Thyroid Hormone Receptors Suppress Pituitary Tumor Transforming Gene 1 Activity in Hepatoma

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Abstract

Pituitary tumor transforming gene 1 (PTTG1) is expressed in most tumors. However, whether thyroid hormone (T3) and its receptors (TR) regulate PTTG1 in human hepatocellular carcinomas (HCC) remains unclear. Previous cDNA microarrays revealed PTTG1 is down-regulated by T3/TR. This study investigated the significance of PTTG1 regulation by T3 in HCC cells. The PTTG1 mRNA and protein expression were repressed by T3 in HCC cell lines overexpressing TR. However, after knockdown of TRs by RNA interference, PTTG1 repression by T3 was abolished. Similar results were observed in thyroidectomized rats. To localize the regulatory region in the PTTG1 promoter, serial deletions within the PTTG1 promoter region were constructed. The promoter activity of the PTTG1 gene was repressed (25–51%) by T3. Additionally, these findings indicate that PTTG1 may be regulated by Sp1. The critical role of the −594 and −520 Sp1 binding sites was confirmed by electrophoretic mobility shift assay. Transfection with Sp1 expression vector enhanced the activity of the PTTG1 promoter fragment reporter. Also, Sp1 was down-regulated in HCC cells and in thyroidectomized rat after T3 treatment. Additionally, ectopic expression of PTTG1 promotes cell proliferation in Hep3B hepatoma cells. Conversely, knockdown of PTTG1 or Sp1 expression reduced cell proliferation in HepG2 cells. Notably, the expression of PTTG1 and Sp1 was inversely correlated with the expression of TR proteins in HCC. Together, these findings indicate that PTTG1 gene expression is mediated by Sp1 and is indirectly down-regulated by T3. Finally, overexpression of PTTG1 or Sp1 in HCCs is TR-dependent and crucial in the development of HCC.

Introduction

Thyroid hormone (T3) regulates growth, development, and differentiation in vertebrates. Trithyronine binds to specific receptors (thyroid hormone receptors, TR), which belong to the superfamily of nuclear receptors. These receptors function as ligand-dependent transcription factors by binding to sequences known as thyroid hormone response elements (TRE), which are usually located in the promoter regions of target genes (1). The human TRs are encoded by the TRα and TRβ genes located on human chromosomes 17 and 3, respectively. By alternative splicing and different promoter usage, these two genes yield at least four proteins: TRα1, TRα2, TRβ1, and TRβ2 (2).

Materials and Methods

Cell culture. The human hepatoma cell lines HepG2, Hep3B, Huh7, J7, and Mahlavu were routinely grown in DMEM supplemented with 10% (v/v) fetal bovine serum. Serum was depleted of T3 as described elsewhere (11).

The liver is well recognized as a target organ for T3. Human hepatocytes express equal amounts of TRα1 and TRβ1 proteins (3). Normal human hepatocytes are difficult to maintain in vitro and become senescent soon after isolation. Therefore, T3 regulation was examined in several human hepatoma cell lines. Using a cDNA microarray technique, this laboratory previously identified 148 genes positively regulated by T3 in a TRα1 overexpressing hepatoma cell line (HepG2-TRα1). However, the other genes negatively regulated by TR were largely unexamined. Increasing evidence suggests that aberrant TR regulation or mutant TR genes may be associated with human neoplasia (4). Previously, Lin et al. (5) reported truncated TRα1 and TRβ1 cDNA in 53% of human hepatocellular carcinomas (HCC). A high incidence of multiple point mutations was observed in these mutated TRs. Other groups (6, 7) have reported mutated TRs in HCC and cultured cells. This study focused on a set of genes (i.e., oncogenes) normally repressed by TR but aberrantly induced because of reduced TR expression or mutation during carcinogenesis.

The pituitary tumor transforming gene 1 (PTTG1) is a multifunctional gene encoding a 23-kDa, 202–amino acid protein that induces cell transformation in NIH3T3 cells and tumor formation in nude mice (8). Furthermore, PTTG1 is involved in liver regeneration (9) and is overexpressed in the hepatoma cell lines (10). Additionally, previous microarray analysis showed that TR negatively regulated PTTG1. However, PTTG1 regulation by T3 has not been fully characterized, and its pathogenic implications in hepatomas are largely unknown. This study therefore examined the transcriptional regulation of the PTTG1 gene by TR and its physiologic significance.

