EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 33, No. 3, 156-163, September 2001

Protein kinase C modulates telomerase activity In human cervical cancer cells

Yong Wook Kim[1*], Soo Young Hur[1*], Tae Eung Kim[1], Joon Mo Lee[1], Sung Eun Namkoong[1], In-Kyung Kim[2,3] and Jin Woo Kim[1,3,4]

[1] Department of Obstetrics and Gynecology
[2] Department of Biochemistry
[3] Molecular Genetic Laboratory, Catholic Research Institutes of Medical Science, College of Medicine, The Catholic University of Korea, 505 Banpodong, Seochoku, Seoul, Korea
[4] Corresponding author: Tel, +82-2-590-2389; Fax, +82-2-593-2389; E-mail, jinwoo@cmc.cuk.ac.kr
*These authors contributed equally to this work.

Accepted 18 September, 2001

Abbreviations: PKC, protein kinase C; TPA, 12-*0*-tetradecanoyl phorbol 13-acetate; hTR, human telomerase RNA; hTERT, human telomerase reverse transcriptase; RT-PCR, reverse transcription-polymerase chain reaction

Abstract

Telomerase, a ribonucleoprotein reverse transcriptase that extends telomeres of eukaryotic chromosomes is repressed in normal somatic cells but is activated during development and neoplasia. The regulation mechanism of telomerase activity in cancer cells is not clearly known. In this report, a possible affect of PKC on telomerase activity was examined using HeLa and CUMC-6 cervical cancer cell lines. Exposure of cells to PKC inhibitor, bisindolylmaleimide I and Gö6976, and high levels of PKC activator, 12-*0*-tetradecanoyl phorbol 13-acetate (TPA) resulted in the inhibition of PKC activity in both cells. Telomerase activities were also inhibited by bisindolyl-maleimide I and Gö6976, respectively, in a time-dependent manner. As PKC activity changes in TPA-treated cervical cancer cells, telomerase activities were increased at low dose of TPA and decreased at high dose. The expression levels of human telomerase subunits, human telomerase RNA (hTR) were not influenced by PKC modulating drugs. In contrast, the expression of full-length human telomerase reverse transcriptase (hTERT) was decreased after exposure to bisindolylmaleimide I and Gö6976 in a time-dependent manner. hTERT expression was not affected by low dose of TPA. In contrast, high dose of TPA inhibited hTERT expression level. But the expression patterns of β-deletion transcript of hTERT after 72 h of treatment with PKC inhibitors or high dose of TPA exposure were not discernable as compared with those of full-length hTERT transcripts to PKC modulating drugs. These results suggest that PKC-modulating drugs altered telomerase activities by affecting full-length hTERT expression profile in human cervical cancers.

Keywords: Cervical cancer, PKC, telomerase, hTERT, hTR

Introduction

Telomerase, a specialized ribonucleoprotein reverse transcriptase that extends hexanucleotides (TTAGGG) telomeres onto chromosomal ends is repressed in normal somatic cells but is activated during development and neoplasia. Telomerase activity has been detected in germline cells (Meeker and Coffey, 1997) and in the majority of immortal and tumor cells (Shay and Bacchetti, 1997; Park *et al.,* 1998), but not in normal somatic cells except in some self-renewing cells with high regenerative potential such as hematopoietic (Hiyama *et al*., 1995) and endometrial cells (Kyo *et al*., 1997). These findings have led to the hypothesis that telomerase reactivation represents a critical step in the neoplastic process.

Three major components of human telomerase have been cloned: the RNA component of human telomerase which was first identified and functions as a template for telomere elongation (Feng *et al*., 1995), telomerase-associated protein 1/telomerase protein component 1 (TP1/TLP1) which is similar to the *Tetrahymena* telomerase protein p80 (Nakayama *et al*., 1997), and the catalytic subunit of human telomerase, hTERT, which confers the reverse transcriptase activity (Lingner *et al*., 1997).

Some studies have shown that the expression of hTR is higher in more progressive cancers (Soder *et al*., 1997; Yashima *et al*., 1997). But the expression of hTR is not a predictor of telomerase activity, because hTR is expressed in all cells (Avilion *et al*., 1996). Similarly, TP1/TLP1 is also ubiquitously expressed (Nakayama *et al*., 1997). In contrast, the expression of hTERT mRNA is associated with telomerase enzyme activity and concomitant immortalization, and introduction of hTERT cDNA into normal human epithelial cells and fibroblasts confers telomerase activity in these cells (Weinrich *et*

al., 1997; Counter *et al*., 1998). hTERT-expressing normal cell clones have an extended life span without any changes in karyotype (Bodnar *et al*., 1998). These findings strongly suggest that hTERT is the limiting component for telomerase activity. hTERT transcript undergoes alternative splicing in several tissues and cell lines (Kilian *et al*., 1997; Ulaner *et al*., 1998). Many of the alternatively spliced transcripts do not code for functional reverse transcriptases. Although the function of these transcripts is unknown, alternative splicing of the hTERT transcript may be important for the regulation of telomerase activity and may give rise to proteins with different biochemical functions. Human telomerase has been proposed as a novel and potentially highly selective target for diagnostic and therapeutic application against tumors (Raymond *et al*., 1996). However, little is known concerning the molecular mechanisms by which telomerase is activated in human tumors.

