Journal of Enzyme Inhibition and Medicinal Chemistry, April 2009; 24(2): 524–530

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Thyroglobulin may affect telomerase activity in thyroid follicular cells

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(*Received 12 December 2007; revised 25 April 2008; accepted 15 May 2008*)

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Abstract

Telomerase (TA) activity is known to be present in malignant tumor cells, but not in most somatic differentiated cells. TA shows relatively high activity in thyroid cancer cells, but reports vary. This fact prompted us to elucidate whether cell component inhibitors of TA in the thyroid follicles can modulate its activity. The activity of TA extracted from Hela cells was inhibited by mixing with the supernatant fraction of human thyroid tissue extract. To examine the effect of iodine, thyroid hormones (ℓ-T3 and ℓ-T4) and human thyroglobulin (hTg) contained in the thyroid follicles, ℓ-T3, ℓ-T4 and hTg were added to the TRAP assay system *in vitro*, using TA from Hela cells. Iodine, ℓ-T3 and ℓ-T4 did not affect TA activity, but hTg inhibited the TA activity in a dose-dependent manner (IC_{50} of hTg: ca 0.45 μM: inhibiting concentration of hTg was from 0.15 μM to 3.0 μM). The hTg inhibition was not evident in the RT-PCR system, suggesting no effect of hTg on Taq DNA polymerase activity. The hTg inhibition of TA activity was attenuated by dNTP but not significantly by TS primer. These data suggest that hTg contained in thyroid follicular cells of various thyroid diseases may affect the TA activity measured in biopsied thyroid specimens, and that the reduction of the TA activity by hTg may induce slow progression and growth, and low grade malignancy of thyroid cancer, particularly differentiated carcinoma.

Keywords: *Telomerase, thyroglobulin and thyroid tumor*

Introduction

Telomerase (TA), which synthesizes the repetitive hexameric sequence on the end of chromosomes, and which subsequently maintains telomere length is expressed in human cancer cells, germ line cells, lymphocytes and stem cells, but not in non-neoplastic, differentiated somatic cells [1]. TA is required for cell immortalization [2,3].

Recently, human TA has been cloned and sequenced [4,5], and reconstituted with the template ribonucleic acid component (hTR) [6], catalytic component (hTERT) [4,5,7,8] and TA-associated protein (hTEP1) [9].

It has been reported that the gene expression of the hTERT catalytic subunit correlates with TA activity of immortal cell lines [10], and that tumorigenesis may represent an earlier event, rather than the reactivation of the TA enzyme.

The associated mechanism of TA enzyme activity and cell proliferation is proposed to be involved in tumorigenesis, and subsequently in thyroid carcinogenesis [11]. Thus, the measurement of the reactivation of TA activity and hTERT gene expression in thyroid tissue specimens may be a potential tool to discriminate benign from malignant thyroid nodules [12–15], or to define the aggressive nature of thyroid tumors and shorter survival time [16,17].

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ISSN 1475-6366 print/ISSN 1475-6374 online © 2009 Informa UK Ltd.
DOI: 10.1080/14756360802218920

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False-positive fine needle aspiration biopsy results in benign thyroid lesion specimens may be the result of lymphocyte infiltration in molecular detection methods [18,19]. Similarly, false-negative results may be due to the presence of TA inhibitors or other factors. The presence of TA inhibitor has been proposed to be a potent problem for the analysis of cell extracts [20,21], though it might be negligible in thyroid specimens [22,23].

We therefore investigated the effects of some factors present in thyroid specimens in a TA activity assay system. These factors may prevent detection of slow progressiveness and low grade malignancy, especially in follicular or papillary carcinoma, as there is relatively low TA activity in the thyroid gland [19].