This study shows that T3 down-regulates PTTG1 expression in HCC cells. Moreover, the promoter activity of the PTTG1 is repressed by T3. Serial deletion of the promoter and electrophoretic mobility shift assays (EMSA) were performed to locate the Sp1 sites on the PTTG1 promoter. Finally, the possible physiologic functions of such regulation were elucidated.

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The gel was then blotted onto a nitrocellulose membrane and subjected to Northern blot analysis, as described previously (12).

**Cloning the PTTG1 5′ Flanking region and promoter activity assay.** Fragments of the PTTG1 promoter (nucleotides –2,512 to +52) were amplified via PCR according to the published nucleotide sequence (8) and then inserted into the pASK-Luc vector. Mutations of Sp1 binding sites were also constructed (Δ–620/–512). The promoter construct sequence was verified by automated DNA sequencing. To measure the influence of T3 on the transcriptional activity of the PTTG1 promoter, HepG2-TRα1#1 cells (1 × 10^6 per 12-well plate) were cotransfected via a Lipofectamine protocol using 1.2 µg/well of pASK-Luc vector containing PTTG1 promoter sequences (Invitrogen Corp.). The Sp1 expression plasmid was kindly provided by Hung-Jan-Jong (National Cheng Kung University). Cells were also cotransfected with 0.33 µg of the β-galactosidase expression vector, p5Vj plasmid (Clontech Laboratories, Inc.), as described elsewhere (12).

**Electrophoretic mobility shift assay.** The PTTG1 promoter fragment (–620 to –512) was amplified and labeled with [α-32P]dCTP (3,000 Ci/mmol; Amersham) via PCR for use as a probe. The PTTG1 promoter fragment (–925 to –817) served as a control. For the EMSA, an equal amount of in vitro-translated (Promega) Sp1 protein or nuclear extract (14) was incubated with [α-32P]-labeled PTTG1 fragments, as described elsewhere (13). MOPC21, a mouse monoclonal antibody, was used as a negative control (Sigma-Aldrich).

**Quantitative reverse transcription–PCR.** Total cellular RNA was extracted by Trizol, as described elsewhere. Subsequently, cDNA was synthesized using the SuperScript II kit for reverse transcription–PCR (RT-PCR; Life Technologies), as described previously (15). Real-time quantitative RT-PCR (Q-RT-PCR) was conducted, as described previously (15).

**Experimental animals and human specimens.** In accordance with techniques described elsewhere, thyroidectomy (Tx) was performed on two groups of 6-week-old male Sprague-Dawley rats (n = 10), and sham operations were performed on one group of 6-week-old male rats (n = 6; ref. 12). All animal experiments in this study complied with NIH guidelines and the Chang-Gung Institutional Animal Care and Use Committee for Care and Use of Laboratory Animals.

Additionally, 65 patients with HCC diagnosed between 2000 and 2003 were consecutively selected for this study. The study protocol was approved by the Medical Ethics and Human Clinical Trial Committee at Chang-Gung Memorial Hospital.

**Recombinant adenovirus and infection.** The PTTG1 cDNA was subcloned into the XhoI site of the vector pAdTrack-CMV (16), a gift from B. Vogelstein (Johns Hopkins University). The resulting construct was sequenced, linearized, and cotransformed with pAdEasy1 into BJ5183 competent bacteria to generate recombinant pAdEasy1-PTTG1. Recombinant viruses were produced by transfecting linearized pAdEasy1-PTTG1 into 293 cells via a Lipofectamine method. Cells growing in log phase were infected with either empty or PTTG1 adenovirus for 40 h. Subsequently, the cell proliferation rate was assayed.

**Establishing TRs/PTTG1/Sp1 knockdown stable HCC cell lines.** The clones (V2HS_28556) of short hairpin RNA (shRNA) targeting PTTG1, and shRNA (NM_004219) for Sp1 were obtained from GENDISCOVERY (Open Biosystems). Additionally, the clone (TRCN000002039) of shRNA targeting TRα1 and the clone shRNA (TRCN000002034) for TRβ1 were purchased from National RNA Interference Core Facility (Institute of Molecular Biology, Academia Sinica). Transfection of shRNA for the targeting of endogenous PTTG1/Sp1/TRs genes was performed using Lipofectamine reagent (Invitrogen). After 24 h of incubation, cells were transferred to medium containing puromycin for selection. After 2 wk of selection, specific repression of the targeted gene was confirmed by Western blot analysis.