PKC plays an important role in transmembrane signal transduction and induction of many cellular responses including cell proliferation, differentiation, and gene expression (Nishizuka, 1995). Some evidences are provided that alterations in PKC activity are involved in malignant transformation processes (Persons *et al*., 1988). In addition, several inhibitors of PKC have been reported to exert antitumor activity *in vitro* and *in vivo* (Minana *et al*., 1991). It has been shown that PKC inhibitors produce a significant inhibition of telomerase activity in treated cells (Ku *et al*., 1997), and PKC mediates the phosphorylation of hTERT and induces a marked increase in telomerase activity (Li *et al*., 1998). These findings suggest that PKC can directly modulate telomerase activity in tumor cells, including nasopharyngeal and breast cancers. Accordingly, it is possible that PKC may alter the expression of full-length hTERT or alternative splicing of the hTERT transcript in some types of cancers.

But, up to date, it has not been reported whether PKC can alter the expression profiles of hTERT in uterine cervical cancer. Accordingly, we have investigated the telomerase activities and the expressions of hTR, hTERT, and alternative splicing of the hTERT transcript in cervical cancer cells after treatment with PKC-modulating drugs.

Materials and Methods

Cell culture

Human cervical cancer cell line, HeLa, was obtained from American Type Culture Collection (Rockville, MD). Human cervical cancer cell line, CUMC-6, was established *in vitro* from primary tumors (Kim *et al*., 1997). Cells were maintained at 37°C in humidified atmosphere of 5% CO_2 in Waymouth's MB 752/1 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (Gibco-BRL).

Drug treatment

PKC inhibitors, bisindolylmaleimeide I was purchased from ICN Biomedicals (Aurora, OH) and Gö6976 from Calbiochem (La Jolla, CA). PKC activator, TPA was obtained from Sigma Chemicals (St. Louis, MO). Exponentially growing cells were treated with bisindolylmaleimeide I (5 µM and 10 µM), Gö6976 (1 µM and 5 µM), and TPA (10 nM and 1 µM) for indicated times, respectively.

PKC activity assay

PKC activity was measured using the SignaTECT™ Protein Kinase C Assay System kit (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, 1×10^7 cultured cells were washed with cold phosphate-buffered saline (PBS) and suspended in 500 µl of cold extraction buffer containing 0.5 mM EDTA, 0.5 mM EGTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 25 mM Tris-HCl (pH 7.4). Cells were homogenized with 20 strokes of a cold Dounce homogenizer and centrifuged at 14,000 *g* for 7 min at 4°C. The supernatant was passed over DEAE-cellulose ion exchange column (Whatman DE52; 0.25 g/column) that was pre-equilibrated in extraction buffer and the PKC-containing fraction was eluted with same buffer containing 200 mM NaCl. Protein concentration in supernatant was determined by the Bio-Rad protein assay system (Bio-Rad, Hercules, CA). The reaction was initiated by the addition of enzyme samples to a reaction mixture (total volume 25 µl) consisting of 100 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 1.25 mM EGTA, 2 mM $CaCl_2$, 0.5 mg/ml BSA, 1.6 mg/ml phosphatidylserine, 0.16 mg/ml diacylglycerol, PKC biotinylated peptide substrate, 0.5 mM ATP, and 10 µCi/µl [γ-^{32}P] ATP (3,000 Ci/mmol; Amersham). After incubation for 5 min at 30°C, reactions were terminated by addition of 7.5 M guanidine hydrochloride. Samples were spotted onto SAM^{2TM} biotin capture membrane and then washed with 2 M NaCl in H_3PO_4, and the deionized water. Radioactivity was counted using a liquid scintillation counter. The PKC activity was defined as the difference in counts per minute incorporated into substrate in the absence and presence of phospholipid.

Telomerase assay

Telomerase activity was determined by using the telomerase PCR-enzyme-linked immunosorbent assay (ELISA) kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. In brief, cells were washed in cold PBS, trypsinized, counted and spun at 3,000 *g*. The cells were resuspended at a density of