Materials and methods

Chemicals: Commercially available potassium iodide disolved in distilled water, and ℓ-triiodothyronine (T3), ℓ-thyroxine (T4) disolved in 0.5% dimethylsulfoxide (DMSO) or 0.001 N NaOH were obtained from Sigma chemical Co. (St. Louis, MO, USA). Human thyroid thyroglobulin (hTg) (homodimeric glycoprotein which does not contain HBcAg and antibody to HIV and HCV. purity: >96%) were obtained from Calbiochem (EMD Biosciences Inc. San Diego, CA, USA).

TA enzyme samples were obtained from harvested and counted Hela cells which were transferred in ice-cold lysis buffer and homogenized according to the protocol of the TA detection kit.

Thyroid tissue specimens were obtained from patients with Graves' disease or normal thyroids on thyroidectomy after informed consent.

The TA activity assay was performed according to the TA PCR ELISA (developed by Roche Diagnostics, Mannheim, Germany) in an extension of the original method described by Kim et al. [1]. Briefly, a TS primer (AATCCGTCGAGCAGAGTT) from the kit was labeled using biotin. TS was elongated during 10–30 min at 25°C using TA extracted from 10^5 cells of Hela cells.

PCR amplification was performed at 94°C for 30 s, 50°C for 30 s and 72°C for 90 s with 32 cycles, ended by a 10-min step at 72°C with an internal standard of 36 base pairs (bp). A sample heated at 100°C for 5 min was used as negative control. The PCR products were assayed in ELISA kit or analysed by electrophoresis in a non-denaturing 12.5% polyacrylamide gel at 400 V. The gel was then applied to the detection process. The inter- and intra-assay coefficient of variances were ca. 5%.

The effect of thyroglobulin on the PCR system was confirmed as follows: extracted-DNA was prepared from human blood samples by conventional methods. Using UCSNP19 primer for ordinary PCR, UCSNP19 (Calpain-10 gene) was amplified [24].

UCSNP19 was amplified with the following primers: forward, 5′-GTTTGGTTCTCTTCAGC-GTGGAG-3′; reverse, 5′-GTGAGCCTCTGGCAT-TGAGC-3′.

PCR was carried out using a Perkin Elmer GeneAmp PCR System 9700 (Applied Biosystems, Foster city, CA, USA), and the conditions of PCR for each primer pair were as follows: initial denaturation at 94°C for 5 min, and then 35 cycles of 94°C for 30 s, 60°C for 30 s. 72°C for 1 min, and a final extension at 72°C for 10 min. At the beginning of each PCR experiment, hTG dissolved in distilled water was

