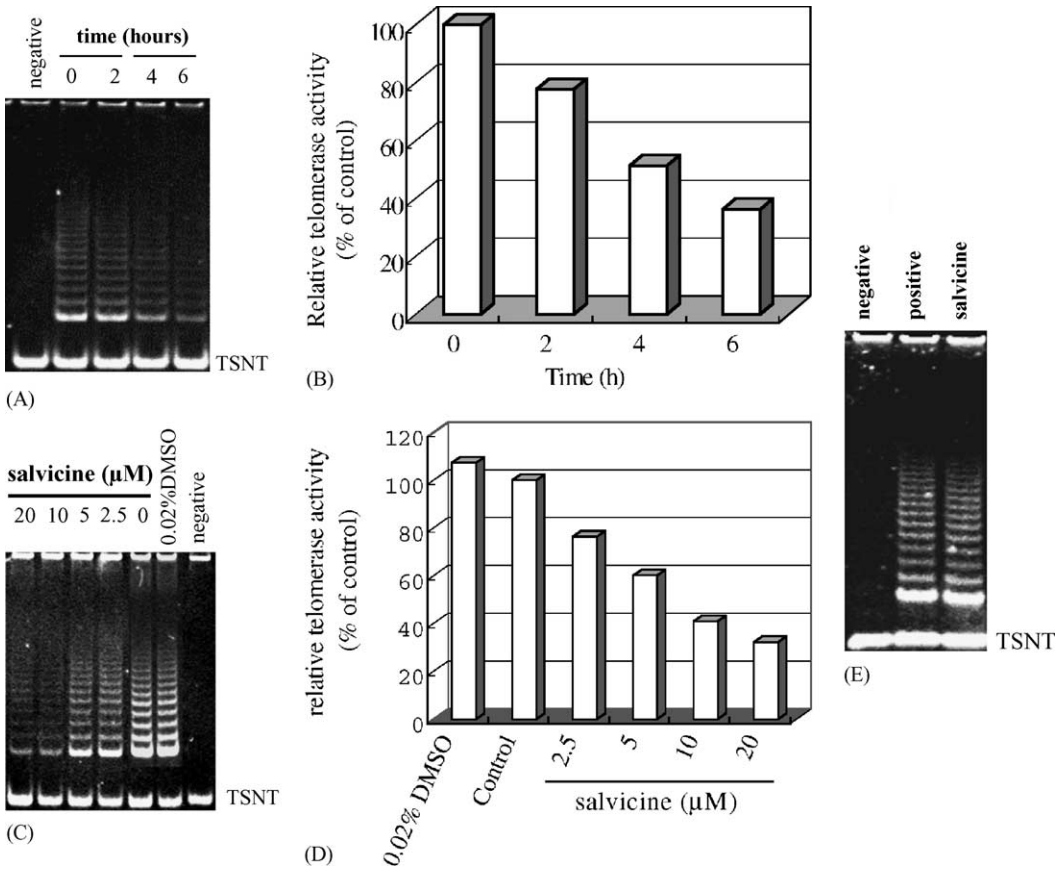

Fig. 2. Effect of salvicine on telomerase activity in HL-60 cells. Telomerase was prepared from cells treated with salvicine (10 μM) for different periods (A, B) or at various concentrations for 4 hr (C, D), as described in Section 2. The data presented are representative of three independent experiments. Telomerase activities were determined by the TRAP assay. Negative: no telomerase extract was added. The relative telomerase activity was determined by calculating the ratio of the intensity of the area under the bands representing telomeric repeats in treated cells to the untreated control (taken as 100%). (E) Effect of salvicine on telomerase activity in a cell-free system. The TRAP assay was performed without (negative) or with (positive) telomerase extracts, or with both the telomerase extract and 10 μM salvicine (salvicine). TSNT: internal control.

