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SHORT COMMUNICATION

Modulation of Telomerase Activity by Zinc in Human Prostatic and Renal Cancer Cells

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ABSTRACT. Because the up-regulation of telomerase in most cancer tissues is considered to be responsible for the unlimited proliferation of cancer cells, suppression of telomerase activity is an attractive potential target for cancer therapy. The mechanism for the activation of telomerase in cancer cells, however, is still unclear. In the present study, we demonstrated that Zn induces an enhancement of telomerase activity in the human renal cell carcinoma (NRC-12) and prostatic cancer (DU145) cell lines. The maximum elevation of the activity was observed 6 hr after treatment with 100 μM Zn; it was diminished by the addition of either metal chelator or cycloheximide. Other metals such as Cd and Cu also enhanced telomerase activity but to a lesser extent, and no correlation between the activation of telomerase and the induction of metallothionein was observed. Our findings provide the first evidence that metals, especially Zn, can modulate telomerase activity in cancer cells. BIOCHEM PHARMACOL **59**;4:401–405, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. telomerase activation; zinc; prostatic cancer cell; renal cell carcinoma

The telomere is the structure of the chromosomal end, characterized by repeated sequences of the TTAGGG motif [1]. The function of the telomere has been considered to be the protection of chromosomes against degradation and rearrangement. Due to the inability of DNA polymerase to replicate the 5′ end of the lagging strand, the length of telomeres is shortened progressively with each cell division, which eventually leads to cellular senescence when the telomere length is reduced beyond the critical level [2]. Telomerase is an enzyme responsible for the extension of telomeric repeats, thereby compensating for the shortening of telomeres [1, 3, 4]. This enzyme was found to be a reverse transcriptase containing an integral RNA component, a small segment of which acts as a template for the synthesis of telomeric repeats [4]. Normal human somatic cells express low or undetectable telomerase activity, but the activity of telomerase has been found to be up-regulated in most cancer cells, suggesting a role of telomerase in the unlimited proliferation of cancer cells [1, 3, 5]. Thus, telomerase is a target for diagnostic and therapeutic application. To date, most studies have been focused on how to depress telomerase activity, expecting a potential role of telomerase inhibitors in cancer therapy [5, 6].

However, how telomerase is activated at specific stages of carcinogenesis is still unknown. Furthermore, it remains unclear how telomerase activity is regulated post-transcriptionally in cancer cells in which the gene for telomerase has already been activated. Recently, human TERT§ was identified as a catalytic subunit in human telomerase, and it is considered to play an essential role in the activation of telomerase in cancer cells [7]. However, other regulatory mechanisms have not been clarified.

Zn is an essential trace element and is involved in a number of cellular functions as a constituent of more than 200 enzymes and Zn-finger motifs in transcription factors [8]. Zn deficiency is known to suppress the proliferation of tumor cells [9], suggesting that Zn has an important role in cell proliferation. The involvement of Zn in the proliferation of lymphocytes and other non-cancer cells also has been documented [10]. The effects of Zn on telomerase activity, however, have not been investigated. Therefore, we examined the modulating effects of Zn and other metals on the activity of telomerase in renal cell carcinoma (NRC-12) and human prostatic cancer (DU145) cell lines. Here, we demonstrate an enhancement of telomerase activity by treatment with Zn in both of these cancer cell lines.

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§ *Abbreviations*: TERT, telomerase reverse transcriptase; TPEN, *N*,*N*,*N*′,*N*′-tetrakis (2-pyridylmethyl) ethylenediamine; CHX, cycloheximide; TRAP, Telomeric Repeat Amplification Protocol; TPG, total product generated; PCR, polymerase chain reaction; MT, metallothionein; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

MATERIALS AND METHODS

Cell Lines and Cell Culture

NRC-12 (human renal cell carcinoma) cells were provided by Dr. Yoshihiko Tomita (Niigata University). DU145 (human prostate cancer) cells were obtained from the American Type Culture Collection. Both cell lines were cultured at 37° in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2.

Treatment of Cells

Cells were plated at a density of 1×10^6 in 10-cm dishes. After incubation for 24 hr, the cells were treated with $ZnSO_4$ (30 and 100 μM), $CdCl_2$ (3 and 10 μM), and $CuSO_4$ (1, 3, and 10 μM) for 3, 6, and 12 hr and then harvested for the analysis of telomerase activity. For the Zn-chelating study, 100 μM TPEN dissolved in PBS was added to the medium simultaneously with Zn, and the culture was incubated for 6 hr. In the experiment with CHX, the cells were preincubated with 100 μM CHX for 6 hr and then treated with Zn for 6 hr.

