Biotechnology and Bioprocess Engineering **2006**, 11: 7-12

Inhibition of Telomerase Activity in U937 Human Monocytic Leukemia Cells by Compound K, a Ginseng Saponin Metabolite

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Abstract Telomerase activation is detected in most cancerous cells; hence, telomerase is a highly selective target for cancer therapy, which plays an important role in the apoptotic process. We have previously reported that the ginseng saponin metabolite, Compound K (20-*O*-D-glucopyranosyl-20(*S*)-protopanaxadiol, IH901), inhibits cell proliferation by inducing apoptosis and cell cycle arrest at the G_1 phase. The present study investigated the regulation of telomerase activity in Compound K treated U937 cells. Compound K treatment caused a reduction in telomerase activity and down-regulated the human telomerase reverse transcriptase (hTERT) gene, resulting in the decreased expressions of its protein, and of the c-Myc and Sp1 proteins (transcription factors of hTERT). These results indicate that the anticancer activity of Compound K could be mediated by inhibition of the telomerase activity.

Keywords: Compound K, telomerase, human telomerase reverse transcriptase

INTRODUCTION

Telomerase is a cellular RNA-dependent DNA polymerase, which serves to maintain the tandem arrays of telomeric TTAGGG repeats at eukaryotic chromosome ends [1]. Telomeres are highly conserved in organisms, ranging from unicellular eukaryotes to mammals, indicating the widespread utility of their protective mechanisms for preventing chromosomal ends from undergoing degradation and ligation with other chromosomes. Without telomeric caps, human chromosomes would undergo end-to-end fusion, with the formation of dicentric and multicentric chromosomes [2,3]. These abnormal chromosomes would break during mitosis, resulting in severe damage to the genome and the activation of DNA damage checkpoints, leading to cell senescence or the initiation of apoptotic cell death pathways [4]. It has been demonstrated that telomerase inhibition can result in telomere shortening, followed by proliferation arrest and cell death by apoptosis [5-9]. This makes telomerase as a target not only for cancer diagnosis, but also for the development of novel therapeutic agents [10].

Recently, ginseng saponin metabolites formed by intestinal bacteria have been identified after the oral administration of ginseng extract in both humans and rats [11]. 20-*O*-(β-D-glucopyranosyl)-20(*S*)-protopanaxadiol(Compound K, IH-901; Fig. 1), one of the major metabolites of ginseng, was detected in the urine and blood after the administration of total ginseng saponins to rat. We recently reported that Compound K showed cytotoxicity by the induction of apoptosis through a caspase depen-dent pathway via mitochondria disruption [12] and by the induction of cell cycle arrest at the G_1 phase [13]. Compound K is also known to inhibit glucose uptake by tumor cells [14], reverse multidrug resistance in tumor cells [15], possess chemo-preventive activity against chemical carcinogens [16], show anti-metastatic effect *in vivo* [17] and exhibit anti-tumor effect due to the inhibition of TPA-induced COX-2 expression [18,19].

The present study investigated whether Compound K inhibits the telomerase activity, and the possible mechanisms of telomerase regulation within this process.

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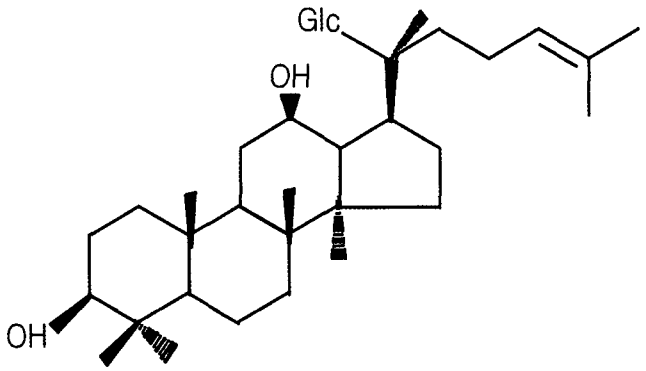

Fig. 1. Chemical structure of Compound K, (20-*O*-(β-D-glucopyranosyl)-20(*S*)-protopanaxadiol, IH-901).