2×10^5 cells per 200 μl ice-cold lysis buffer provided in the kit and incubated for 30 min on ice. The lysates were centrifuged at 16,000 *g* for 20 min at 4°C, and supernatants were transferred into fresh Eppendorf tubes and stored at -80°C until use. Volumes of cell extracts, equivalent to 2×10^3 cells, were used in the telomerase PCR-ELISA assay. Cell extracts were incubated in the presence of biotin-labeled P1-TS primers at 25°C for 20 min. Telomerase present in cell extracts was allowed to elongate telomeric primers during incubation, and the telomerase activity was inactivated at 94°C for 5 min. The extended products were then amplified in a Gene Amp PCR System 9600 thermal cycler (Perkin-Elmer) for 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, polymerization at 72°C for 90 s, and a final cycle at 72°C for 10 min. Five microliters of each amplification product were denatured, hybridized to a digoxigenin (DIG)-labeled detection probe complementary to telomeric repeat sequences, and bound to a streptavidin-coated microtiter plate. Afterward, wells were incubated with anti-DIG-peroxidase-labeled polyclonal antibody for 30 min at room temperature. Finally, the immobilized PCR products were then visualized by color development after the addition of peroxidase substrate (3,3',5,5'-tetramethylbenzidine) and the absorbance of samples was measured with ELISA reader (LB 9260 MicroScreener, EG&G, Berthold, Germany) at a wavelength of 450 nm (reference wavelength, 620 nm). Telomerase activity was measured as duplicates and a negative as well as a positive control was run each time. Mean optical density (OD) values of duplicated measurements were recorded as the telomerase activity. A negative control was provided for each extract by heat inactivation of the telomerase enzyme at 95°C for 10 min. Immortalized human kidney cells (293 cells) provided in the kit were used as the positive control.

RT-PCR analysis

The expression of hTR and hTERT was analyzed by RT-PCR. Total RNAs from cervical cancer cell lines were reverse transcribed using random hexamer (Perkin Elmer) and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI). Twenty microliter of the cDNA reaction mixture was incubated at 42°C for 60 min, heated at 95°C for 5 min, and then slowly cooled to 4°C. PCR was carried out in a 20 μl reaction mixture containing 1.5 μl RT reaction mixture, 100 μM dNTPs, 10 pmol each oligonucleotide primer, 10 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and 0.5 units *Taq* polymerase. The PCR mixture was overlaid with mineral oil and then amplified in a thermal cycler (Perkin-Elmer). The amplification profiles were as follows: 28 cycles of denaturation at 95°C for 20 s, primer annealing at 68°C for 40 s, and extension at 72°C for 30 s for hTR; 33 cycles of denaturation at 95°C for 20 s, primer annealing at 68°C for 40 s, and extension at 72°C for 30 s for the first hTERT; and 35 cycles of denaturation at 95°C for 25 s, primer annealing at 68°C for 50 s, and extension at 72°C for 50 s for the second hTERT. β-actin was used to control differences in amplification or losses of cDNA sample during loading. For β-actin amplification, temperature for primer annealing was reduced to 58°C. The sequences of oligonucleotide primers, synthesized according to the published hTR (GenBank accession number: U86046), hTERT (GenBank accession number: AF015950) (Ulaner *et al*., 1998), and β-actin cDNA sequences (Ng *et al*., 1985), were as follows: for hTR, forward, 5'-CTAACCCTAACTGAGAAGGGCGTAG-3' and reverse, 5'-GAAGGCGGCAGGCCGAGGCTTTTCC-3'; for the first hTERT, forward (hTERT-1784S), 5'-CGGAAGAGTGTCTGGAGCAA-3' and reverse (hTERT-1928A), 5'-GGATGAAGCGGAGTCTGGA-3'; for the second hTERT, forward (hTERT-2164S), 5'-GCCTGAGCTGTACTTTGTCAA-3' and reverse (hTERT-2620A), 5'-CGCAAACAGCTTGTTCTCCATGTC-3'; for the β-actin, forward, 5'-AGGCCAACCGCGAGAAGATGACC-3' and reverse, 5'-GAAGTCCAGGGCGACGTAGCAC-3'. Amplified products were electrophoresed through 1.2% agarose gels and visualized by ethidium bromide staining. The predicted fragments amplified by PCR for hTR, the first hTERT, and β-actin transcripts were 103, 145, and 350 bp, respectively. For the second hTERT, two reported splice sites (α and β site) are within the region spanned by this primer set and four PCR products (457, 421, 275, and 239 bp) were found depending upon alternative splicing of hTERT transcript in the sample (Kilian *et al*., 1997; Ulaner *et al*., 1998).

Results

PKC activity assay

To determine whether the PKC modulating drugs influence PKC activities in two human cervical cancer cell lines, we measured total PKC activities in HeLa and CUMC-6 cells following exposure to PKC inhibitors, bisindolylmaleimide I and Gö6976, and PKC activator, TPA, respectively. In HeLa cells (Figure 1A), the basal level of PKC activity was 7.3 ± 0.67 pmol/min/mg protein $\times 10^{-2}$, and PKC activity was reduced to 6.5 ± 0.52, 2.0 ± 0.55, 3.5 ± 0.84 and 2.2 ± 0.31 pmol/min/mg protein $\times 10^{-2}$ after exposure to 5 μM bisindolylmaleimide I, 10 μM bisindolylmaleimide I, 1 μM Gö6976 and 5 μM Gö6976 for 24 h, respectively. In CUMC-6 cells (Figure 1A), the basal level of PKC activity was 5.2 ± 0.58 pmol/min/mg protein $\times 10^{-2}$ and PKC activity was reduced to 0.5 ± 0.74, 0.5 ± 0.81, 1.1 ± 0.52 and 0.3 ± 0.41 pmol/min/mg protein $\times 10^{-2}$ after exposure to 5 μM bisindolyl-