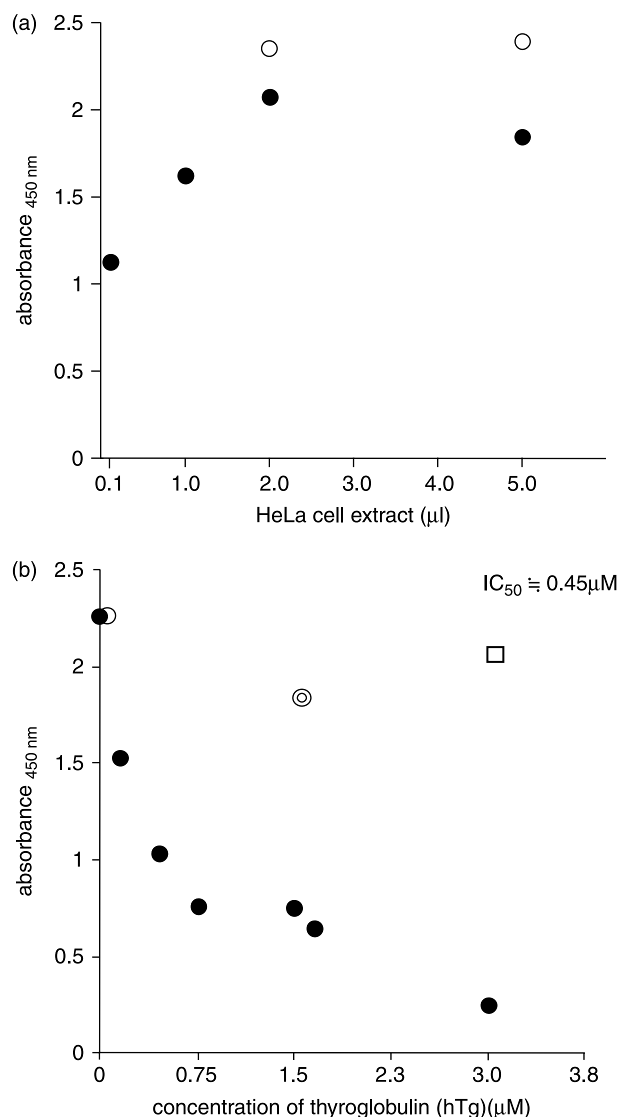

Figure 1. (a) Telomerase activities in HeLa cell extract. Each value is the mean of duplicate experiments. ○, positive control in the assay kit; ●, HeLa cell extract. (b) Effect of human thyroglobulin on telomerase activity *in vitro*. Each value is the mean of duplicate experiments. 10^6 HeLa cells extract/assay tube were used as telomerase enzyme. ○, 0% 66,000 MW bovine serum albumin (BSA) instead of thyroglobulin was added as a control; ◎, 0.1% (final) BSA; □, 1% (final) BSA.

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added in order from 10^{-8} to 10^{-5} M (ten-fold serial dilutions).

The ordinary PCR products were applied to electrophoresis for 1.5 hours at 400 volts with 10% polyacrylamide gel, which was then transferred to detection process, i.e. the gel is stained with ethidium bromide according to the protocol.

Simmilarly, to confirm whether or not hTG interferes with the activity of tag DNA polymerase. The real-time quantitative PCR system was performed as follows: TaqMan Gene Expression Assay (ID:Hs00355773-ml), Applied Biosystems Inc., Japan) was used for primer set to carry out the PCR. This primer set can amplify for DNA of insulin gene. The template DNA was used for cDNA of insulin gene. The condition of real-time PCR carried out using a ABI7000 Sequence Detection System (Applied Biosystems Inc., Japan) was as follows: initial denaturation at 95°C for 10 min, and then 40 cycles of 95°C for 15 s, 60°C for 60 s. At the beginning of the real-time PCR experiment, hTg resolved in distilled water was added in order from 10^{-7} M to 10^{-5} M.

Results

The TA activity in Hela cells prepared with Lysis Buffer was measured by the detection kit. The TA activity was linearly increased in proportion to the enzyme volume up to 2.0 μl (Figure 1a).

As shown in Table I, the mixing of μg quantities of Hela cell extract having a high TA activity with the thyroid tissue extracts from 2 or 5% thyroid homogenate (the amount of thyroglobulin (measured by immunoradiometric assay [25]. sensitivity: 5-1000 ng/ml): 10.3 to16.0 μg/mg thyroid tissue) resulted in a significant reduction of TA activity.

Since these data suggest the possibility of the existence of internal inhibitors in the thyroid tissue, we investigated the effect of iodine, thyroid hormones and thyroglobulin contained in the supernatant fraction on the TA activity in the assay system.

Iodine, ℓ-T3, ℓ-T4 and albumin did not affect the TA activity (Table I). However, hTg caused a dose-dependent inhibition of TA activity from 0.15 to 3.0 μM (ten-fold serial dilution) (50% inhibitory concentration, IC_{50}: ca. 0.45 μM) (Figure 1b).

It is difficult to study the kinetics of thyroglobulin inhibition of TA activity because the assay system includes PCR. We, therefore, tried to add TS primers (100 pmoles) or dNTP (100 pmoles) at 10 and 20 min after starting the TA assay reaction with hTg. As shown in Figures 2a and b, TS primers had partially and not significantly reversed the effect, but dNTP released the hTg inhibition of TA activity, suggesting that it may be a reversible and competitive inhibition.

Table I. Effect of iodine or thyroid hormones and or human thyroid extract on telomerase activity.