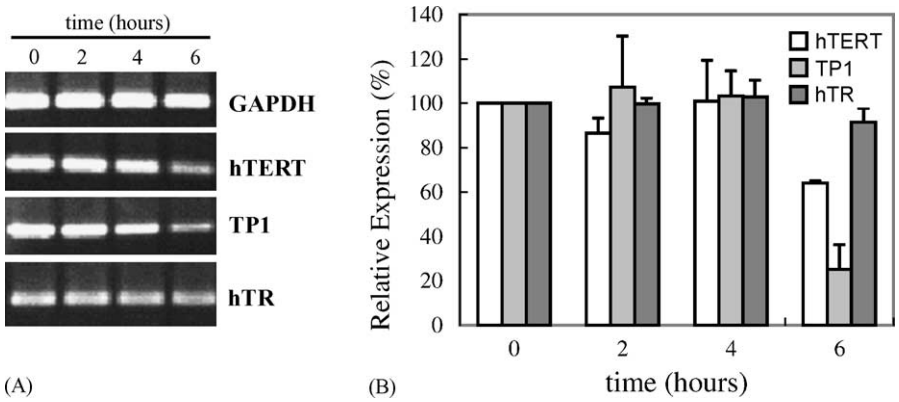

Fig. 3. Expression of telomerase subunits at the mRNA level in salvicine-induced HL-60 cell apoptosis. Total cellular RNA from HL-60 cells, untreated or treated with 10 μM salvicine for 2, 4, and 6 hr, respectively, was analyzed using RT–PCR for GAPDH, hTERT, TP1, and hTR mRNA expression. (A) Representative photo from three independent experiments. (B) Quantification of the DNA bands from three independent experiments. Results are presented as means ± SD.

mechanisms by which salvicine decreased telomerase activity, we examined the expression of telomerase subunits in apoptotic HL-60 cells at the mRNA level. Salvicine-treated HL-60 cells did not exhibit obvious changes in hTR expression (Fig. 3). However, although TP1 and hTERT mRNA expression levels remained stable 4 hr after salvicine exposure, down-regulation of these two subunits was observed at the 6-hr time point. These changes are not analogous with alterations in telomerase activity, which decreased as early as 2 hr after salvicine treatment. The delayed decrease in the transcription of hTERT and TP1 suggests that telomerase subunit transcription does not play a crucial role in regulating telomerase activity in salvicine-induced HL-60 cell apoptosis.

3.4. Effect of OA on salvicine-induced apoptosis and down-regulation of telomerase activity

Phosphorylation and dephosphorylation of proteins are fundamental mechanisms utilized by cells to regulate cellular processes, including proliferation and apoptosis. Protein phosphorylation is essential for telomerase activity. Since the above results suggest a possible link between telomerase activity and salvicine-induced HL-60 cell apoptosis, we postulated that this interrelationship is mediated by protein phosphorylation/dephosphorylation. To verify this hypothesis, the effect of a protein phosphatase inhibitor, OA, on salvicine-induced HL-60 cell apoptosis and telomerase activity was examined. Using DNA agarose electrophoresis and flow cytometry, we observed that 200 nM OA was the minimum concentration required to inhibit apoptosis markedly in HL-60 cells exposed to 10 μM salvicine for 4 hr (Fig. 4A and B). As illustrated in Fig. 4B, the background level of apoptotic HL-60 cells for a 4-hr incubation was 5.13 ± 0.80%. After a 4-hr exposure to salvicine, apoptosis was induced significantly in HL-60 cells (50.67 ± 6.98%); OA treatment alone slightly increased the percentage of apoptotic cells (15.20 ± 8.30%). Upon co-treatment with OA (200 nM) and salvicine (10 μM), the percentage of apoptotic cells was 3.82 ± 1.30%, indicating complete blockage of cell death. The effect of OA on telomerase activity in HL-60 cells was investigated simultaneously. Incubation of cells with 200 nM OA alone for 4 hr increased telomerase activity slightly (120% that of control). When OA was added to cells treated with 10 μM salvicine, the relative telomerase activity increased significantly, from 50% of control to 118% (Fig. 5). These results clearly imply that OA opposes the influence of salvicine on HL-60 cells, and that dephosphorylation may play an important role in the modulation of telomerase activity during salvicine-induced apoptosis.