**Detection of cell cycle phase by propidium iodide staining.** Cells were harvested after 12 h of culture in the 6-cm dishes, washed once in PBS, centrifuged, and fixed in 70% ethanol for 24 h at 4°C. The nuclei were stained with propidium iodide (50 µg/mL) after treatment with 0.5% Triton-X100/PBS containing 0.05% DNase-free RNase, and the DNA content was analyzed by flow cytometry using the fluorescence-activated cell sorting Ari cell sorting system (Becton Dickinson). The percentage of cells in each phase of the cell cycle was determined using the Modfit LT program (Verity Software House).

**Cell proliferation assay.** Cells (3 × 10^5) were grown in a 96-well plate at 37°C with 5% CO2. At each time point, growth rate of cell was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method or by cell number counting. Absorbance was read at 570 nm, and background absorbance at 690 nm was subtracted in a Titertek Multiscan Plus MKI ELISA reader (Labsystems and Life Sciences International Ltd.) Results are given in fold change of each control value.

**Statistical analysis.** Statistical analysis was carried out with the help of the Statistical Package for Social Science, version 13.0 (SPSS, Inc.).

**Results**

**Effects of T3 on PTTG1 mRNA and protein expression in HepG2 cells.** In the four HepG2 cell lines investigated in this study (HepG2-TRα1#1, HepG2-TRα1#2, HepG2-TRβ1, and HepG2-Neo), overexpression of TR protein was ~10-fold, 3-fold, and 3.2-fold that of the HepG2-Neo control cell line (Fig. 1A). Overexpressed PTTG1 protein and mRNA were compared between various HepG2 cell lines after incubation with or without T3 for varying durations (Fig. 1B and C). The thyroid hormone T3 suppressed expression of PTTG1 in HepG2-TRα1#1, HepG2-TRα1#2 (data not shown), and HepG2-TRβ1 cells to a greater extent than in HepG2-Neo cells (Fig. 1B). Protein levels in PTTG1 decreased 14% to 84% after incubation of HepG2-TRα1#1, HepG2-TRα1#2 (data not shown), and HepG2-TRβ1 cells with T3 (10 and 100 nmol/L) for 24, 48, and 72 hours, respectively. These experimental results show the time-dependent properties of T3. However, the repression of PTTG1 by T3 was maximized at ∼10 nmol/L (Fig. 1B).

The effect of T3 on the expression of PTTG1 mRNA was examined by Northern blot analysis. A 0.6-kb PTTG1 mRNA was detected in three examined cell lines (Fig. 1C). Exposure of HepG2-TRα1#1 cells and HepG2-TRβ1 cells to 10 and 100 nmol/L T3 for 24, 48, and 72 hours, respectively, produced a dose-dependent decrease (63–87%) in PTTG1 mRNA. Furthermore, the repression by T3 was almost maximal at 24 h. Additionally, the effect of T3 on PTTG1 mRNA expression in HepG2-Neo cells was minimal (Fig. 1C). These experimental results thus suggest that the effect of T3 on PTTG1 protein expression is mediated, at least partly, at the transcriptional level.

**Knockdown of TRs expression abolishes repression of PTTG1 by T3.** To determine whether the PTTG1 repression is indeed TR dependent, TB protein expression was knocked down by RNA interference in four HCC cell lines, HuH7, J7, and Mahlavu expressed endogenous TRα1 and TRβ1 proteins (Fig. 1A). The small interfering RNA (siRNA) expression vectors encoding anti-sense TRα1 or TRβ1 sequences and a control plasmid with a scrambled sequence were transfected into each cell line. Figure 1A depicts the expression of TR proteins in four TR knockdown (KD) and control (Neo) cell lines. The data indicate that RNA interference repressed the expression of almost all the TR proteins. Subsequently, the repression of Sp1 or PTTG1 by T3 was diminished in the KD but not in the control Neo cell lines (Fig. 1D). These experimental results show that TRs are a crucial determinant of T3 repressive ability.