Telomerase in the assay	Final concentration of T_3, T_4, iodine and albumin and or solvents or thyroid extract	T_3		T_4		Iodine	Thyroid extract*	Albumin	Distilled water
		in DMSO	in NaOH	in DMSO	in NaOH				
+	10^{-3} M	2.25 (A450 nm)	2.30	2.87	3.32				
+	10^{-4} M	2.75	3.30	2.53	2.99				
+	10^{-5} M	3.18	2.92	2.43	3.21				
+	10^{-6} M	3.11	3.10	2.36	3.30				
+	10^{-7} M	3.01	–	2.82	–				
+	0.5% DMSO only	3.20	–	3.20	–				
+	0.001 N NaOH only	–	3.33	–	3.33				
+	10^{-3} M					3.01			
+	10^{-5} M					2.54			
+	10^{-7} M					3.24			
+	2% extract						0.07		
+	5% extract						0.06		
+	0.1% (final)							3.01	
+	1% (final)							2.88	
+ (posit.)	0								3.35
− (neg.)	0								0.02

Each value is the mean of duplicate or triplicate experiments. DMSO: dimethylsulfoxide, T_3: B-triiodothyronine, T_4: B-thyroxine. T_3 and T_4 were dissolved in 0.5% DMSO or 0.001 N NaOH. Telomerase was extracted from 10^6 Hela cells harvested. Posit, or neg: positive or negative control prepared in the assay kit. A: absorbance. *Three thyroid tissues of Graves' disease or 2 thyroid tissues adjacent to thyroid adenoma or 2 normal thyroid tissues were homogenized with 20% or 50% (W/V) lysis buffer (phosphate buffer and saline (pH 7.5), or saline) and the homogenates were centrifuged at l000 × g for 10 min. The supernatants were used for the assay. The values are the mean of duplicate or triplicate experiments.

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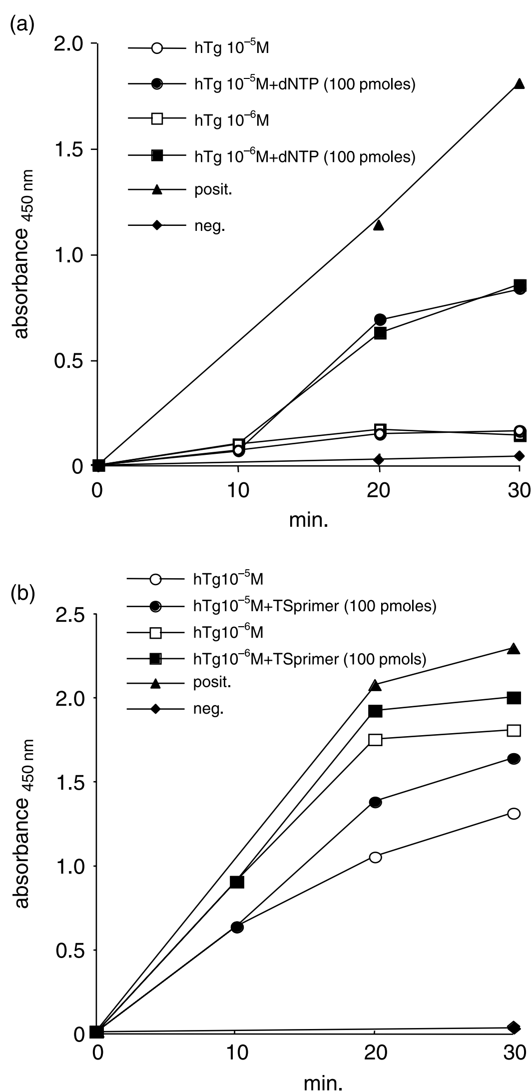

Figure 2. Effect of intermediate addition of dNTP(a) or TS primer(b) on the telomerase assay. Each value is the mean of duplicate experiments. HTg, human thyrogrobulin; posit., positive control; neg., negative control.

