Mediation of the inhibitory effect of thyroid hormone on proliferation of hepatoma cells by transforming growth factor-beta

Chun-Che Yen, Ya-Hui Huang, Chu-Yu Liao, Cheng-Jung Liao, Wan-Li Cheng, Wei-Jan Chen¹ and Kwang-Huei Lin

Department of Biochemistry, Chang-Gung University, 259 Wen-hwa 1 Road, Taoyuan, Taiwan, Republic of China

¹First Cardiovascular Division, Chang Gung Memorial Hospital, Taoyuan, Taiwan, Republic of China

(Requests for offprints should be addressed to K-H Lin; Email: khlin@mail.cgu.edu.tw)

Abstract

Thyroid hormone (triiodothyronine, T₃) regulates growth, development and differentiation. To examine the influence of T₃ on hepatoma cell growth, thyroid receptor (TR) α 1 or TR β 1 over-expressing HepG2 cell lines were used. Growth of the HepG2-TR stable cell line was inhibited by over 50% following treatment with T₃. However, transforming growth factor (TGF)- β neutralizing antibody, but not the control antibody can reverse the cell growth inhibition effect of T₃. Flow cytometric analysis indicated that the growth inhibition was apparent at the transition point between the G1 and S phases of the cell cycle. The expression of major cell cycle regulators was used to provide further evidence for the growth inhibition. Cyclin-dependent kinase 2 (cdk2) and cyclin E were down-regulated in HepG2-TR cells. Moreover, p21 protein or mRNA levels were up-regulated by around 5-fold or 7.3-fold respectively following T₃ treatment. Furthermore, phospho-retinoblastoma (ppRb) protein was down-regulated by T₃. The expression of TGF- β was studied to delineate the repression mechanism. TGF- β was stimulated by T₃ and its promoter activity was enhanced six- to eight-fold by T₃. Furthermore, both T₃ and TGF- β repressed the expression of cdk2, cyclin E and ppRb. On the other hand, TGF- β neutralizing but not control antibody blocked the repression of cdk2, cyclin E and ppRb by T₃. These results demonstrated that T₃ might play a key role in liver tumor cell proliferation.

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Introduction

The thyroid hormone, 3,3',5-triiodo-L-thyronine (T₃), mediates numerous physiological processes, including embryonic development, cellular differentiation, metabolism and the regulation of cell proliferation (Hulbert 2000, Aranda & Pascual 2001). T₃ controls these processes in most organs. The effects of T₃ are mediated by nuclear thyroid hormone receptors (TRs). Moreover, TRs bind to the thyroid hormone response elements (TREs) located upstream from the promoters of target genes to regulate their expression transcriptionally (Hulbert 2000, Aranda & Pascual 2001). The nature of the transcriptional response is determined by cell type, promoter context, and hormone status (Hulbert 2000, Aranda & Pascual 2001). In most cases, TRs are transcriptional repressors without their cognate hormone (T₃ or thyroxine (T₄)) and are turned into activators by ligand binding (Hulbert 2000, Aranda & Pascual 2001).

Two main types of TRs have been identified, termed TR α and TR β , which are encoded on human chromosomes 17 and 3 respectively (Cheng 2000,

Aranda & Pascual 2001). Transcripts of each of these genes undergo alternative promoter choice for generating both the TR α 1 and α 2 and the TR β 1 and β 2 receptor isoforms (Cheng 2000, Hulbert 2000, Aranda & Pascual 2001).