3.5. Effect of salvicine on PP2A activity in HL-60 cells

PP2A, a negative regulator of telomerase activity [25], is one of the cellular targets of OA. To obtain direct evidence

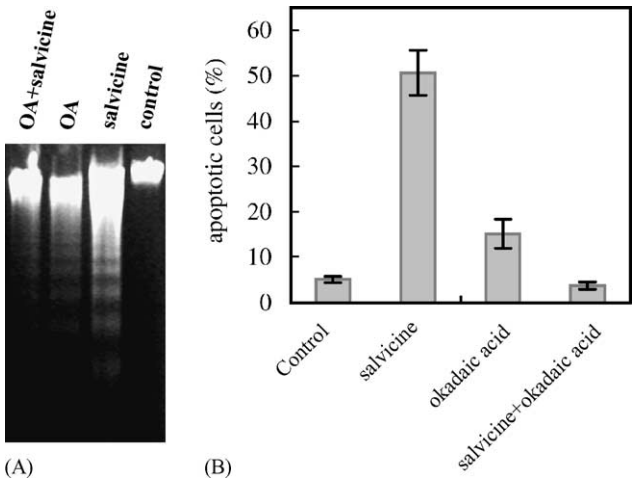

Fig. 4. Inhibitory effects of OA on salvicine-induced HL-60 cell apoptosis. Cells were treated with 10 μM salvicine, in the absence or presence of 200 nM OA for 4 hr. Apoptosis was detected by DNA agarose gel electrophoresis (A) and was quantitated by flow cytometry (B). Results are expressed as means ± the range for two independent flow cytometry experiments.

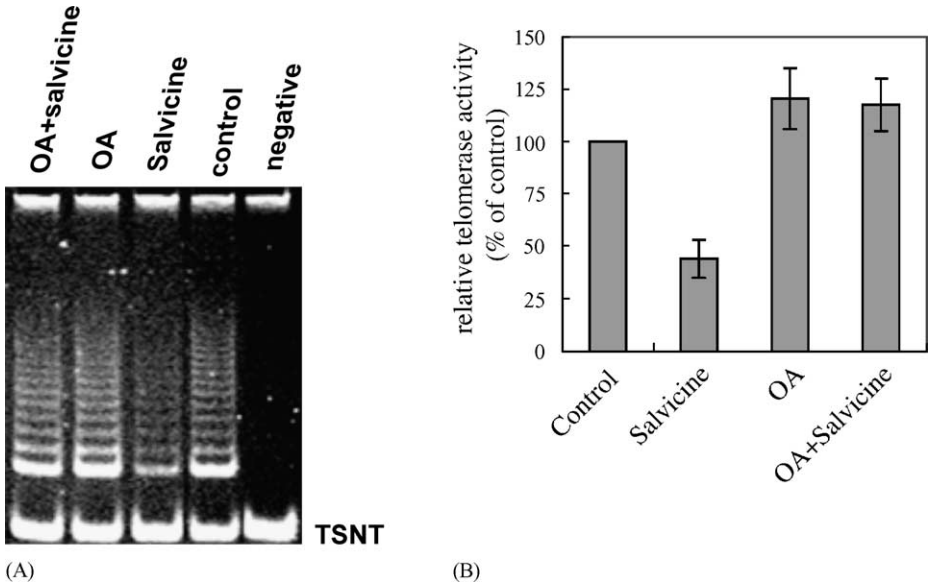

Fig. 5. Reversal of the down-regulation of telomerase activity in salvicine-induced HL-60 cells by OA. Cells were treated with 10 μM salvicine in the absence or presence of 200 nM OA for 4 hr. Telomerase activity was determined by the TRAP assay as described in Section 2. (A) Representative photo of telomerase activity. Negative: no telomerase extract was added; control: untreated HL-60 cells; salvicine, OA, and OA + salvicine indicate telomerase activity in HL-60 cells treated with salvicine (10 μM), OA (200 nM), and the two agents together, respectively. TSNT: internal control. (B) Graphic representation of the experiment in panel A. The relative telomerase activity was determined by calculating the ratio of the intensity of the area under the bands representing telomeric repeats in treated cells to that of the untreated control (taken as 100%). Results are expressed as means ± the range for two independent experiments.

that salvicine causes down-regulation of telomerase activity via PP2A, the effect of salvicine on PP2A activity in HL-60 cells was examined. Cells were treated with various concentrations of salvicine for 4 hr, and lysed in a low detergent lysate buffer. The PP2A activity in protein lysates was determined with a kit purchased from Promega that selectively tests for PP2A activity. Since direct addition of 10 nM OA to the assay buffer resulted in >90% inhibition of phosphatase activity with IC_{50} values within a range of 0.6 to 1.5 nM, we conclude that the phosphatase observed is likely PP2A or a PP2A-like phosphatase. Salvicine increased PP2A activity in a concentration-dependent manner, in that low concentrations (1 μM) only slightly elevated PP2A activity, while at 5 and 10 μM activity was increased markedly to about 1.6- and 2.3-fold that of the control, respectively. The effect of OA on the salvicine-induced decline in PP2A activity was observed simultaneously. Co-treatment of cells with OA completely prevented activation of PP2A activity by salvicine (Fig. 6). We conclude from the above results that down-regulation of telomerase activity during salvicine-induced apoptosis may result from PP2A activation.