MATERIALS AND METHODS

Chemicals

Compound K was prepared by the incubation of protopanaxadiol type ginsenosides with *Bacteroides* JY-6, a human intestinal bacterium, sub-cultured in a general anaerobic medium for 24 h at 37°C. The incubated medium was extracted with BuOH, the supernatant concentrated *in vacuo* and processed using silica gel column chromatography, with $CHCl_3$-MeOH-H_2O (65:35:10, v/v) as the mobile phase. The isolated Compound K was characterized by mass spectroscopy and ^1H-and ^{13}C-nuclear magnetic resonance (NMR) spectrometries. Co-mpound K was freshly dissolved in dimethyl sulfoxide (DMSO), to a final concentration not exceeding 0.2%.

Cell Line and Cell Culture

U937 (a human monocytic leukemia cell line), ob tained from the American Type Culture Collection (ATCC, Rockville, MD, USA) was cultured at 37°C in RPMI1640 (Gibco BRL, Gaithersburg, MD, USA), containing 2 mM glutamine, 10% heat-inactivated fetal calf serum, penicillin (100 units/mL) and streptomycin (100 μg/mL), under an atmosphere of 5% CO_2.

Flow Cytometry

Flow cytometric analysis of the DNA content was performed to assess the sub-G_1 hypo-diploid cells (apoptotic cells) [20,21]. 1×10^6 cells were harvested at various times after treatment with Compound K, and fixed for 1 h at 4°C in 1 mL of 70% ethanol. The cells were then washed twice with phosphate buffered saline (PBS) and incubated in the dark at 37°C for 30 min, with 100 μg/mL propidium iodide and 100 μg/mL RNase A. The flow cytometric analysis was performed using a FACSCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA). The effect of Compound K on apoptosis was determined by the degree of increase in the population of sub-G_1 hypo-diploid cells.

Telomeric Repeat Amplification Protocol (TRAP) Assay

The telomerase activity was measured using the telomere repeat amplification protocol (TRAP), with a Telosay™ Telomerase assay kit (Intron Biotechnology, Sungnam, Korea). After PCR, electrophoresis was performed on a 15% non-denaturing acrylamide gel, with the bands subsequently detected using a silver staining kit (Intron Biotechnology).

RNA Extraction and Reverse Transcription

Total RNA was extracted using TRIzol reagent (Gibco BRL), according to instructions of manufacturer. cDNA synthesis was performed at 42°C for 30 min, 99°C for 5 min, and 4°C for 5 min in 20 μL reaction mixture, with 1 μg total RNA, using the reverse transcription kit (Promega, Madison, WI, USA).

PCR Amplification

Primers used for the hTERT-PCR were those present in the Light Cycler TeloTAGGGhTERT Quantification kit (Roche Diagnostics, Mannheim, Germany), with the amplification carried out according to the instructions of the manufacturer [22]. Relative expression levels were calculated by quantifying the hTERT levels normalized to the porphobilinogendeaminase gene (PBGD).

Western Blot Analysis

Cells were harvested at various times after treatment with Compound K, and washed twice with PBS. The harvested cells were then lysed, on ice for 30 min, in lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), and 0.1% NP 40] and centrifuged at 13,000 × g for 15 min. The supernatant was collected, and the protein concentration determined. Cellular proteins were boiled for 5 min, with 40 μg subsequently subjected to electrophoresis on a 10% SDS-polyacrylamide gel. Blots were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA), which was then incubated with primary rabbit polyclonal anti-caspase 3, -TERT, -c-Myc, and -Sp1 antibodies. The gel was further incubated with goat anti-rabbit immunoglobulin G-horseradish peroxidase-conjugates (Pierce, Rockford, IL, USA), and then exposed to X-ray film. The protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

RESULTS

The ability of Compound K to induce apoptosis was determined by flow cytometric analysis, which showed that the apoptotic populations were 2, 15, 40, and 58% at 0, 24, 48, and 72 h, respectively, after treatment with 30 μg/mL of Compound K, which is the IC_{50} value of Compound K on U937 (Fig. 2A). The expression of caspase 3