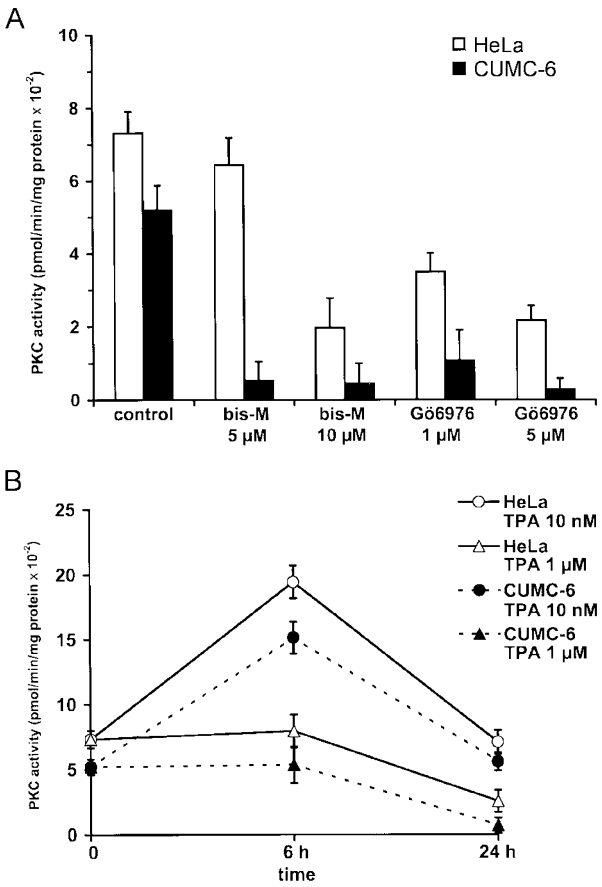

Figure 1. Effects of PKC inhibitors or activator on PKC activities in HeLa and CUMC-6 cells. (A) Cells were treated for 24 h with 5 μM and 10 μM bisindolylmaleimide I (bis-M) and 1 μM or 5 μM Gö6976, respectively. (B) Cells were treated with 10 nM and 1 μM TPA for 6 h and 24 h, respectively. Each bar represents the mean ± SD of three independent experiments.

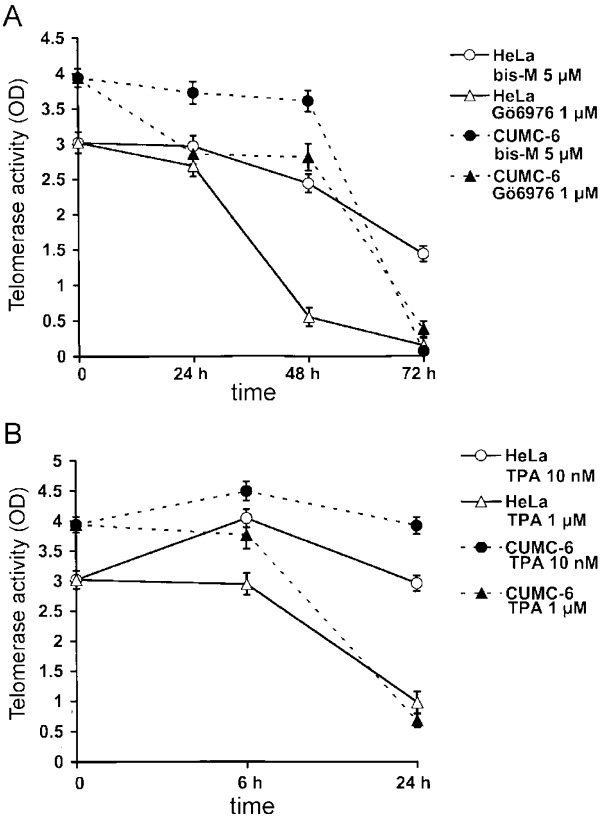

Figure 2. Telomerase activities in HeLa and CUMC-6 cells treated with 5 μM bisindolylmaleimide I (bis-M) and 1 μM Gö6976 for 24 h, 48 h and 72 h (A), 10 nM and 1 μM TPA for 6 h and 24 h (B), respectively. Each bar indicates the mean ± SD of three independent experiments.

maleimide I, 10 μM bisindolylmaleimide I, 1 μM Gö6976, and 5 μM Gö6976 for 24 h, respectively. These results demonstrate that both bisindolylmaleimide I and Gö6976 inhibited PKC activity effectively in HeLa and CUMC-6 cells.

Although TPA is an activator of PKC activity, it has previously been shown that TPA at maximal or sub-maximal concentration elicits down-regulation of PKC in response to continuous stimulation (Blobe *et al.,* 1996; Liu, 1996). To test this possibility, we measured PKC activities after exposure to low dose (10 nM) and high dose (1 μM) of TPA for 6 h and 24 h, respectively (Figure 1B). PKC activity was increased in both cell lines after exposure to 10 nM TPA for 6 h. However, no significant change of PKC activity was observed in both cell lines treated with 10 nM TPA for 24 h and 1 μM TPA for 6 h. But PKC activities were decreased in both cell lines treated with 1 μM TPA for 24 h (Figure 1B). These results suggest that although low dose (10 nM) of TPA increased PKC activities for the first few hours, continuous stimulation with high dose (1 μM) of TPA decreased PKC activities in both cell lines.