3.6. Effect of caspase inhibitors on salvicine-induced PP2A activation

Caspases possibly play a central role in mediating various apoptotic responses, and activated caspase-3 induces PP2A activation during anti-Fas antibody-induced Jurkat cell apoptosis [26]. To examine whether caspase-3 is involved in salvicine-induced PP2A activation, we initially observed the role of caspase-3 in salvicine-induced HL-60 cell apoptosis. Results confirmed that salvicine induces HL-60 cell apoptosis in a caspase-3-dependent manner. Salvicine treatment increased caspase-3, rather than caspase-1 activity in HL-60 cells. Treatment with salvicine for 2 hr resulted in a 4-fold increase in caspase-3 activity. After 4 and 6 hr, 4.7- and 6.4-fold increases in caspase-3 activity were observed, respectively. However, caspase-1 activity was relatively constant during this time (Fig. 7A).

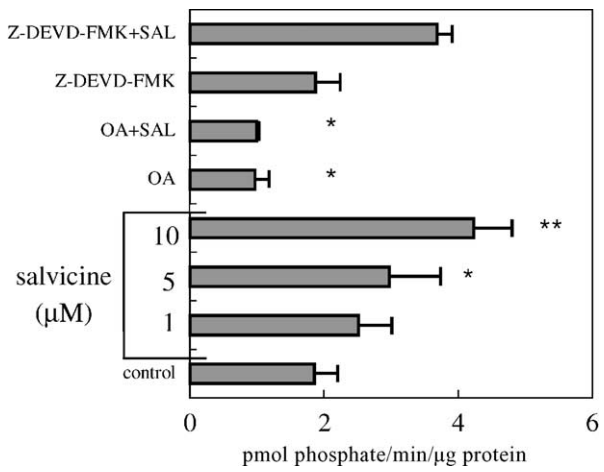

Fig. 6. Effects of salvicine (SAL), OA, and Z-DEVD-FMK on PP2A activity in HL-60 cells. HL-60 cells were treated with 10 μM salvicine (unless indicated otherwise) in the presence or absence of OA (200 nM) or Z-DEVD-FMK (200 μM) for 4 hr. PP2A activity was determined as described in Section 2. Results are expressed as means ± the range for two independent experiments. Key: (*) $P < 0.05$, and (**) $P < 0.01$ compared with the control.

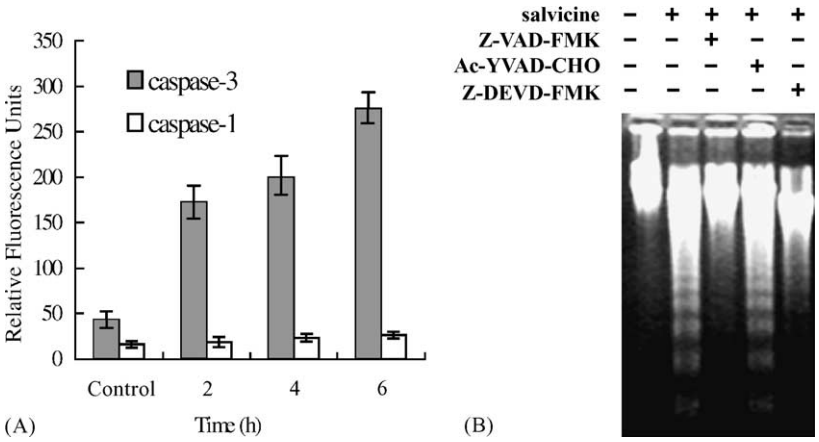

Fig. 7. Involvement of caspase-3 in salvicine-induced HL-60 cell apoptosis. (A) Changes in caspase-1 and caspase-3 activities in salvicine-induced HL-60 cell apoptosis. HL-60 cells were exposed to 10 μM salvicine for 0, 2, 4, and 6 hr, respectively. Data are shown as means ± SD (N = 3). (B) Suppression of salvicine-induced DNA fragmentation by Z-VAD-FMK and Z-DEVD-FMK, but not by Ac-YVAD-CHO.