Telomerase Assay

Detection of telomerase activity was carried out by the TRAP assay using a TRAPeze® Telomerase Detection Kit. Briefly, telomeric repeats (GGTTAG) were added onto the 3′ end of a substrate oligonucleotide by the telomerase extracted from the sample, and the extended products were subsequently amplified by PCR, which generated a ladder of products with 6-base increments in the gel after electrophoresis. The extraction of telomerase, telomerase extension reaction, and PCR amplification were performed according to the instructions of the manufacturer. In PCR amplification, 2 units of *Taq* DNA polymerase (Takara) were used, and 30 cycles of 3-step PCR (94° for 30 sec, 50° for 30 sec, and 72° for 60 sec) were performed. PCR products were subjected to electrophoresis on a 12.5% polyacrylamide gel. The gels were stained with SYBR® Green I nucleic acid gel stain (Molecular Probes) for 45 min, and the intensity of the TRAP products was quantitated by a FluoroImager SI™ equipped with IMAGEQUANT (Molecular Dynamics). Each peak was quantitated in terms of peak height and peak area. The quantitation of telomerase activity was done by applying the following formula: {(measured total area of peaks that represented telomerase activity)/(measured area of the peak of the internal control)}/{(measured total area of peaks of telomerase activity in the positive control)/(measured area of the peak of the internal control in the positive control)} × 100 = TPG units.

Measurement of MT

Cells were plated at a density of 1×10^6 in 10-cm dishes. After incubation for 24 hr, the cells were treated with 100 μM $ZnSO_4$, 30 μM $CdCl_2$, or 3 μM $CuSO_4$ for 6 hr, and then harvested for the analysis of MT using the Cd binding assay previously reported [11].

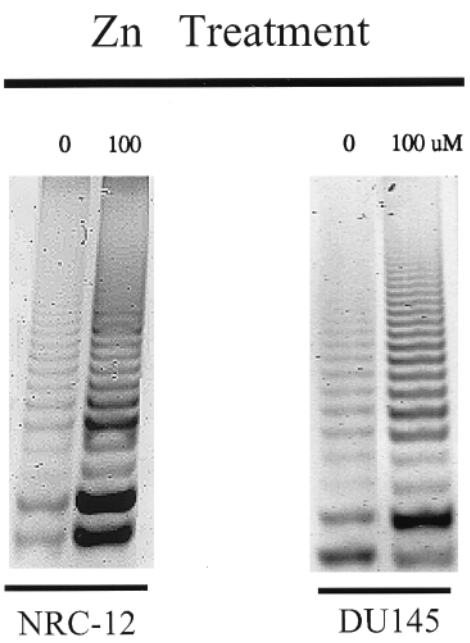

FIG. 1. Telomerase activity detected by the TRAP assay in control and Zn-treated NRC-12 and DU145 cells. Both cell lines were treated with 100 μM $ZnSO_4$ or with Zn-free medium (control) for 6 hr, and then telomerase activity was assayed using a TRAPeze® Telomerase Detection Kit. The TRAP products amplified by PCR were subjected to electrophoresis and stained with SYBR® Green I. Note that the ladders of the cells treated with 100 μM Zn for 6 hr exhibited higher intensity.

Cytotoxicity

The sensitivity of the cells to Zn, Cd, and Cu was determined by microtiter assay. Cells (1×10^4) were cultured in 96-well microtiter plates, incubated for 24 hr, and exposed to 1–1000 μM $ZnSO_4$, $CdCl_2$, or $CuSO_4$ in DMEM with fetal bovine serum for 12 and 24 hr. The viability of the cells was assayed by determining the color development caused by the reduction of MTT using a microplate reader (Reader 340 ATTC, SLT Labinstruments).