**Localization of a negative regulatory region in the PTTG1 promoter.** To further clarify the regulatory effect of T3 on PTTG1 mRNA expression, cycloheximide, a protein synthesis inhibitor, was introduced. The effect of T3 on PTTG1 mRNA expression in the presence or absence of cycloheximide was determined in HepG2-TRα1#1 cells. Blocking of protein repression with cycloheximide
effectively abolished transcriptional repression of \( PTTG1 \) by 10 nmol/L T3. This experimental result suggests that the effect of T3 on \( PTTG1 \) regulation may be indirect (data not shown). To determine whether the T3-induced repression of \( PTTG1 \) mRNA in HepG2 cells is mediated by an indirect effect of TR on \( PTTG1 \) transcription, the fragment of the \( PTTG1 \) promoter encompassing nucleotides −2,512 to +52 (relative to the translational initiation site) was cloned and inserted into a pA3TK-luc vector (containing a minimum thymidine kinase promoter). Serial truncated mutants of the \( PTTG1 \) promoter fragment (based on the −2,512/+52 construct; Fig. 2A) were also constructed and transfected into HepG2-TRα1 cells. The transcriptional activity of the −2,512/+52, −1,000/+52, and −1,000/−500 constructs was reduced by 45% to 58.5% in the presence of T3 (Fig. 2A) compared with the activity of the pA3TK-luc vector. To further delineate the promoter region involved in this marked inhibition of transcriptional activity by T3, the \( PTTG1 \) promoter sequences from nucleotides −1,000 to −500 were analyzed to identify binding sites from the transcription factor database (TFSEARCH: Searching Transcription Factor Binding Sites). This region includes two AP1 binding sites (−921 and −733), two Sp1 binding sites (−594 and −520), and one NF1 binding site (−511; Fig. 2B). Additionally, computer analysis revealed no TREs in this \( PTTG1 \) promoter region. To further determine the pivotal nuclear transcription factor binding sites on the \( PTTG1 \) promoter regulated by T3, the promoter was further divided into seven fragments including −1,000/−553, −1,000/−595, −620/−553, −553/−487, −620/−512, −513/+52, and −500/+52. As Fig. 2A shows, T3 reduced by 29.5% to 55% the promoter activity of only the −1,000/−553, −620/−553, −553/−487, and −620/−512 fragments (in HepG2-TRα1 cells; Fig. 2A). Those fragments contain at

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**Figure 1.** Thyroid hormone represses \( PTTG1 \) expression in HepG2 cell lines at the protein and mRNA levels. **A,** the TR protein expression level in four TR stable lines (HepG2-Neo, HepG2-TRα1#1, HepG2-TRα1#2, and HepG2-TRβ1). Four HCC cell lines (Huh7, J7, Mahlavu, and HepG2-TRα1#1) were transfected with TRα1-specific or TRβ1-specific RNA interference as described in Materials and Methods. Expression of TR proteins in four cell lines without (Neo) or with (KD) specific TR RNA interference transfection. \( PTTG1 \) expression was repressed by T3 at protein (B) or mRNA levels (C). The TR stable lines were incubated with T3-depleted (Td) medium in the absence or presence of 10 or 100 nmol/L T3 for 24, 48, or 72 h. Cell lysates (100 μg of protein) were then subjected to immunoblot analysis with polyclonal antibody to \( PTTG1 \). Alternatively, total RNA (20 μg/lane) was then isolated and sequentially analyzed by Northern blot using the \(^{32}\)P-labeled cDNA probe of \( PTTG1 \) and 18S rRNA. **D,** TR-dependent repression of \( PTTG1/Sp1 \) expression by T3. Cells were incubated with T3-depleted medium in the absence or presence of 10 nmol/L T3 for 48 h. Cell lysates (100 μg of protein) were then subjected to immunoblot analysis with polyclonal antibody to \( PTTG1 \) or Sp1. Actin was used as a loading control.

least one Sp1 binding site. However, deletion of the Sp1 binding at −594 (Fig. 2B; −553−648, Fig. 2A) or −520 (Fig. 2B; −620−553, Fig. 2A) did not significantly affect the transcription repression activity of T₃. Notably, transcription activity was induced rather than abolished completely when two Sp1 sites (−1,000−595, −513−526, −500−552) were deleted. Furthermore, deletion of AP1 and NF1 binding sites (−620−512) did not alter the repressive effect of T₃ on the PTTG1 promoter. However, the repression effect was diminished by mutation of these critical sites (Δ−620−512). These experimental results strongly suggest the involvement of Sp1 or its related factor and show the important role of Sp1 in T₃ regulation of PTTG1 promoter activity. Taken together, our data indicate that the repression of PTTG1 promoter activity by T₃ is mediated by Sp1.

**T₃-reduced Sp1 expression in HepG2-TRα1 cells.** Immunoblotting or Q-RT-PCR was performed to determine whether Sp1 or Sp1-related Sp2, Sp3, and Sp4 transcription factor is repressed by T₃/TR and subsequently represses PTTG1 expression. Clearly, no T₃ repression of Sp2 or Sp4 was observed by Q-RT-PCR, whereas Sp3 expression was reduced after 72 h of T₃ treatment (Fig. 3A). The Sp1 protein levels decreased ~71.5% after incubation with 10 nmol/L T₃ for 48 hours. Furthermore, cells incubated in 100 nmol/L T₃ for 48 hours revealed slightly increased (88%) repression of Sp1 protein expression. Moreover, after 72 hours of incubation in 100 nmol/L T₃, Sp1 expression was almost completely repressed (>90%; Fig. 3B). Concomitantly, PTTG1 protein levels also decreased ~70% to 85% after incubation of HepG2-TRα1 cells with 10 or 100 nmol/L T₃ for 48 or 72 hours (Fig. 3B). However, this phenomenon was not observed in HepG2-Neo cells (Fig. 3C).