Telomerase activity

Telomerase activity was detected at high level in untreated cervical cancer cell lines, HeLa (mean OD: 3.02) and CUMC-6 (mean OD: 3.94). Five micromoles of bisindolylmaleimide I and 1 μM of Gö6976 produced a significant inhibition of telomerase activities in a time-dependent manner in the both cell lines (Figure 2A).

Treatment of the HeLa and CUMC-6 cells with 10 nM TPA for 6 h resulted in an enhanced telomerase activity compared to the control (Figure 2B). However, no significant changes of telomerase activities were observed in both cell lines treated with low dose (10 nM) of TPA for 24 h and high dose (1 μM) of TPA for 6 h. But telomerase activity after 24 h of treatment with 1 μM TPA was significantly decreased in both cell lines. Taken together, these results showed that the inhibition of telomerase activity with bisindolylmaleimide I, Gö6976 and high dose (1 μM) of TPA, and the activation of telomerase activity with low dose (10 nM) of TPA corresponded to the PKC activity changes.

Expression of hTR and hTERT

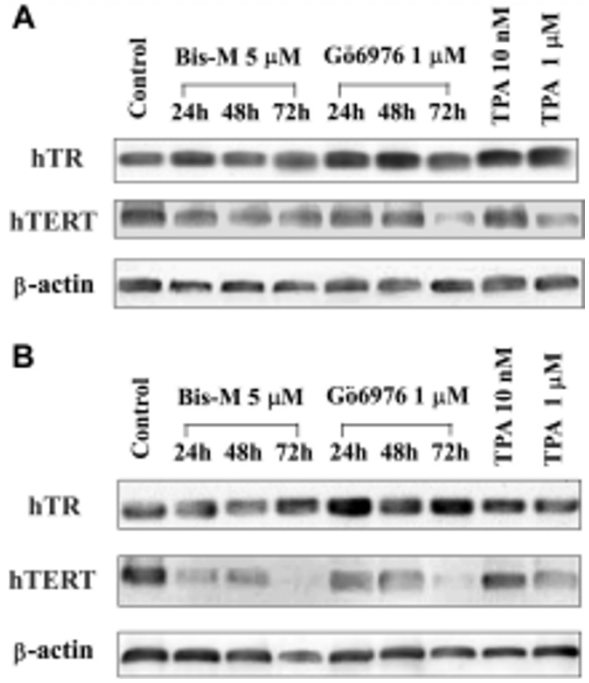

Figure 3. Expression of hTR and hTERT mRNA after exposure to PKC activator or inhibitors in HeLa (A) and CUMC-6 cells (B). hTERT mRNA expression was amplified by RT-PCR with primers hTERT-1784S and hTERT-1928A. The control amplification of β-actin mRNA is also shown.

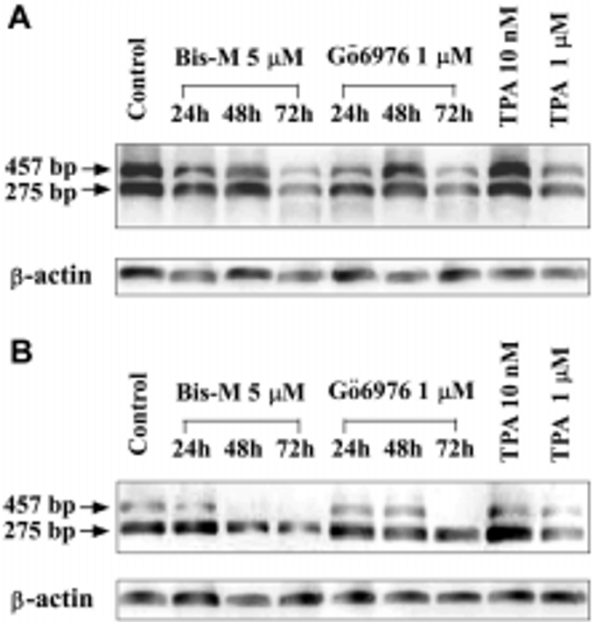

Figure 4. Expression of alternatively spliced hTERT transcripts after exposure to PKC activator or inhibitors in HeLa (A) and CUMC-6 cells (B). hTERT mRNA expression was amplified by RT-PCR with primers hTERT-2164S and hTERT-2620A, which covers the region of two potential splice sites. The control amplification of β-actin mRNA is also shown.