RESULTS AND DISCUSSION

We investigated the modulatory effects of Zn on the activity of telomerase in NRC-12 and DU145 cells. The activity of telomerase was visualized by the ladders formed by the TRAP assay as displayed in Fig. 1. In non-treated (control) DU145 cells, the activity of telomerase was clearly detected, which was in accordance with the report by Sommerfeld *et al.* [12] showing up-regulation of telomerase activity in DU145 cells. NRC-12 cells also exhibited detectable activity of telomerase without Zn treatment (Fig. 1). Treatment of NRC-12 and DU145 cells with Zn

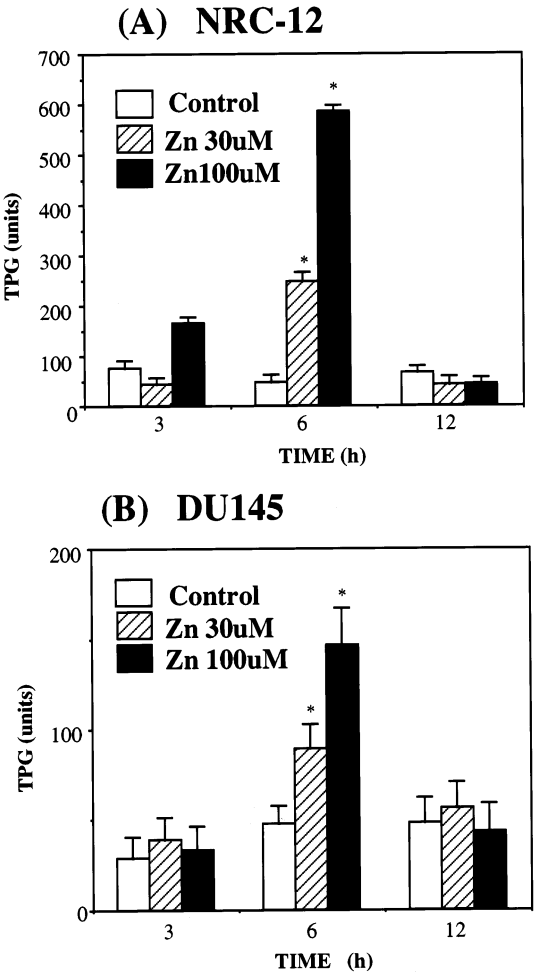

FIG. 2. Time- and concentration-dependent changes in telomerase activity in NRC-12 and DU145 cells treated with Zn. NRC-12 (A) and DU145 (B) cells were treated with 30 or 100 μM $ZnSO_4$ for 3, 6, and 12 hr, and then the TRAP assay was performed as described in Fig. 1. The intensity of the ladders formed by the TRAP assay was quantitated by densitometry and expressed as TPG units using an internal standard. Values, obtained from three independent experiments, are expressed as means ± SD. (*) significantly different from the control at the same time point ($P < 0.01$, *t*-test).

resulted in an increased intensity of the ladders at 6 hr. When the telomerase activities in Zn-treated cells were quantitated by densitometry and compared with the control cells at each time point (Fig. 2), a significant increase in the activity was observed 6 hr after the treatment with Zn in both NRC-12 and DU145 cells. Zn increased telomerase activity in a concentration-dependent manner, and treatment with 100 μM Zn resulted in a 12- and 3-fold increase in NRC-12 and DU145 cells, respectively, compared with the control cells at 6 hr. In NRC-12 cells, treatment with 100 μM Zn increased telomerase activity also at 3 hr.

To examine whether treatment with 30 and 100 μM Zn caused cytotoxic effects in these cells, an MTT assay was performed. As shown in Table 1, both NRC-12 and DU145 cells treated with 30 or 100 μM Zn for 12 and 24 hr exhibited no decrease in cell survival. These results indicate that the enhancing effect of Zn on the telomerase activity in NRC-12 and DU145 cells may not be caused by its cytotoxicity, but rather by some biological events, such as induction of Zn-binding proteins or activation of transcription factors containing Zn-finger motifs.