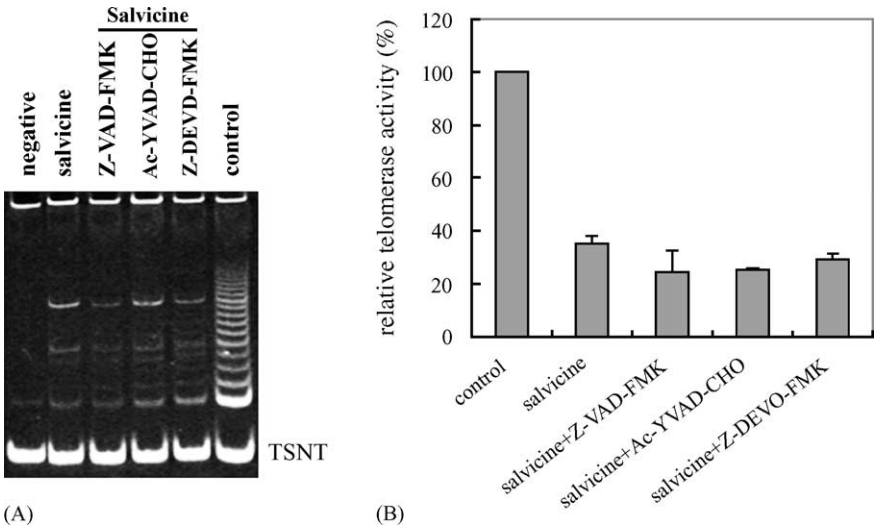

Fig. 8. Effect of caspase inhibitors on telomerase activity in HL-60 cells treated with 10 μM salvicine for 4 hr. (A) HL-60 cells were treated with 10 μM salvicine alone or in combination with a 200 μM concentration of caspase inhibitors for 4 hr. Telomerase activity was determined by the TRAP assay. Negative: no telomerase extract was added. Control: telomerase extract from untreated cells was added. TSNT: internal control. (B) Graphic representation of the experiment in panel A. The relative telomerase activity was determined by calculating the ratio of intensity of the area under the bands representing telomeric repeats in treated cells to that of the untreated control (taken as 100%) (mean ± range, N = 2).

Moreover, a general caspase inhibitor, Z-VAD-FMK, and a specific caspase-3 inhibitor, Z-DEVD-FMK, were able to block salvicine-induced DNA fragmentation. In contrast, Ac-YVAD-CHO, an inhibitor of caspase-1, had little effect (Fig. 7B).

We next determined the effect of a caspase-3 inhibitor on PP2A activity in HL-60 cells treated with salvicine. As shown in Fig. 6, Z-DEVD-FMK did not exhibit any influence on PP2A activity compared with the control, nor could it block salvicine-induced PP2A activation. Furthermore, a telomerase activity assay demonstrated that none of the three caspase inhibitors examined reversed salvicine-induced down-regulation of telomerase activity (Fig. 8). These data rule out a role for caspase-3 in PP2A activation, and suggest that down-regulation of telomerase activity is probably not the consequence of salvicine-induced apoptosis in HL-60 cells.

4. Discussion

In this study, we found a pronounced down-regulation of telomerase activity induced by salvicine in HL-60 cells, accompanied by increased rates of apoptosis. Moreover, both the decline in telomerase activity and apoptosis induction were prevented by OA. In addition, we demonstrated that 10 μM salvicine does not directly inhibit telomerase activity in a cell-free system, indicating that down-regulation of telomerase activity is closely related to salvicine-mediated apoptotic cell death.