RT-PCR was performed to determine whether there were detectable changes in RNA expression of hTR or hTERT that might correlate with changes in telomerase activity after treatment of PKC modulating drugs. The level of expression of hTR and hTERT in each sample was determined by counting band intensity and normalizing to that of β-actin. The high level of expression of hTR was detected in untreated HeLa (Figure 3A) and CUMC-6 (Figure 3B) cells, respectively. After exposure to PKC inhibitors (bisindolylmaleimide I and Gö6976) or TPA, the level of hTR was not changed in both cell lines. hTERT expression was initially measured by RT-PCR using primers for region of the transcript upstream of the reverse transcriptase domain (the first hTERT primer). hTERT was expressed in untreated HeLa (Figure 3A) and CUMC-6 (Figure 3B) cells. The expression of hTERT was inhibited by bisindolylmaleimide I and Gö6976, respectively, in a time-dependent manner. hTERT expression was not affected by low dose (10 nM) of TPA. In contrast, high dose (1 μM) of TPA inhibited hTERT expression level in both cell lines.

Recently, it has been reported that variants of hTERT transcripts are expressed in human fetal tissue and tumor cell lines, presumably arising from alternative splicing (Kilian *et al*., 1997; Ulaner *et al*., 1998). To examine the presence and alteration of splicing variants, we performed RT-PCR using the primer set (the second hTERT primer) covering the region of two potential splice sites. The full-length hTERT transcripts (457 bp) would be expected to code for an active reverse transcriptase and the deletion of α- and β-splicing site result in a loss of 36 bp and 182 bp, respectively. The full-length hTERT transcripts and the β-deletion transcripts of hTERT (275 bp) were found in HeLa (Figure 4A) and CUMC-6 (Figure 4B) cell lines, respectively. In contrast to the change of the expression profiles of full-length hTERT transcripts, the change of the expression patterns of splicing variants (275 bp β-deletion transcripts) of hTERT after exposure to PKC modulating drugs was not prominent.

Discussion

Although telomerase activation is thought to be involved in cancer cell immortalization, little is known about how telomerase is activated and controlled in human cancers. The mechanism by which telomerase is regulated has attracted considerable interest. Although the control of telomerase expression and activity is still unclear, some molecular mechanisms have been described that may contribute to the regulation of telomerase activity in humans. First, it has been proposed that the regulation of telomerase activity in human fibroblasts and some epithelial cells is controlled at the level of transcription of hTERT. This idea is supported by the fact that hTERT is expressed only in cells positive for telomerase activity

and transfection of plasmids directing the expression of hTERT into telomerase-negative cell types results in the appearance of telomerase activity (Weinrich *et al*., 1997; Counter *et al*., 1998). Second, it has been reported that posttranscriptional alternative splicing of hTERT may serve as another mode for regulation of telomerase (Kilian *et al*., 1997; Ulaner *et al*., 1998). Third, tertiary and quaternary structures of telomerase holoenzyme are modulated by protein phosphorylation in such a way that the enzyme is activated.

These are based on some experimental evidences: PKC and protein kinase B (Akt kinase) enhance telomerase activity through phosphorylation of hTERT, and protein phosphatase 2A inhibits nuclear telomerase activity in breast cancer cells (Li *et al*., 1998; Kang *et al*., 1999). Taken together, these facts suggest that the regulation of telomerase activity is controlled at multiple levels, including both transcriptional and posttranscriptional mechanisms.

PKC, an enzyme family of serine/threonine protein kinases, is involved in the signal transduction pathway elicited by a variety of extracellular stimuli, such as hormones, mitogens and neurotransmitters (Nishizuka, 1995). Many studies have suggested that PKC play a role in processes relevant to neoplastic transformation, carcinogenesis and tumor cell invasion (Liu, 1996). Recently, the evidence for involvement of PKC in the regulation of telomerase activity was supported by studies on the inhibition of telomerase activity in cells treated with PKC inhibitors, bisindolylmaleimide I and H-7 (Caponigro *et al*., 1997; Ku *et al*., 1997). One possible explanation for the involvement of PKC is that PKC may be required for posttranscriptional or posttranslational modification of telomerase subunit, such as phosphorylation. This postulation was supported by studies that protein phosphorylation reversibly regulates the function of telomerase and protein kinase Cα mediates the phosphorylation of telomerase-associated protein 1 and hTERT (Li *et al*., 1998).

In the present study, we demonstrated that PKC inhibitors decreased telomerase activity and PKC activator increased telomerase activity in concurrence with the hTERT expression in the treated cells. In contrast, the hTR expression exhibited no association with telomerase activity and PKC modulating drugs did not affect the hTR expression. These findings indicate that the regulation of telomerase is determined by transcription of hTERT but not hTR. This is in agreement with findings of other investigators (Kilian *et al*., 1997; Weinrich *et al*., 1997; Counter *et al*., 1998; Ulaner *et al*., 1998).

Also, our findings indicate that PKC differentially affects gene expression for each telomerase component and suggest that PKC may be involved in the regulation of telomerase activity through transcriptional control of the hTERT gene as well as protein phosphorylation. In addition, these findings provide the evidence that hTR transcription is not affected by feedback regulation result from alteration of hTERT transcription.