Next, we investigated whether elevation of telomerase activity could be induced by other metals such as Cd and Cu. Since Zn elevated telomerase activity at concentrations showing no cytotoxicity, we first determined non-toxic concentrations of Cd and Cu using the MTT assay (Table 1). As a result, we used 3 and 10 μM concentrations of $CdCl_2$ for both cell lines, 1 and 3 μM concentrations of $CuSO_4$ for NRC-12 cells, and 3 and 10 μM concentrations of $CuSO_4$ for DU145 cells. Interestingly, Cd and Cu also induced telomerase activity in both cell lines in a concentration-dependent manner. The direct effects of metals on the PCR step can be ignored, since no increase in PCR products was seen when these metal ions were added to the reaction mixture for PCR. The maximum activation of telomerase also was observed 6 hr after treatment with Cd or Cu, as shown in Table 2. When the extent of activation of telomerase was compared among the three metals, Zn exhibited the highest activation in both cell lines. One possible cellular component inducible by Zn and other metals may be MT, which is a cysteine-rich metal-binding protein located in the nucleus in cancer cells [11]. Since these three metals are inducers of MT, we determined the levels of MT 6 hr after the addition of metals to the medium. As shown in Table 2, MT levels induced by Zn, Cd, or Cu at 6 hr were 1.4- to 2.5-fold of the control. Since MT induction by metals is known to reach maximum levels at 12–24 hr after treatment [13], the maximal enhancement of telomerase activity preceded the peak of MT induction. Furthermore, there was no correlation between the levels of MT induction at 6 hr and telomerase activation by each metal. Thus, further study is required to clarify the effect of MT induction on the activation of telomerase.

We also examined the effects of a metal chelator, TPEN, on the elevation of telomerase activity induced by Zn. Treatment of NRC-12 cells with 100 μM TPEN alone did not affect telomerase activity (Table 3). Treatment of DU145 cells with 100 μM TPEN reduced telomerase activity (data not shown), but 30 μM TPEN did not affect the activity. Therefore, we used 100 μM TPEN for NRC-12 cells and 30 μM for DU145 cells to examine the effects of TPEN on Zn-induced telomerase elevation. As shown in Table 3, the telomerase activities of NRC-12 and DU145 cells treated with 100 μM Zn were reduced to the control levels by the simultaneous addition of TPEN. These results suggested that the increase in the telomerase activity might be caused by TPEN-chelatable Zn.

The maximum enhancement of telomerase activity by Zn was achieved 6 hr after treatment with Zn, but then the activity returned to the control level when the cells were exposed continuously to Zn for 12 hr (Fig. 2). This suggests

TABLE 1. Sensitivity of NRC-12 and DU145 cells to Zn, Cd, and Cu as determined by the MTT assay*

Treatment	Concentration (μM)	Viability of cells (% of control)			
		NRC-12		DU145	
		12 hr	24 hr	12 hr	24 hr
Control		100.0 ± 6.9	100.0 ± 5.2	100.0 ± 5.6	100.0 ± 5.5
$ZnSO_4$	30	109.2 ± 9.1	110.3 ± 6.6	106.6 ± 2.9	107.2 ± 1.9
	100	110.8 ± 8.3	118.3 ± 8.7	108.7 ± 5.7	112.3 ± 1.6
	300	71.6 ± 2.1†	49.6 ± 0.9†	72.1 ± 3.8†	57.9 ± 0.7†
$CdCl_2$	3	102.6 ± 5.7	101.5 ± 2.6	98.3 ± 7.3	100.1 ± 4.1
	10	99.6 ± 4.8	103.5 ± 8.7	99.2 ± 4.6	97.3 ± 3.2
	30	88.6 ± 7.1	84.3 ± 6.6	90.3 ± 6.1	87.6 ± 5.5
$CuSO_4$	1	99.1 ± 2.9	97.3 ± 4.4	102.7 ± 2.3	96.4 ± 5.9
	3	95.2 ± 6.0	95.9 ± 9.2	101.6 ± 6.5	98.6 ± 5.2
	10	88.3 ± 9.4	61.3 ± 2.1†	97.7 ± 8.3	96.1 ± 6.7
	30	67.3 ± 3.3†	38.8 ± 9.4†	98.5 ± 10.2	95.9 ± 9.7

*Cells (1×10^4) were plated in 96-well microtiter plates, cultured for 24 hr, and exposed to metal compounds in DMEM with fetal bovine serum for 12 and 24 hr. The viability of the cells was assayed by determining the color development caused by the reduction of MTT, and is expressed as a percent of the control cells. Values (means ± SD) were obtained from three independent experiments.

†Significantly different from the control ($P < 0.05$, *t*-test after logarithmic transformation).

that the activation of telomerase may not simply reflect the change in the cellular concentration of Zn. Possibly, either induction of early-response proteins or post-transcriptional modification of proteins by Zn may be involved in the activation of telomerase. To test this hypothesis, we added CHX to the medium 6 hr prior to, or simultaneously with, the treatment with Zn, and examined the activity of telomerase. As shown in Table 3, CHX treatment for 6 hr prior to Zn exposure completely inhibited the Zn-induced enhancement of telomerase activity at 6 hr in both NRC-12 and DU145 cells, suggesting that *de novo* synthesis of protein may be required for the Zn-induced activation of telomerase.