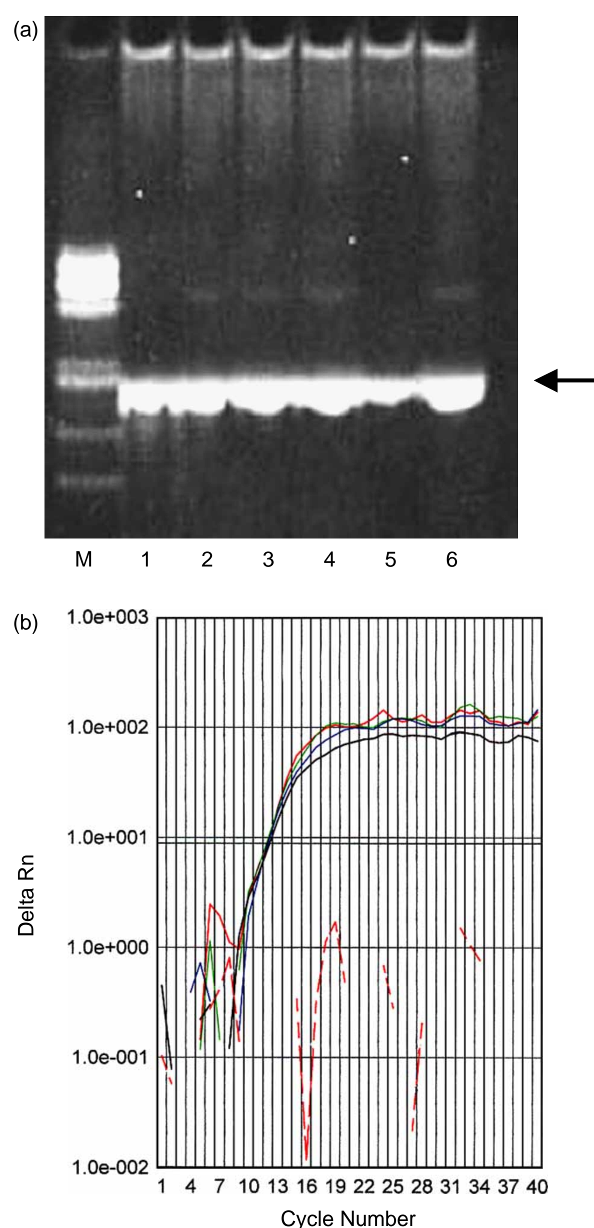

Figure 3. Effect of human thyroglobulin on ordinary or real time quantitative PCR product (particularly taq DNA polymerase). (a) ordinary PCR. Lane 1 ~ 6, UCSNP 19 is used as a primer (←); M, size marker; 1: control: without addition of human thyroglobulin. 2 ~ 6: addition of $10^{-9} \sim 10^{-5}$ (ten-fold serially diluted) M of human thyroblobulin at the beginning of PCR. (b) real time quantitative PCR. The primer used was TaqMan Gene Expression Assay (ID: Hs00355773-ml). At the beginning of the real time PCR 10^{-5} M (red line) or 10^{-6} M (green line) and or 10^{-7} M (blue line) of human thyroglobulin. Purple line: without addition of human thyroglobulin at the beginning of the PCR (positive control). Dotted red line under the threshold line (dark green): without addition of template DNA at the beginning of the PCR (negative control). Both positive and negative control experiments were successfully proceeded.

To clarify the inhibitory mechanism of hTg in the assay system in which PCR is used to amplify the DNA fragment elongated by TA, we added 10^{-8} to 10^{-5} M of hTg at the start of the ordinary PCR step. As shown in Figure 3a, PCR product was not affected by hTg under these conditions. And also confirming this in the real-time quantitative PCR the threshold point was not affected by hTg from 10^{-7} to 10^{-5} M (Figure 3b), suggesting that hTg may not inhibit Taq DNA polymerase in the PCR step.