The regulation of telomerase activity involves control at multiple levels, including gene transcription, post-translational protein–protein interactions, and protein phosphorylation/dephosphorylation [27]. The catalytic subunit of telomerase, hTERT, is possibly the rate-limiting determinant of enzymatic activity. Many studies demonstrate a correlation between hTERT mRNA expression and telomerase activity in several cell lines and tissues [22,28,29]. Moreover, in the differentiation of human leukemia cells induced by various agents, the pattern of repression of telomerase activity is comparable with decreased hTERT mRNA expression [30,31]. However, hTERT mRNA expression does not always correlate with telomerase activity. Liu *et al.* [32] reported a negative relationship between telomerase activity and hTERT mRNA expression in human lymphocytes. A recent investigation also revealed that decreased telomerase activity caused by radiation and chemotherapeutic agents parallels increased hTERT mRNA and protein expression [33]. Our data displayed a delayed decline in hTERT mRNA expression, consistent with the above observations. Taken together, these results suggest that transcriptional regulation of hTERT is not sufficient to explain the regulation of telomerase activity, and indicate a possible role for post-transcriptional factors in the control of telomerase function.

In addition to the observed delay in the decrease of hTERT mRNA expression, our studies led to the novel and unexpected finding that TP1 mRNA also was down-regulated, although it was preceded by telomerase decrease and apoptosis. TP1 cDNA encodes a 2629 amino acid sequence and produces the TP1 proteins p240 and p230. It is known that p240 is modified to p230 *in vivo*, which is the dominant form in telomerase-positive cells, suggesting that the post-transcriptional modification of TP1 is involved in the regulation of telomerase activity [34]. It is noteworthy that TP1 functions as a negative regulator of telomerase activity, as verified by its increased expression in conjunction with the down-regulation of telomerase activity in HL-60 cells by differentiating agents [35]. The present study reports a possible association between telomerase activity and repression of TP1 mRNA expression, suggesting that TP1 may function as an important regulatory component for telomerase activity. However, the function of TP1 in telomerase regulation needs to be investigated further. As salvicine is the first agent reported to decrease the expression of TP1 mRNA, at least in the context of apoptosis, it may be used as a tool to study the role of TP1 in the regulation of telomerase activity.

In contrast to the down-regulation of the two subunits, TP1 and hTERT, no change in hTR mRNA expression was observed in apoptotic HL-60 cells, suggesting that its expression does not correlate with telomerase activity. This result is in accordance with a previous report that hTR is not a predictor of telomerase activity, since it is expressed in both telomerase-negative and -positive cells [36].

Phosphorylation of hTERT and TP1 protein at unknown sites is reported to play an important role in the regulation of telomerase activity [27,37]. The reverse phosphorylation of proteins regulated by protein kinase and phosphatase plays a crucial role in a variety of cellular processes, such as differentiation, proliferation, and apoptosis [38]. PP2A is the most abundant serine/threonine-specific phosphatase in mammals, involved in the oncogenic process [39]. Some agents induce cell apoptosis by increasing PP2A activity [40,41]. Moreover, PP2A plays an important role in the regulation of telomerase activity. Li *et al.* [25] showed that PP2A inhibited nuclear telomerase activity in human breast cancer cells by dephosphorylation of a telomerase component, which was prevented by OA. Based on these experimental data, we hypothesized that salvicine-induced down-regulation of telomerase activity in apoptotic cells may be mediated by increasing PP2A activity. The results presented in this study confirm this hypothesis as follows: first, PP2A activity increased markedly in salvicine-induced HL-60 cell apoptosis in a concentration-dependent manner; and second, inhibition of PP2A activity by OA blocked salvicine-induced HL-60 cell apoptosis completely, and markedly reversed the down-regulation of telomerase activity. OA is a crude inhibitor of protein phosphatases. However, a selective inhibition of PP2A activity can be obtained by limiting its concentration. OA can be applied to cells up to a level of 1 μM without any detectable inhibition of protein phosphatase 1 (PP1) or other major serine/threonine-phosphatase activities (i.e. protein phosphatase 2B and 2C) [38,42]. In this study, the inhibition rate of 200 nM OA on PP2A activity was about 50%, suggesting that the effect of 200 nM OA on PP2A is specific, at least relatively. Therefore, although we did not examine the effects of OA treatment on any protein phosphatases other than PP2A, we assume that PP2A plays a more important role than PP1 in salvicine-induced HL-60 cell apoptosis. The role of PP2A in mediating the inhibition of telomerase activity is ascertained, since the other major serine/threonine-phosphatases have no effect on telomerase activity [25].