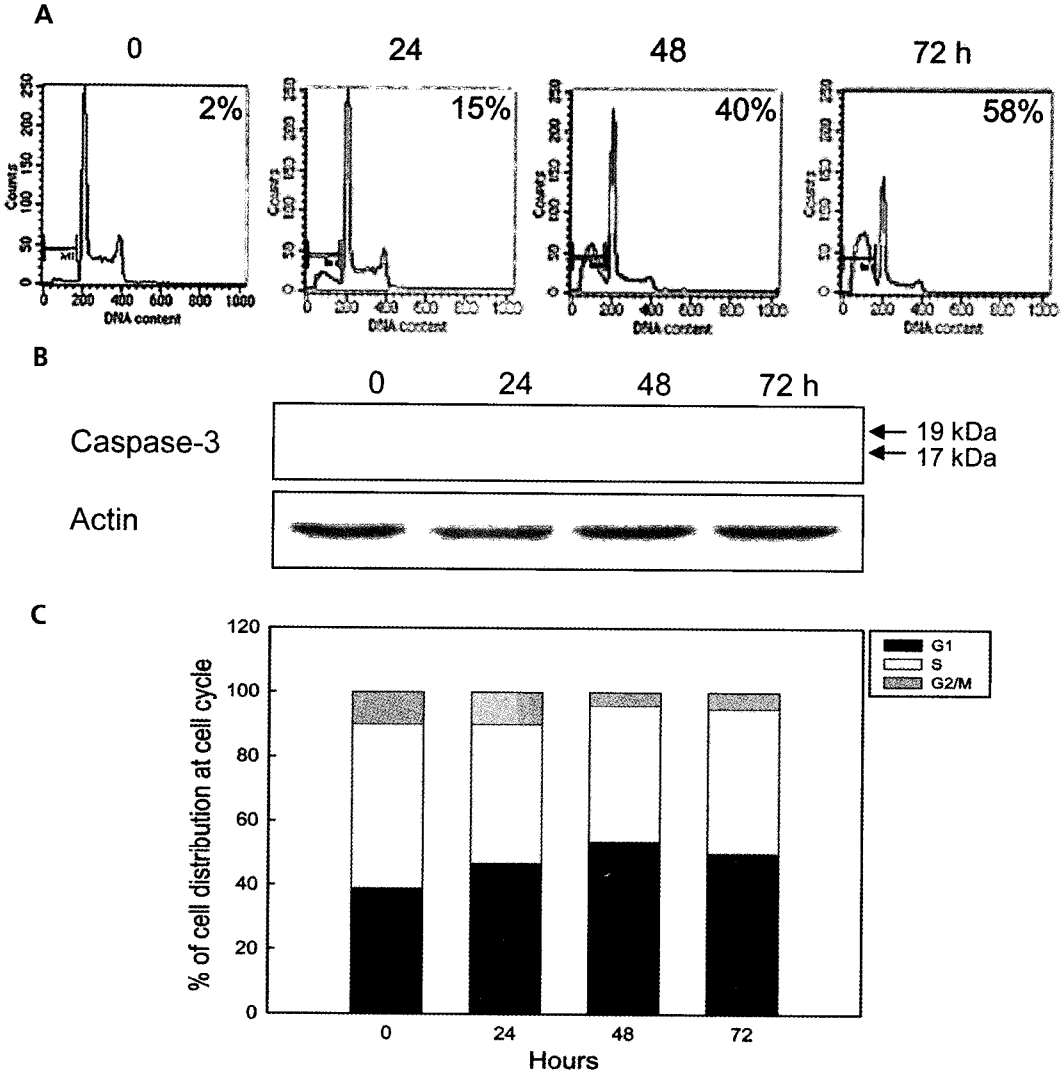

Fig. 2. Patterns of apoptosis and cell cycle arrest induced by Compound K in U937 cells. Cells were cultured for 24, 48, and 72 h, with 30 μg/mL of Compound K. Apoptotic cells and the distribution of the cell cycle, respectively, were quantified by flow cytometry (A and C), and the change in caspase 3 detected using a Western blot assay (B).