Of the three PKC modulating drugs used in our study, bisindolylmaleimide I and Gö6976 are more selective for PKC relative to other cellular protein kinases and show some isoenzyme specificity for classical PKC isoenzymes (PKCα, βI, βII, γ), TPA has also yield the selectivity for PKC and does not influence the atypical PKC isoenzymes (PKCζ, ι, λ) (Wilkinson *et al*., 1993). However, all of these modulators are not one isoenzyme specific inhibitor. Although we excluded the possibility that other protein kinases are potentially involved in transcriptional regulation of hTERT, we have not defined the role of the individual PKC isoenzymes in transcriptional regulation of hTERT. Further study will be required to determine which PKC isoenzymes are involved in the regulation of telomerase activity through transcriptional control of hTERT.

In addition, we show the distinct effect of different concentrations of TPA on cervical carcinoma cell lines. A concentration of 10 nM TPA increased the PKC activity after short treatment time but a higher concentration (1 μM) showed a little effect on PKC activity. Furthermore, 1 μM TPA decreased the PKC activity after prolonged treatment. This result is consistent with the results of previous reports (Blobe *et al*., 1996; Liu, 1996; Hsu *et al*., 1998). These results demonstrate that TPA at different dosages for different lengths of time exerts biphasic regulation of PKC activity in HeLa and CUMC-6 cells.

Alternative mRNA splicing is a common mechanism for regulating gene expression in higher eukaryotes and there are many examples of tissue-specific, development-specific and sex-specific alteration in splicing events (Adams *et al*., 1996). Recently, several variants of hTERT mRNA have been identified in human fetal tissue and tumor cell lines, presumably arising from alternative splicing. These variants transcripts either resulted in deletion of 12 amino acid from reverse transcriptase domain A (α splice deletion) or 182 nucleotide deletion that cause a reading frame truncation (β splice deletion). Because expression of full-length hTERT, but not splice variants, correlated with telomerase activity, it was suggested that alternative splicing may play a role in regulating telomerase activity. However, the biological properties of the spliced transcripts remain unclear (Kilian *et al*., 1997; Ulaner *et al*., 1998).

More recently, it has been reported that no alternative splicing of hTERT was detected in human B and T lymphocyte (Liu *et al*., 1999). This report indicated that alternative splicing of hTERT might be cell-type-specific.

In the present study, two RT-PCR products, which represent the full-length hTERT transcript and the β deletion transcript, were found in both of cervical cancer cell lines. These results support that alternative splicing of hTERT may serve as a potential mechanism for

regulation of telomerase activity in cervical tumors. In addition, our results showed that the down-regulation levels of full-length hTERT after 72 h of treatment with PKC inhibitors or high dose of TPA exposure were more prominent as compared with those of the alternative splicing of hTERT. The meaning of these discrepancies in expression profiles between full-length and splicing variants should be probed in the future.

Recent study demonstrated that activating PKC results in upregulation of c-myc expression (Xu *et al*., 1998). It also has been reported that myc induces telomerase by increasing hTERT transcription (Wang *et al*., 1998), and the hTERT promoter contains myc binding sites, as well as potential binding sites for other transcription factors that may be involved in its regulation (Cong *et al*., 1999). Taken together, these reports raise the possibility that PKC may affect hTERT expression through myc or other transcription factor. However, it was reported that PKC activity did not correlate with c-myc expression levels in K562. Thus, the issue of whether the c-myc levels correlate with PKC activity has been subjected to controversy (Lerga *et al*., 1999). It remains to be determined whether PKC affects the expression of the telomerase-related genes through a secondary mechanism, such as myc or other transcription factors.

Acknowledgements

The authors wish to acknowledge the financial support of the Korea Research Foundation made in the program of year of 1998 (98-005-F00124).

References

Adams MD, Rudner DZ, Rio DC. Biochemistry and regulation of pre-mRNA splicing. Curr Opin Cell Biol 1996;8:331-39

Avilion AA, Piatyszek MA, Gupta J, Shay JW, Bacchetti S, Greider CW. Human telomerase RNA and telomerase activity in immortal cell lines and tumor tissues. Cancer Res 1996;56: 645-50

Blobe GC, Stribling S, Obeid LM, Hannun YA. Protein kinase C isoenzymes: regulation and function. Cancer Surv 1996;27: 213-48

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. Extension of life-span by introduction of telomerase into normal human cells. Science 1998;279:349-52

Caponigro F, French RC, Kaye SB. Protein kinase C: a worthwhile target for anticancer drugs? Anticancer Drugs 1997;8: 26-33

Cong YS, Wen J, Bacchetti S. The human telomerase catalytic subunit hTERT: organization of the gene and characterization of the promoter. Hum Mol Genet 1999;8:137-42

Counter CM, Meyerson M, Eaton EN, Ellisen LW, Caddle SD, Haber DA, Weinberg RA. Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. Oncogene 1998;16:1217-22

Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J. The RNA component of human telomerase. Science 1995;269: 1236-41

Hiyama K, Hirai Y, Kyoizumi S, Akiyama M, Hiyama E, Piatyszek MA, Shay JW, Ishioka S, Yamakido M. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. J Immunol 1995;155:3711-15

Hsu SL, Chou YH, Yin SC, Liu JY. Differential effects of phorbol ester on growth and protein kinase C isoenzyme regulation in human hepatoma Hep3B cells. Biochem J 1998; 333:57-64