**Overexpression of Sp1 activates PTTG1 promoter activity.** To clarify whether Sp1 is crucial in T₃ regulation of PTTG1 promoter activity, a Sp1 expression vector (in pcDNA3 vector) was cotransfected with the PTTG1 promoters noted in Fig. 2A. As Fig. 4A shows, the transfection of Sp1 expression vector in T₃-depleted as well as in 10 nmol/L T₃ medium increased PTTG1 promoter activity ~4-fold to 6-fold in PTTG1 promoter fragments containing Sp1 (−1,000/+52; −1,000−500; −1,000−553; −620−553, 513−553, 513−620, 620−553, 512−520, 500−552) but had no effect on PTTG1 promoter fragments containing non-Sp1 or mutated Sp1 binding site (−1,000−595, Δ−620−512, −513−526, and −500+/552; Fig. 4A). These data indicate that overexpressed Sp1 may activate the PTTG1 promoter in HepG2-TRα1 cells with or without T₃. Also, Sp1 apparently does not increase PTTG1 promoter activity if Sp1 binding sites have undergone mutation (Δ−620−512).

To verify that Sp1 protein target Sp1 sites directly, a fragment containing two Sp1 sites was used as an EMSA probe. Reactions performed with [α-³²P]-labeled −620 to −512 fragment and in vitro-translated Sp1 protein produced one predominant band (Fig. 4B, lane 3). Sp1 antibody (Fig. 4B, lane 4) but not control

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**Figure 2.** Regulation of PTTG1 expression by Sp1 at the transcriptional level. A. Serial deletion of the PTTG1 5′ or 3′ flanking region reporter constructs in the pA3TK-luc vector (1.2 µg/well) were transfected in HepG2-TRα1 cells. After transfection, cells were harvested after 24 h with or without 10 nmol/L T₃ treatment. All the promoter constructs were inserted into the pA3TK-luc vector that contains a minimal thymidine kinase (TK) promoter. The designation Δ−620−512 indicates mutated Sp1 binding site. The promoter activities were calculated relative to each 0 nmol/L T₃ (+T3/C0) and further normalized to the pA3TK-luc control, as well as β-galactosidase activities (T3-induced change in normalized β-gal activity). Columns, mean obtained from at least three independent experiments performed in triplicate; bars, SE. The negative value indicates the percentage of repression. B, schematic representation of the PTTG1 promoter with potential transcription factor binding sites.
Figure 3. Regulation of Sp1 family by T3 in HepG2-TR cells. A, regulation of Sp1, Sp2, Sp3, and Sp4 mRNA expression by T3 was analyzed by Q-RT-PCR. Western blot in HepG2-TRa1#1 cells (B) or in HepG2-Neo cells (C) incubated in T3-depleted medium in the presence or absence of 10 or 100 nmol/L T3 for 24, 48, or 72 h. Total cell lysate was then subjected to immunoblot analysis with polyclonal antibody to Sp1 or PTTG1. Columns, mean obtained from at least three independent experiments performed in triplicate; bars, SE. Values are shown as fold induction of the control (24 h, 0 nmol/L T3) value and differences were examined using the Student’s t test. **, *P* < 0.01; *, *P* < 0.05.
Figure 4. Sp1 binds to PTTG1 promoter and enhances PTTG1 expression. A, the PTTG1 reporter constructs (1.2 μg/well) from Fig. 2A were individually transfected into HepG2-TRX1 cells. Activation of reporters was assayed by cotransfection with or without Sp1 expression vector (0.3 μg/well) into HepG2-TRX1 cells in the presence or absence of 10 nmol/L T3. Promoter activity was calculated relative to the pA3TK-luc control. Columns, mean obtained from at least three independent experiments performed in triplicate; bars, SE. B, in vitro–translated Sp1, TRs, or RXRa was incubated in a final reaction volume of 20 μL containing ~10⁶ cpm of [α-³²P]-labeled oligonucleotide probes of the PTTG1 promoter (hot probe) for 40 min at room temperature. To determine the binding specificity of the Sp1/PTTG1 complex, 10-fold molar excess of specific (cold probe) or nonspecific (ns) probe was included in the reaction mixture. The MOPC21 antibody is a nonspecific monoclonal antibody. ΔSp1 indicates the mutated Sp1 probe. SS, Sp1 protein/PTTG1 oligonucleotide complexes were supershifted by Sp1 but not C4 antibody. C, nuclear extracts (NE) were isolated from HepG2-TRX1 cells, and 5 μg of protein were incubated in a final reaction volume of 20 μL as described in B.
Figure 5. The PTTG1 was regulated by TR through Sp1 in both thyroidectomized rats and human HCC specimens.