Terashima *et al.* [14] reported an elevation of telomerase activity in human lymphoma and myeloma cells after irradiation. They also showed an activation of telomerase at non-toxic doses of irradiation, although the underlying mechanism has not been demonstrated clearly. It has been well documented that Zn plays significant roles in cell growth and cell cycling [15] as a constituent of Zn-containing proteins, including DNA polymerase and Zn-finger motifs in the transcription factors. Recently, Wu *et al.* [16] demonstrated that the expression of TERT, the catalytic subunit of telomerase, can be activated by c-MYC. The effect of Zn on the activation of TERT expression should be examined. Elucidation of the mechanism by which Zn modulates the activity of telomerase might reveal not only a new aspect of the mechanism of regulation of telomerase activity in cancer cells but also a new role of Zn in the regulation of the growth and senescence of cells. Further study on the mechanism of activation of telomerase by Zn, as well as screening of other enhancers of telomerase, is required.

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TABLE 2. Activation of telomerase and induction of metallothionein by Zn, Cd, and Cu in NRC-12 and DU145 cells

Treatment	Cell line			
	NRC-12		DU145	
	Telomerase (TPG units)	MT (μg/mg protein)	Telomerase (TPG units)	MT (μg/mg protein)
Control	48.1 ± 14.3 (100.0)*	0.90 ± 0.06 (100.0)	47.7 ± 9.8 (100.0)	1.26 ± 0.06 (100.0)
Zn†	585.6 ± 28.4 (1217.4)	2.02 ± 0.06 (223.9)	146.6 ± 20.4 (307.3)	2.02 ± 0.17 (160.5)
Cd‡	187.9 ± 85.4 (390.7)	2.26 ± 0.10 (251.2)	108.9 ± 29.5 (228.3)	2.76 ± 0.14 (219.6)
Cu§	261.5 ± 38.7 (543.7)	1.85 ± 0.06 (205.1)	66.4 ± 14.0 (139.1)	1.71 ± 0.30 (136.0)

Values (means ± SD) were obtained from three independent experiments.

*Numbers in parentheses are percent of control.

†Zn treatment was performed with 100 μM $ZnSO_4$ for 6 hr in both cell lines.

‡Cd treatment was performed with 10 μM $CdCl_2$ for 6 hr in both cell lines.

§Cu treatment was performed with 3 μM $CuSO_4$ for 6 hr in the NRC-12 cell line and with 10 μM $CuSO_4$ for 6 hr in the DU145 cell line.

TABLE 3. Effects of TPEN and CHX on Zn-induced activation of telomerase in NRC-12 and DU145 cells

	Telomerase activity (TPG units)	
	Cell line	
Treatment	NRC-12	DU145
Control	48.1 ± 14.3 (100.0)*	47.7 ± 9.8 (100.0)
Zn†	585.6 ± 28.4 (1217.4)	146.6 ± 20.4 (307.3)
TPEN alone	50.5 ± 6.0 (105.6)	53.2 ± 3.3 (111.5)
TPEN + Zn‡	56.1 ± 5.4 (116.6)	50.7 ± 6.8 (106.3)
CHX (−6 hr)	45.4 ± 18.6 (94.3)	40.4 ± 5.9 (84.7)
CHX (−6 hr) + Zn§	46.0 ± 14.3 (95.7)	37.3 ± 7.7 (78.4)
CHX (0 hr)	52.3 ± 9.1 (108.8)	50.8 ± 13.9 (106.5)
CHX (0 hr) + Zn‖	37.8 ± 14.2 (78.5)	52.4 ± 3.0 (109.8)

Values (means ± SD) were obtained from three independent experiments.

*Numbers in parentheses are percent of control.

†Zn treatment was performed with 100 μM $ZnSO_4$ for 6 hr in both cell lines.

‡TPEN (100 μM in NRC-12 or 30 μM in DU145) was added to the medium simultaneously with Zn (100 μM) for 6 hr.

§CHX (100 μM) was added to the medium 6 hr prior to Zn treatment.

‖CHX (100 μM) was added to the medium simultaneously with Zn (100 μM) for 6 hr.

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