one of the active forms of apoptosis executors, increased with time (Fig. 2B). These findings suggest that Compound K induces apoptosis of U937 cell in a time-dependent manner. As shown in Fig. 2C, Compound K also induced the cell cycle arrest at the G_1 phase. The percentage of G_1 phase of Compound K treated cells at 24 h increased to 47%, relative to the 39% of the control, and this pattern was retained for 72 h. These findings show that Compound K inhibits cell growth through apoptosis and via cell cycle arrest of the G_1 phase. Next, whether the telomerase activity was altered in the Compound K induced growth inhibition of U937 cells was determined. The TRAP data showed that the inhibition of the telomerase activity in Compound K treated cells was decreased in a time dependent manner (Fig. 3A). As shown in Fig. 3B, when the relative intensity of the telomerase activity in untreated U937 cells was taken as 1.0, the intensity of the telomerase activities in the cells treated with Compound K for 24, 48, and 72 h were 0.45, 0.28, and 0.21, respectively. To understand the mechanism of the telomerase activity inhibition due to treatment with Compound K, the effects of Compound K on the expression of hTERT, one of telomerase components, were examined at the mRNA level using real time quantitative RT-PCR employing a Light Cycler TeloTAGGGhTERT Quantification kit. As shown in Fig. 4A, Compound K treatment resulted in a decrease of the hTERT mRNA levels at 24 h, and this pattern was consistent with the expression of the hTERT protein level (Fig. 4B). This data suggests that decreased hTERT transcription plays a crucial role in the regulation of the telomerase activity in Compound K induced growth inhibition. To determine the effect of Compound K on the expression of the c-Myc and Sp1 proteins; the transcription factor of hTERT, the

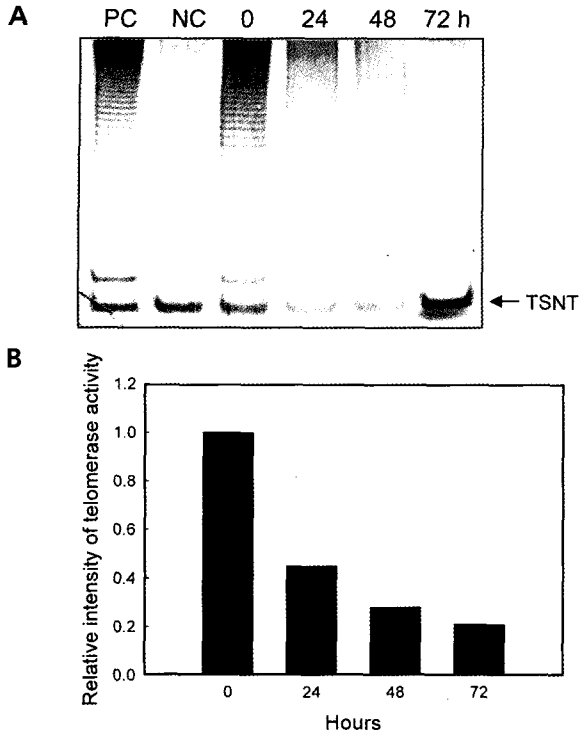

Fig. 3. Inhibition of telomerase activity in Compound K treated U937 cells. (A) Cells were cultured for 24, 48, and 72 h, with 30 μg/mL of Compound K. The telomerase activity was measured using the TRAP method. Positive control (PC): 0.02 μg telomerase protein, negative control (NC): 0.02 μg heat-inactivated telomerase protein. TSNT: internal standard. (B) The relative intensity of telomerase activity was determined by calculating the ratio of the intensity of the area under the bands representing telomeric repeats in Compound K treated cells to that of the untreated control (taken as 1.00).