A. repression of PTTG1 and Sp1 expression by Tx in rat livers. Expression of PTTG1 protein in sham, Tx (thyroidectomized), or Tx + T3 male Sprague-Dawley rat livers was determined by Western blot analysis, as described in Materials and Methods. Intensities of PTTG1 and Sp1 bands on blots were quantified. Actin was used as a loading control. Values are shown as fold induction of the control (Tx) group, and differences were examined using the Student t test. Columns, mean of values from three independent experiments (n = 10 rats per group); bars, SE. **, P < 0.01; *, P < 0.05. B, reduced TR expression and overexpression of Sp1/PTTG1 protein in 23 representative pairs of human HCC specimens. T, tumor tissue; N, noncancerous adjacent tissue. Equal loading was confirmed by Coomassie blue staining after SDS-PAGE (data not shown).

T₃ represses PTTG1 and Sp1 protein expression in vivo. Surgical Tx was performed in two groups of 6-week-old male Sprague-Dawley rats (n = 10 per group) to determine the in vivo responses of PTTG1 and Sp1 to T₃ treatment. One group (Tx + T₃) was injected with T₃ daily for 2 weeks; the second group (Tx) received no T₃ injections; the third group was sham-operated controls. The rats were sacrificed at the end of the experiment (4 weeks after thyroidectomy), and serum was collected for T₃ and TSH determination. The livers were removed for Western blot analysis. The T₃ serum levels in the Tx group were ~0.022-fold (12.3 versus 548.1 ng/dL) than those in the group that received T₃ treatment (Tx + T₃). Levels of TSH in the Tx group were ~67.2-fold (2.42 versus 0.036 ng/mL) than those of the T₃-treated group. The T₃ and TSH serum levels in the sham group were ~48.1 ng/dL and 0.197 ng/mL, respectively. Western blot analysis showed repression of PTTG1 and Sp1 protein levels in the Tx + T₃ group were 76% and 61%, respectively, that of the Tx group. The PTTG1 and Sp1 protein levels in the sham group were 32% and 28%, respectively, that of the Tx group (Fig. 5A).

Furthermore, the clinicopathologic significance of PTTG1 expression in HCC was also investigated. In total, 65 patients with HCC were consecutively selected for this study. An equal amount (100 µg) of protein from each specimen was loaded for electrophoresis and Western blot analysis. Equal loading was confirmed by Coomassie blue staining after SDS-PAGE (data not shown). The PTTG1 and Sp1 proteins were detected in most of the cancerous tissues. The results from 23 representative paired HCC specimens are shown in Fig. 5B, indicating the increased PTTG1/Sp1 expression and the concurrently decreased TR protein expression in.
HCC tissues. The percentage of all paired samples with upregulated PTTG1 and Sp1 was 47.7% (31 of 65) and 64.6% (42 of 65) in the cancerous tissues, respectively, relative to the matched noncancerous adjacent tissues. Additionally, Trx1 and Trβ1 were reduced in 70.8% (46 of 65) and 55.4% (36 of 65) of the cancerous tissues, respectively. Linear regression analysis revealed a significantly positive correlation between the tumor:normal (T/N) ratios of PTTG and Sp1 [β = 0.531; 95% confidence interval (95% CI) of β, 0.232–0.940; P < 0.0001]. Concomitantly, significantly negative correlations were observed between the T/N ratios of (a) PTTG and Trα (β = −2.778; 95% CI of β, −4.125 to −1.431; P < 0.0001), (b) PTTG and Trβ (β = −1.373; 95% CI of β, −2.172 to −0.573; P = 0.001), (c) Sp1 and Trα (β = −2.824; 95% CI of β, −4.139 to −1.510; P < 0.0001), and (d) Sp1 and Trβ (β = −1.334; 95% CI of β, −2.123 to −0.544; P = 0.001). Thus, the expression of both PTTG1 and Sp1 was inversely correlated with the expression of TR proteins in HCCs (Fig. 5B).