Regarding previously published results (Lin *et al.* 2004), transforming growth factor-beta (TGF- β) was stimulated by T₃ at the mRNA level. TGF- β regulates cell growth and proliferation, and has been shown to block the growth of numerous cell types (De Caestecker 2004). The TGF- β receptor includes type 1 and type 2 subunits. These subunits comprise serine-threonine kinases that signal through the smad family of transcriptional regulators. T₃/T₄ have been shown to stimulate the proliferation of eukaryotic cells (Barrera-Hernandez *et al.* 1999, Aranda & Pascual 2001). Several studies demonstrated that cyclin D1 induction is an early event in T₃-induced hepatocyte proliferation (Pibiri *et al.* 2001, Alisi *et al.* 2004). These previous studies indicate that this cyclin may be a common target responsible for mitogenic activity of ligands of nuclear receptors. However, the influence of T₃ on human liver tumor cell proliferation is currently unknown although a similar

observation has been reported in rats (Ledda-Columbano *et al.* 2000).

The liver has long been recognized as a target organ for thyroid hormones. In fact, Chamba *et al.* (1996) reported that roughly equal quantities of TR α 1 and TR β 1 protein occur in human hepatocytes (Chamba *et al.* 1996). HepG2 is a well-differentiated hepatocellular carcinoma cell line without detectable TR protein expression. However, it secretes all 15 plasma proteins and preserves numerous liver-specific functions and thus can serve as an *in vitro* model (Chang *et al.* 1983). Consequently, the HepG2 cell line provides a useful model system for studying the influence of T_3 on the proliferation of liver tumor cells. The system was recently used to demonstrate that TGF- β is regulated by T_3 (Lin *et al.* 2004). This work shows that T_3 up-regulates the expression of TGF- β and subsequently suppresses liver tumor cell proliferation.

Materials and methods

Cell culture

Human hepatoma cell lines, HepG2-TR α 1#1, HepG2-TR α 1#2, HepG2-TR β 1 and HepG2-Neo, and rat pituitary tumor GC cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum. Three TR over-expressing lines, and the control cell line, HepG2-Neo, have been described previously (Lin *et al.* 2004). The serum was depleted of T_3 (Td) as described by Samuels *et al.* (1979). Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂.

Flow cytometry

Flow cytometric analysis was performed as described by Fan *et al.* (1995). Briefly, cells were harvested via trypsinization and fixed in 75% ethanol for at least 24 h at 4 °C. The cells were then washed with PBS containing 1% BSA (Life Technologies, Inc., Rockville, MD, USA) and incubated with 100 µg/ml RNase A (Sigma, St Louis, MO, USA) and 50 µg/ml propidium iodide (Sigma) for 2 h at room temperature. Finally, the stained cells were analyzed on a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Cell proliferation assay

Cells were plated on 6-cm dishes at 2×10^5 cells/dish, with each sample being plated in triplicate. Cells were counted using the Coulter Counter ZM (Coulter Electronics Inc., Luton, Beds, UK).

Immunoblot analysis

Cell lysates were fractionated using SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% gel, and the separated proteins were transferred to a nitrocellulose membrane (Pall Life Sciences, Ann Arbor, MI, USA) and subsequently visualized via chemiluminescence using an ECL detection kit (Amersham Inc., Piscataway, NJ, USA) as described previously (Shih *et al.* 2004). The antibodies used were rabbit polyclonal antibodies to cyclin E, and retinoblastoma (Rb) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse monoclonal antibody to cdk2 (1:1000 dilution in PBS) (Santa Cruz Biotechnology) or TGF- β (1:500–1000 dilution in PBS) (Serotec Ltd, Oxford, Oxon, UK). TGF- β ₁ was purchased from Pepro Tech (London, UK).

Northern blot analysis

Total RNA was extracted from the cells using TRIzol Reagent (Life Technologies) and equal amounts of total RNA (20 µg) were analyzed on a 1.2% agarose-formaldehyde gel as described previously (Lin *et al.* 2000, 2002). The separated RNA molecules were then transferred to a nylon membrane (Amersham) and subjected to northern blot analysis as described previously (Lin *et al.* 2004, Shih *et al.* 2004).

Quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR)

Total RNA was extracted from cells using TRIzol, as described above. Subsequently, cDNA was synthesized using the Superscript II kit for RT-PCR (Life Technologies) as described previously (Shih *et al.* 2004).