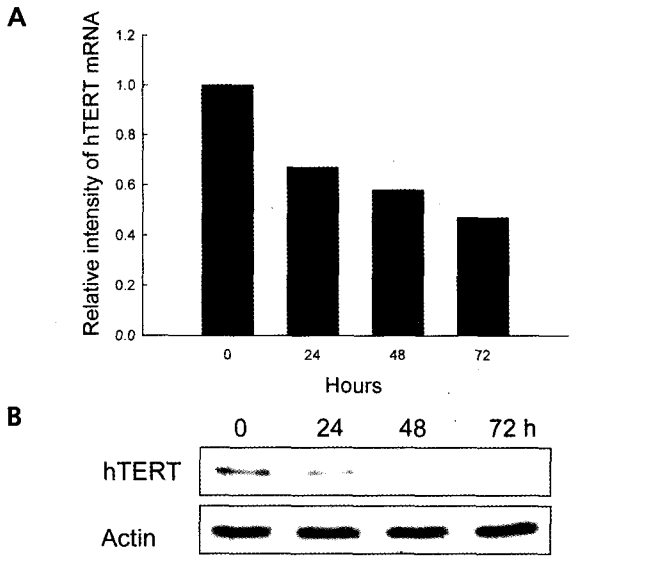

Fig. 4. Down-regulation of hTERT mRNA and telomerase protein by Compound K treatment. Cells were cultured for 24, 48, and 72 h, with 30 μg/mL of Compound K. (A) Expression of hTERT mRNA was examined by real time quantitative RT-PCR, using a Light Cycler TeloTAGGGhTERT Quantification kit. The relative intensity of hTERT mRNA was determined by calculating the ratio of the intensity of fluorescence representing hTERT mRNA in Compound K treated cells to that of the untreated control (taken as 1.00). (B) Expression of TERT protein was examined by Western blot analysis.

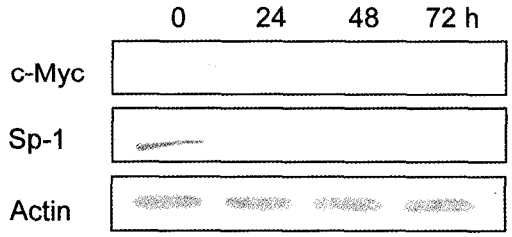

Fig. 5. The decreased expressions of c-Myc and Sp1 proteins by Compound K treatment. Cells were cultured for 24, 48, and 72 h, with 30 μg/mL of Compound K. Expressions of the c-Myc and Sp1 protein were examined by Western blot analysis.

protein levels of c-Myc and Sp1 in Compound K treated cells were examined by Western blot assay. As shown in Fig. 5, significantly decreased expressions were observed for both proteins in Compound K treated cells. These findings suggest a correlation between the expression of c-Myc and Sp1 and the level of hTERT transcription in Compound K treated cells.

DISCUSSION

Telomerase is an RNA-containing reverse transcriptase, responsible for elongation of shortened telomere, with the maintenance of the telomere length being crucial for cell survival [23-25]. Telomerase inhibitors are being considered as potential anticancer agents due to the high levels of telomerase activity detected in various cancer cells, but not in normal tissue [26,27]. Recent studies have revealed that telomerase plays an important role in suppressing the apoptotic cascade [28,29]. Apoptosis, often synonymously used with the term "programmed cell death", is genetically controlled form of cell death, which appears to be involved in tumor cell killing by anticancer drugs and cellular targeting [30,31]. Alterations in the expression of genes affecting cell cycle progression occur in human cancers [32]. Recently, we reported that Compound K showed cytotoxicity due to the induction of apoptosis through a caspase dependent pathway, via mitochondria disruption and cell cycle arrest at the G_1 phase [12,13].

hTERT (human telomerase reverse transcriptase), a telomerase-component gene, plays a pivotal role in telomerase activity, due to its high level of expression in cancer cells [26,27,33,34]. Compound K was found to reduce the expression of hTERT mRNA and its protein. These results suggest that inhibition of the functional telom-

erase activity by Compound K may have resulted from the down-regulation of hTERT. The levels of c-Myc and Sp1 expression have been reported to correlate with the transcriptional activity of hTERT, and the expressions of c-Myc and Sp1 increased during malignant progression, in association with the telomerase activity [35]. This report suggested that c-Myc and Sp1 cooperatively function to activate transcription of the hTERT gene, with the up-regulation of these expressions possibly being critical for telomerase activation during carcinogenesis. Compound K modulated the expressions of c-Myc and Sp1 proteins. This finding implies that Compound K may, in turn, affect certain regulatory elements within the hTERT promoter, thereby leading to the down-regulation of hTERT expression.

CONCLUSION

The present study has demonstrated that Compound K reduced the activity of telomerase via down-regulation of the hTERT gene in U937 human leukemia cells, resulting in the decreased expressions of its protein, and of the c-Myc and Sp1 proteins. These results indicate that the anticancer activity of Compound K could be mediated by inhibition of the telomerase activity as well as by induction of apoptosis and arrest of cell cycle.

Acknowledgements This work was supported by a Korea Research Foundation Grant (KRF-2004-002-E00027).

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[Received August 2, 2005; accepted September 24, 2005]