Knockdown of Sp1 or PTTG1 expression inhibits HepG2-Trα1 cell growth. To further study PTTG1 function in HCC, PTTG1 expression was repressed by siRNA. The siRNA expression vectors encoding antisense PTTG1 sequences and a control plasmid with a scrambled sequence were transfected into HepG2-Trα1 cells. Figure 6A depicts the expression of PTTG1 in two HepG2-Trα1 knockdown (PTTG1 KD1 and PTTG1 KD2; lanes 2 and 3) and one control (HepG2-Trα1-Neo; lane 1) stable cell line. Additionally, Sp1 knockdown (Sp1 KD1 and Sp1 KD2; Fig. 6A, lanes 5 and 6) and control (HepG2-Trα1-Luc; lane 4) stable cell lines were prepared. Reduced Sp1 concomitantly suppressed PTTG1 expression (Fig. 6A, lanes 5 and 6), demonstrating that Sp1 is specifically down-regulated by T3 and subsequently decreases PTTG1 expression in HepG2-Trα1 cells. The two PTTG1-knockdown stable lines (#1 and #2), as well as the two Sp1 KD stable lines (#1 and #2) grew significantly (P < 0.01) more slowly than did the control HepG2-Trα1-Neo or HepG2-Trα1-Luc cell lines (Fig. 6B), suggesting that PTTG1 or Sp1 positively regulates cell proliferation.

Ectopic overexpression of PTTG1 promotes cell proliferation in Hep3B hepatoma cells. The Hep3B cells expressing low levels of endogenous PTTG1 were infected with adenovirus encoding PTTG1. After 40 hours of infection, PTTG1 expression in infected cells was determined. Figure 6A shows that PTTG1 expression in two Hep3B-PTTG1 sublines (#1 and #2; Fig. 6A, lanes 9 and 10) was higher than in noninfected cells or in cells infected with control GFP vector (Fig. 6A, lanes 7 and 8). To determine the effect of PTTG1 overexpression in Hep3B cells, cell proliferation was determined by MTT assay or cell number counting (data not shown) at 24 to 120 h after adenovirus-PTTG1 infection. The adenovirus-PTTG1-infected cells exhibited significantly higher proliferation rates than uninfected cells or cells infected with control GFP vector, whereas cell proliferation was very similar in both control cells (Fig. 6C). These experiments indicate that overexpression of PTTG1 can induce cell proliferation in Hep3B hepatoma cells.

Figure 6D illustrates that knockdown PTTG1 or Sp1 expression increased the percentage of cells in the G0-G1 phases by ~1.3-fold or 1.6-fold, respectively. The increase in cell number in G0-G1 phases was simultaneous with the decrease in the percentage of S-phase cells in PTTG1 KD and Sp1 KD. Conversely, overexpression of PTTG1 in Hep3B cells accelerated cell growth by reducing the number of cells in G0-G1 phases roughly 71% (43.8 of 61.82).

Discussion

This study characterized PTTG1, which was previously identified in cDNA microarray screening for T3-responsive genes in HepG2-Trα1 cells. The regulation of PTTG1 by T3 and the significance of this regulation in HCC have not been reported. This study examined the molecular mechanism of PTTG1 regulation by T3 in isogenic HepG2 cell lines and its physiologic significance. The experimental results indicate that PTTG1 regulation by T3 is indirect and mediated by Sp1. Further study showed that T3 down-regulates Sp1 expression. Additionally, exogenous transfection of Sp1 expression vector into HepG2-TR cells increased PTTG1 promoter activity. Furthermore, promoter activity analysis showed that two important Sp1 sites at –594 and –520 upstream of human PTTG1 participate in T3-induced change in human PTTG1 gene transcription. Observation of a similar regulatory process observed in thyroidecotomized rat specimens is consistent with these findings. Notably, the expression of PTTG1 and Sp1 was inversely correlated with the expression of TR proteins in HCCs. Moreover, the experimental data clearly show that negative regulation of PTTG1 by T3 is mediated by reduced Sp1 expression. Transcription factors other than Sp1, such as NF-Y, also regulate PTTG1 expression (17). Both Sp1 and NF-Y are essential in the transcriptional regulation of the human PTTG gene. However, the regulation of NF-Y by T3 was not observed (data not shown); therefore, NF-Y is not involved in T3 repression.