Discussion

Recent work has demonstrated that some normal cells exhibit hTERT gene expression [10,26], and that benign thyroid nodules, which occasionally have concomitant lymphocyte infiltration, also have TA activity [27] and gene expression [28]. These findings required *in situ* hybridization techniques to detect TA activity or hTERT gene expression [29]. Conversely, there are also reports that some malignant cells could have another mechanism for elongated telomere

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maintenance in the absence of TA activity [30]. Others have reported that the TA activity from cytological specimens from thyroid aspiration biopsy is less than that from tissue samples, and also that the TA enzyme itself may be sensitive to temperature fluctuations and may be easily degraded by proteinase and nuclease [31]. In clinically less aggressive papillary thyroid carcinoma which has low TA activity, or negative alternative lengthening of telomeres [32], colloid protein in the test sample may lead to an increased risk for false-negative results [33] Whether or not these effects have crucial consequences, thyroid tissue specimens seem to have less TA expression.

Matthews et al. have reported that TA activity was detected 22–38% in papillary and follicular thyroid carcinomas and have suggested the possibilities of TA false-negatives, and the TA-negative thyroid tumors had in part long Telomeres and vice verse [34].

Thyroglobulin inhibition might be reversible for the TA activity, but thyroglobulin may not affect the gene expression of the enzyme. However, in the TRAP assay or the gene expression analysis the relation between TA and thyroglobulin has been still inconclusive [35–37].

Surveys of human tumor oncogene expression have focused on thyroid tumors because of the low incidence and mortality from thyroid diseases, except for anaplastic thyroid carcinoma, in which the transformation occurs in a cooperative manner [38] by the overexpression of C-myc and N-ras oncogenes, which may result in more aggressive disease [39]. In slowly progressing papillary thyroid carcinomas, the existence of a new transforming gene has been confirmed [40]. Such characteristics may affect prognosis.

Thyroglobulin may affect TA activity during the shift of TA enzyme from the endoplasmic retriculum in which TA enzyme and thyroglobulin occasionally different contents of thyroid hormone or iodide in malignant tumor [41] are synthesized, to the nuclear membrane, or through the nuclear pore complex and nuclear localization signals [42]. Thyroglobulin autoregulation of thyroid-specific gene transcription [43] and the translocation of ER (endoplasmic reticulum)-accumulated substances, etc. to the nucleus through ER-nuclear signal transduction pathway [44] were reported. Kohn et al. [45] has demonstrated thyroglobulin-induced signaling, namely, thyroglobulin suppression of thyroid-restricted genes through binding to apical membrane asialoglycoprotein receptor and then active on 5′-flanking region of TTF (thyroid transcription factor) −1, pax 8, or TTF-2. However, the nature of the actual transcriptional effecter is not clarified.

We have reported that thyroglobulin inhibits thyroidal DNA polymerase β [46] and reverse transcriptase [47], both of which, like TA, are RNA-dependent DNA polymerases. This suggests that a similar mechanism may be involved in the inhibitory action of thyroglobulin on these enzymes.

Several inhibitors of TA activity have been proposed [48], but therapeutically useful compounds have not yet been developed.

Recently, using an antisense strategy for the protein component of TA, the inhibition of thyroid cancer cell growth and an increased rate of apoptosis were observed [49]. Thyroid cancer gene therapy using human TA promoter has also been proposed [50].

Our results suggest that TA actibity in the biopsied thyroid tumor tissue which synthesizes thyroglobulin is possibly less than the actual activity or false-negative. This may result that malignant thyroid tumors become slow progressive and low grade malignancy except for anaplastic thyroid carcinoma which does not synthesize thyroglobulin.

Wyllie et al. indicated the therapeutic intervention of Werner syndrome of which cellular life span was extended by forced gene expression of TA [51].

The forced gene expression of thyroglobulin in the tissue of malignant thyroid tumors may suppress the progression of thyroid malignancy and extend life span.

Acknowledgements

We thank Ms. N. Takekawa for preparing this manuscript and her secretarial assistance.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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