The execution stage of apoptosis in mammalian cells is thought to be initiated by specific caspases. Caspases cleave key structural components of the cytoskeleton and nucleus, as well as numerous proteins involved in signaling pathways. Among them, caspase-3 has been implicated as a key mediator of apoptosis in mammalian cells. Santoro *et al.* [26] observed that activated caspase-3 caused up-regulation of PP2A activity by cleavage of PP2A in the process of anti-Fas antibody-induced Jurkat cell apoptosis. However, our data are not consistent with this observation. Although activated caspase-3 was involved in salvicine-induced apoptosis, a specific caspase-3 inhibitor did not reverse induction of PP2A activity and reduction of telomerase activity induced by salvicine, suggesting that caspase-3 may not be an activator of PP2A in this process. Another proposed upstream factor of PP2A

activation in apoptosis is ceramide, one of the lipid-like second messengers [41,43–45]. PP2A has been identified as a member of the ceramide-activated protein phosphatase group, and therefore is implicated in mediating the effects of ceramide on cell growth and apoptosis [45]. Whether PP2A activation is induced by a rise in ceramide levels remains to be elucidated.

To date, published data on changes in telomerase activity during cell death induced by chemotherapeutic agents are conflicting. We previously reported that telomerase activity was down-regulated significantly during camptothecin-induced HL-60 cell apoptosis [9]. Another study also demonstrated both telomerase inhibition and apoptosis induction in hexamethylene bisacetamide-treated human colon carcinoma LoVo cells [10]. However, Sato *et al*. [11] showed that exposure of human pancreatic cancer cells to a topoisomerase inhibitor, etoposide, at various concentrations (1–30 μM), resulted in a 2- to 3-fold increase in telomerase activity. Zhang *et al*. [12] reported that the telomerase activity in apoptotic HL-60 cells, induced by 20 μg/mL of puromycin or 5 μg/mL of actinomycin for 6–12 hr, remained unchanged. These data suggest that down-regulation of telomerase activity is probably not a certain consequence of apoptosis. It has been well recognized that change in telomerase activity by anticancer drugs is not mediated by their direct action on telomerase. We also confirmed that salvicine did not inhibit telomerase activity in a cell-free TRAP assay (Fig. 2E). Changes in telomerase activity induced by anticancer drugs are reported to be probably mediated by some cellular event such as cell cycle [33,46], DNA damage and repair [11,47], or telomere erosion [7,48]. Our study suggests that anticancer drugs that affect a modulator of telomerase activity (such as PP2A) may change telomerase activity. However, is it a common event that PP2A activation caused by anticancer drugs decreases telomerase activity? In addition, some anticancer drugs are known to have no effect on telomerase activity [12], which raises the question of whether their lack of effect stems from these drugs having no effect on a modulator of telomerase activity (such as PP2A), or on other cellular events related to telomerase regulation. These questions are of great interest to us, and relative work is in preparation in our laboratory. Therefore, the above conflicting phenomenon could be explained in part by inducer- and cell-type-specific modulation of telomerase activity. Additionally, our results suggest that the down-regulation of telomerase is not the consequence of salvicine-induced HL-60 cell apoptosis, because the caspase inhibitors could not prevent the decrease of telomerase activity induced by salvicine. It has been reported that the anti-apoptotic action of telomerase is exerted at a relatively early stage in the cell death process, prior to caspase activation [5], suggesting that down-regulation of telomerase activity may be the upstream event of caspase-3 activation or apoptosis. However, we cannot make a decision yet because the distinct relationship of telomerase and caspase is still unclear. There is another possibility that both telomerase down-regulation and apoptosis induced by salvicine are independent secondary events of PP2A activation. Therefore, further studies will be required to delineate whether or how a decrease of telomerase activity contributes to anticancer drug-induced tumor cell apoptosis.

In conclusion, we have provided some evidence that down-regulation of telomerase activity is related to salvicine-induced HL-60 cell apoptosis: (a) treatment with salvicine resulted in a decrease in telomerase activity and apoptosis of HL-60 cells; and (b) co-treatment with OA prevented multiple features of apoptosis and reversed the decline in telomerase activity. Our results suggest that protein dephosphorylation of a telomerase subunit plays a more important role than transcription in the salvicine-induced decrease of telomerase activity. We additionally conclude that PP2A activation by salvicine may not be caused by the activation of caspase-3.

Acknowledgments

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