Furthermore, T3 had a minimal effect on the quantity of PTTG1 mRNA in HepG2-Neo cells. Knockdown of TRs expression abolished the repression of PTTG1 by T3. Thus, the effect of T3 on PTTG1 protein repression is apparently TR-dependent and mediated, at least partly, at the mRNA level. Ying et al. (18) and Furuya et al. (19) reported that PTTG1 is physically associated with TRβ1 or mutant TRβ1 in vivo. Additionally, PTTG1 directly interacts with the ligand-bound TRβ1 and with the steroid receptor coactivator-3 that recruits a proteasome activator (PA28γ). Subsequently, association with the above-mentioned complex induced the degradation of PTTG1 in association with the wild-type TRβ1-dependent receptor but not the mutant receptor. However, this study shows that the reduced expression of PTTG1 was largely due to down-regulation of Sp1 by T3. The experimental results of this study extend and supplement those of Ying et al. The PTTG1 is largely down-regulated at the transcriptional level according to the current findings. The remainder is regulated at the protein level as reported by Ying et al.

Overexpression of PTTG1 enhances the cell proliferation, migration, invasion, and/or tumorigenicity of cells derived from cancers of the human pituitary, esophagus, breast, thyroid, lung, head, and neck, as well as uterine leiomyomas, glioma, and pituitary adenomas (20–28). Earlier studies indicate that T3 significantly suppresses the growth of HepG2-TR overexpressing cells (29). However, T3 repression was not observed in the control cell line (HepG2-Neo), which did not express detectable TR. The suppressive effect is mediated at least partly by down-regulation of PTTG1 observed in this study because overexpression of PTTG1 is associated with cell proliferation, angiogenesis, and poor prognosis in HCC (30). After all, the expression of PTTG1 is very low in normal cells.

The Sp1 protein plays an important role in the growth and invasion of cancer cells (31, 32). The overexpression of Sp1 is often negatively associated with survival rates in several cancers (33, 34). Sp1 has been reported to be overly expressed in gastric cancer.
Figure 6. Functional assay of PTTG1 in hepatoma cells. A, expression of PTTG1 was repressed by siRNA in HepG2-TRα1 cells. PTTG1 knockdown clones (KD#1 and KD#2) expressed low levels of PTTG1 protein. Additionally, the expression of Sp1 was also repressed by siRNA in HepG2-TRα1 cells. Two clones (KD#1 and KD#2) expressed low levels of Sp1 protein. The PTTG1 protein expression levels were also reduced. Furthermore, the Hep3B hepatoma cells were infected by adenovirus or adenovirus-PTTG1 for 40 h. PTTG1 expression was assayed by immunoblot with polyclonal antibody to PTTG1. The PTTG1 or Sp1 knockdown HepG2-TRα1 cells (B) and PTTG1-overexpressed Hep3B cells (C) were incubated in growth medium for 24 to 120 h, and cell proliferation was assayed by MTT or manual cell counting (not shown). Points, mean obtained from at least three independent experiments performed in triplicate; bars, SE. Values are shown as a fold increase compared with the each cell number on day 1, and differences were examined using the Student's t test. **, P < 0.01; *, P < 0.05. D, effect of PTTG1 on the cell cycle in the G0-G1 phase in various PTTG1 knockdown or overproducing cells. Flow cytometric cell cycle histograms. The results are summarized in a table in D.
tissue and negatively expressed in adjacent noncancerous tissue (35). The Sp1 protein is the major transcription factor of several genes associated with proliferation or invasion (36). Recently, Tong et al. (37) indicated that PTTG1 apparently interacts with Sp1, and full-length PTTG1 positively regulates Sp1 activity. Additionally, Sp1 regulates PTTG1. Tong et al. therefore proposed the existence of an auto-feedback mechanism between Sp1 and PTTG1.

Consistent with previous reports (36, 38), the overexpression of PTTG1 in HCC was observed with concomitant overexpression of Sp1 in vitro, as well as in vivo. Moreover, Sp1, but not Sp2, or Sp4 was also negatively regulated by T3, at protein and mRNA levels. Therefore, TRs may play a suppressor role by reducing PTTG1 and Sp1 expression in normal liver. However, when TR expression is reduced in HCC, TRs lose their tumor suppressor role during hepatocarcinogenesis. Thus, alleviation of repression leads to induction of PTTG1 or Sp1 overexpression. Nevertheless, in some cases, the inverse correlation was not observed because of possible deletion or mutation of TR genes (5).

References
Thyroid Hormone Receptors Suppress Pituitary Tumor Transforming Gene 1 Activity in Hepatoma

Ruey-Nan Chen, Ya-Hui Huang, Chau-Ting Yeh, et al.


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