Real-time Q-RT-PCR was performed in a 25-µl reaction mixture containing 50 nM forward and reverse primers, 1 × Syber Green reaction mix (Applied Biosystems, Warrington, UK), and various quantities of template as described previously (Shih *et al.* 2004). Fluorescence emitted by Syber Green was detected using the ABI PRISM 7000 sequence detection system (Applied Biosystem), as described previously (Lin *et al.* 2004, Shih *et al.* 2004).

Cloning the TGF- β 5'-flanking region and promoter activity assay

Fragments of the TGF- β promoter (nucleotides –1362/+101) were amplified via PCR, according to the published nucleotide sequence (Kim *et al.* 1989a,b), and were then inserted into the pGL3 vector (Promega Corp., Madison, WI, USA). The sequence of the promoter construct was confirmed by automated DNA sequencing. To determine the influence of T_3 on the transcriptional activity of the TGF- β promoter, HepG2-TR α 1#1 cells (1×10^5 per 35-mm dish) were

(A) HepG2-Neo **(B) HepG2-TR α 1#1** **(C) HepG2-TR α 1#2** **(D) HepG2-TR β 1**

Figure 1 T_3 represses the proliferation of HepG2-TR cells. (A) HepG2-Neo, (B) HepG2-TR α 1#1, (C) HepG2-TR α 1#2 or (D) HepG2-TR β 1 cells were plated in 60-mm dishes at a density of 2×10^5 cells/dish. Cells were incubated with 0 (■), 10 (●) or 100 (▲) nM T_3 for the indicated time. In some experiments, cells were simultaneously incubated with 100 nM T_3 and 800 ng of the TGF- β neutralizing antibody (Ab, Δ) or non-specific control antibody (nsAb, $\circ$). Subsequently, cell number was determined using the Coulter Counter ZM. Data are expressed as means $\pm$ S.E. of values from three independent experiments. ** $P < 0.01$, 100 nM T_3 -treated vs Td-treated (0) (Student's t -test).

co-transfected, via a Lipofectamine protocol, using 3 μ g pGL3 vector containing TGF- β promoter sequences (Invitrogen) as described previously (Shih *et al.* 2004).

Results

T_3 represses HepG2-TR α 1 and HepG2-TR β 1 cell growth by prolonging the G0/G1 phase

The effect of T_3 on cell proliferation was assayed in three HepG2 stable cell lines with high expression of

TR α 1 or TR β 1 as previously described (Lin *et al.* 2004). Figure 1 shows that without T_3 cells proliferated roughly 2 to 3 times faster than those grown in media containing either 10 or 100 nM T_3 in three TR stable lines. However, this T_3 -repressed effect was not observed in the control cell line, HepG2-Neo, which did not express TR (Fig. 1). Following T_3 treatment, the doubling time for HepG2-TR stable lines increased from 1.03 ± 0.12 to 2.49 ± 0.31 days for TR α 1#1 cells, and from 1.15 ± 0.21 to 2.64 ± 0.25 days for TR β 1 cells; thus T_3 repressed the growth of HepG2-TR cells by around

Table 1 The effect of T₃ on cell cycle distribution in HepG2-TRα1, -TRβ1 and -Neo cells. Data are means±s.e.