Kang SS, Kwon T, Kwon DY, Do SI. Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. J Biol Chem 1999;274:13085-90

Kilian A, Bowtell DD, Abud HE, Hime GR, Venter DJ, Keese PK, Duncan EL, Reddel RR, Jefferson RA. Isolation of a candidate human telomerase catalytic subunit gene, which reveals complex splicing patterns in different cell types. Hum Mol Genet 1997;6:2011-9

Kim JW, Lee CG, Lyu MS, Kim HK, Rha JG, Kim DH, Kim SJ, Namkoong SE. A new cell line from human undifferentiated carcinoma of the ovary: establishment and characterization. J Cancer Res Clin Oncol 1997;123:82-90

Ku WC, Cheng AJ, Wang TC. Inhibition of telomerase activity by PKC inhibitors in human nasopharyngeal cancer cells in culture. Biochem Biophys Res Commun 1997;241:730-36

Kyo S, Takakura M, Kohama T, Inoue M. Telomerase activity in human endometrium. Cancer Res 1997;57:610-14

Lerga A, Crespo P, Berciano M, Delgado MD, Canelles M, Cales C, Richard C, Ceballos E, Gutierrez P, Ajenjo N, Gutkind S, Leon J. Regulation of c-Myc and Max in megakaryocytic and monocytic-macrophagic differentiation of K562 cells induced by protein kinase C modifiers: c-Myc is down-regulated but does not inhibit differentiation. Cell Growth Differ 1999;10:639-54

Li H, Zhao L, Yang Z, Funder JW, Liu JP. Telomerase is controlled by protein kinase C alpha in human breast cancer cells. J Biol Chem 1998;273:33436-42

Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR. Reverse transcriptase motifs in the catalytic subunit of telomerase. Science 1997;276:561-67

Liu JP. Protein kinase C and its substrates. Mol Cell Endocrinol 1996;116:1-29

Liu K, Schoonmaker MM, Levine BL, June CH, Hodes RJ, Weng NP. Constitutive and regulated expression of telomerase reverse transcriptase (hTERT) in human lymphocytes. Proc Natl Acad Sci USA 1999;96:5147-52

Meeker AK, Coffey DS. Telomerase: a promising marker of biological immortality of germ, stem, and cancer cells. A

review. Biochemistry (Mosc) 1997;62:1323-31

Minana MD, Felipo V, Cortes F, Grisolia S. Inhibition of protein kinase C arrests proliferation of human tumors. FEBS Lett 1991;284:60-62

Nakayama J, Saito M, Nakamura H, Matsuura A, Ishikawa F. TLP1: a gene encoding a protein component of mammalian telomerase is a novel member of WD repeats family. Cell 1997;88:875-84

Ng SY, Gunning P, Eddy R, Ponte P, Leavitt J, Shows T, Kedes L. Evolution of the functional human beta-actin gene and its multi-pseudogene family: conservation of noncoding regions and chromosomal dispersion of pseudogenes. Mol Cell Biol 1985;5:2720-32

Nishizuka Y. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 1995;9:484-96

Park YM, Choi JY, Byun BH, Cho CH, Kim HS, Kim BS. Telomerase is strongly activated in hepatocellular carcinoma but not in chronic hepatitis and cirrhosis. Exp Mol Med 1998;30:35-40

Persons DA, Wilkison WO, Bell RM, Finn OJ. Altered growth regulation and enhanced tumorigenicity of NIH 3T3 fibroblasts transfected with protein kinase C-I cDNA. Cell 1988;52:447-58

Raymond E, Sun D, Chen SF, Windle B, Von Hoff DD. Agents that target telomerase and telomeres. Curr Opin Biotechnol 1996;7:583-91

Shay JW, Bacchetti S. A survey of telomerase activity in human cancer. Eur J Cancer 1997;33:787-91

Soder AI, Hoare SF, Muir S, Going JJ, Parkinson EK, Keith WN. Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer. Oncogene 1997; 14:1013-21

Ulaner GA, Hu JF, Vu TH, Giudice LC, Hoffman AR. Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. Cancer Res 1998; 58:4168-72

Wang J, Xie LY, Allan S, Beach D, Hannon GJ. Myc activates telomerase. Genes Dev 1998;12:1769-74

Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, Bodnar AG, Lichtsteiner S, Kim NW, Trager JB, Taylor RD, Carlos R, Andrews WH, Wright WE, Shay JW, Harley CB, Morin GB. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTERT. Nat Genet 1997;17:498-502

Wilkinson SE, Parker PJ, Nixon JS. Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. Biochem J 1993;294:335-37

Xu G, Gai Q, James CB. Protein kinase C inhibits transcription from the RNA polymerase III promoter of the human c-myc gene. Cancer Lett 1998;123:199-205

Yashima K, Litzky LA, Kaiser L, Rogers T, Lam S, Wistuba II, Milchgrub S, Srivastava S, Piatyszek MA, Shay JW, Gazdar AF. Telomerase expression in respiratory epithelium during the multistage pathogenesis of lung carcinomas. Cancer Res 1997;57:2373-77