	Cell cycle distribution (%)	
	G0/G1	S
HepG2-TRα1		
12 h	56.56±1.33	29.56±0.45
12 h+T ₃	66.77±1.07	18.38±0.51
24 h	61.35±3.01	30.27±2.73
24 h+T ₃	83.07±1.51	8.87±0.93
48 h	68.30±1.41	25.06±1.44
48 h+T ₃	91.91±0.87	4.20±0.48
HepG2-Neo		
12 h	63.79±2.45	21.55±0.52
12 h+T ₃	65.56±2.53	19.93±0.12
24 h	57.55±4.37	30.49±3.42
24 h+T ₃	60.74±6.62	28.67±3.29
48 h	74.30±2.61	20.40±1.66
48 h+T ₃	80.41±4.55	15.19±3.70
HepG2-TRβ1		
12 h	51.95±5.43	33.07±3.62
12 h+T ₃	62.40±3.54	18.78±3.58
24 h	69.13±4.27	25.78±6.10
24 h+T ₃	83.26±3.82	10.90±2.16
48 h	65.26±4.91	24.90±2.56
48 h+T ₃	84.66±2.46	8.70±1.46

two- to three-fold. However, T₃ did not influence the doubling time in HepG2-Neo cells (1.58 vs 1.67 days, 0 vs 100 nM T₃). All data indicate that T₃ significantly suppresses the growth of HepG2-TR but not HepG2-Neo cells.

To identify the phase of the cell cycle affected by T₃, cell cycle distribution was assayed via flow cytometry. Table 1 illustrates that the addition of T₃ increased the percentage of cells in the G0/G1 phases by roughly 1.2-, 1.4-, and 1.35-fold following 12, 24, and 48 h respectively. Simultaneously with the increase in cell number in the G0/G1 phases, the percentage of cells in the S phase was reduced in HepG2-TRα1#1 (Table 1). Similar results occurred in HepG2-TRβ1 (Table 1) and HepG2-TRα1#2 cells (data not shown).

T₃ represses the protein expression levels of cdk2 and cyclin E

The major kinase associated with cyclin E in human cells is cdk2. The formation of the cyclin E–cdk2 complex is an important step in the biochemical pathway that controls cell proliferation during G1. Additionally, cyclin E is one of the key regulators of the G1/S transition in the cell cycle. Over-expression of cyclin E has been noted in several malignancies and is associated with high cell proliferation (Keyomarsi & Herliczek 1997, Nielsen *et al.* 1998). Therefore, this study used western blot analysis to determine that the increase in the G0/G1 cell population was inversely associated with the level of cyclin E-cdk2 proteins. Treatment of HepG2-TRα1#1, -TRα1#2 and -TRβ1 cells using 100 nM T₃ led to a down-regulation of approximately 32 and 52% in the level of cdk2 protein following 24, and 48 h compared with T₃-depleted media (Td) in HepG2-TRα1#1 cells (Fig. 2A, B). Similarly, 100 nM T₃ led to down-regulation of approximately 27 and 31% in the protein level of cyclin E following 24 and 48 h (Fig. 2A, B). A similar effect was observed in the HepG2-TRβ1 (Fig. 2C), and HepG2-TRα1#2 cell lines (data not shown). Taken together, repression of both components of the cyclin E–cdk2 complex strongly agrees with the previous result of cell cycle blockage in G0/G1.

T₃-repressed cell proliferation results from stimulation of p21 expression

Levels of the negative regulator of cell cycle progression, p21 protein, increase in senescent cells, while p21 over-expression has been demonstrated to block tumor cell growth (Gong *et al.* 2003). Thus, p21 was investigated as an additional target for controlling cell proliferation. p21 mRNA was strongly induced 1.3-, 3.9- and 7.3-fold in HepG2-TRα1#1 cells at 12, 24, and 48 h respectively, following the addition of 10 nM T₃ to the media (Fig. 3A, B). Similarly, p21 protein was also significantly induced two- to five-fold by T₃ treatment in two TRα1 stable cell lines (Fig. 3C, D). However, T₃ did

Figure 2 Effect of T₃ on cdk2 and cyclin E protein expression in HepG2-TR cells. (A) TRα1#1 expressing HepG2 stable line was incubated using T₃-depleted medium in both the absence or presence of 10 and 100 nM T₃ for 12, 24, and 48 h, after which cell lysates (100 µg protein) were subjected to immunoblot analysis with cdk2 monoclonal or cyclin E polyclonal antibodies. The position of the 29 kDa, 53 kDa, 43 kDa bands for cdk2, cyclin E, and actin respectively are indicated on the left of each blot. (B) The intensities of each protein band were quantified, and the extent of T₃-induced change in protein levels was determined at each time point. The results are expressed as percentage expression relative to the control (Td, 0 nM T₃; open bars) conditions. Data are expressed as means ±s.e. of values from three independent experiments. * *P*<0.05, ** *P*<0.01, T₃-treated vs Td-treated (Student's *t*-test). Solid bars, 10 mM T₃; hatched bars, 100 mM T₃. (C) TRβ1 expressing HepG2 stable line was incubated using T₃-depleted medium in both the absence or presence of 10 and 100 nM T₃ for 12, 24, and 48 h, after which cell lysates (100 µg protein) were subjected to immunoblot analysis with cdk2 monoclonal or cyclin E polyclonal antibodies. The position of the 29 kDa, 53 kDa, 43 kDa bands for cdk2, cyclin E, and actin respectively are indicated on the left of each blot.

Figure 3 Influence of T_3 on p21 expression in HepG2-TR α 1 cells at the mRNA and protein levels. (A) HepG2-TR α 1#1 cells were incubated for 12, 24 or 48 h with or without 10 nM T_3 , after which total RNA was isolated and subjected (20 μ g per lane) to Northern blot analysis with 32 P-labeled p21 or GAPDH cDNA probes. The positions of the 2.3-kb p21 and 1.0-kb GAPDH mRNAs are indicated. (B) The intensities of the p21 mRNA bands on blots similar to that shown in (A) were quantified, and the extent of the T_3 -induced increase in the abundance of p21 transcripts was determined at each point. Data are means $\pm$ S.E. of values from three independent experiments. ** $P < 0.01$, T_3 -treated vs Td-treated (Student's *t*-test). (C) HepG2-TR α 1#1 and HepG2-TR α 1#2 cells were incubated for 24 or 48 h with and without 10 or 100 nM T_3 , after which total protein was isolated and subjected to Western blot analysis. (D) The intensities of the p21 protein bands on blots similar to that shown in (C) were quantified using the two stable lines. Data are means $\pm$ S.E. of values from three independent experiments. * $P < 0.05$, ** $P < 0.01$, T_3 -treated vs Td-treated (Student's *t*-test). Open bars, 0 nM T_3 ; solid bars, 10 nM T_3 ; hatched bars, 100 nM T_3 .

not markedly increase p21 mRNA or protein expression in the control cell line, HepG2-Neo (data not shown).

T_3 treatment impacts the phosphorylation state of Rb protein

Rb tumor suppressor is a critical negative regulator of cellular proliferation. The Rb protein was dephosphorylated (Fig. 4A) in HepG2-TR α 1#1 or two

other TR stable cell lines (data not shown) following T_3 addition, possibly indicating that cyclin E was inactivated by p21. The expression of hyperphosphorylated Rb protein increased significantly following 24 or 48 h T_3 treatment when HepG2-TR α 1 cells were incubated in control conditions (Td) (Fig. 4A). As a further positive control, the phosphorylation status of Rb was investigated in the GC cell line. Consistent with previous studies (Barrera-Hernandez *et al.* 1999), Rb was

(A) HepG2-TR α 1#1 **(B) GC**

Figure 4 Influence of T_3 on Rb protein in HepG2-TR α 1 and GC cells. (A) TR α 1 expressing HepG2 stable line or (B) GC cells were incubated with T_3 -depleted medium with or without 10 or 100 nM T_3 for 12, 24, and 48 h, after which cell lysates (100 μ g protein) were subjected to immunoblot analysis with polyclonal antibodies to Rb. The arrows indicate the hyperphosphorylated (ppRb) and hypophosphorylated (pRb) forms of Rb. Actin served as an internal control. Data were obtained from three independent experiments.

hyperphosphorylated in GC cells after T_3 treatment for 48 h (Fig. 4B). However, T_3 did not significantly change the phosphorylation status of Rb in the control cell line, HepG2-Neo (data not shown). These experimental results indicate that the incubation of HepG2 cells over-expressing TR in media containing T_3 represses the hyperphosphorylation of Rb.

TGF- β is stimulated by T_3

To better understand how T_3 inhibited the proliferation of HepG2-TR α 1 cells, this study investigated the influence of T_3 on the TGF- β expression. Similar to previously published results (Lin *et al.* 2004), TGF- β at the mRNA level was significantly stimulated about 1.5- to 3-fold by T_3 in HepG2-TR α 1#1 cells (Fig. 5A). Additionally, the 12 kDa TGF- β protein was up-regulated about two- to threefold 24 or 48 h following the addition of T_3 (Fig. 5B). To further clarify the influence of T_3 on TGF- β at the transcriptional level,

the TGF- β 5'-flanking region (from -1362 to +101) was cloned into the pGL3 vector and its activity was assayed. T_3 was demonstrated to increase the promoter activity by approximately 7.8- and 5.8-fold at 10 and 100 nM concentrations of T_3 respectively in the TR α 1 stable cell line, compared with its activity in control (Td) media without the addition of T_3 (Fig. 5C). However, T_3 did not considerably increase the promoter activity in the control cell line, HepG2-Neo (Fig. 5C).

TGF- β and its neutralizing antibody influence cyclin E, cdk2 and Rb expression

To clarify the signaling pathways involved in the repression of cyclin E, cdk2 and Rb by T_3 , this study investigated the involvement of TGF- β . The data indicate that treating cells with T_3 for 48 h represses cdk2 expression by at least 40% at the protein level compared with the control (Td) conditions (Fig. 2A, B; Fig. 6A, B, C, lane 1 vs 2). Moreover, TGF- β alone also

repressed the expression of cdk2 following 40 min treatment (Fig. 6A, B, C lane 1 vs 3). Notably, either T_3 or TGF- β repressed the expression of cyclin E, cdk2, and rendered the Rb protein in the hypophosphorylated form (Fig. 6A, B, C lane 1 vs 2 and 3) in the HepG2-TR cells. Importantly, the repression of cyclin E, cdk2, and ppRb by T_3 was blocked by the addition of TGF- β neutralizing antibody (nAb) (Fig. 6A, B, C lane 2 vs 6), but not by the non-specific antibody (nsAb) (Fig. 6A, B, C, lane 2 vs 5) in the HepG2-TR α 1#1, -TR α 1#2, and -TR β 1 stable lines. However, the effects of T_3 and TGF- β were not observed in the Neo cells (Fig. 6D) and did not synergistically repress the expression of cyclin E, cdk2, and ppRb (Fig. 6A, B, C lane 4 vs 2 and 3) in the HepG2-TR cells. Moreover, TGF- β neutralizing antibody, but not the control antibody can reverse the cell growth inhibition effect of T_3 (Fig. 1). Thus, cell proliferation is repressed by T_3 through a TGF- β -mediated mechanism. Additionally, T_3 controls the expression and activity of a number of cell cycle regulators via TGF- β , including p21, pRb and the cyclin E-cdk2 complex.

Discussion

This study identified a novel pathway of T_3 signaling mediated by TGF- β for inhibiting the proliferation of hepatoma cells expressing high levels of TR proteins. The effect of T_3 treatment in promoting the proliferation of normal hepatocytes or GC cells derived from the pituitary has been well documented (Chou *et al.* 1987, Barrera-Hernandez *et al.* 1999). However, HepG2 and Hep3B cells do not express detectable TR proteins (Lin *et al.* 1994). Unlike previous studies, the results of this study indicate that T_3 represses the proliferation of hepatoma cells rather than promoting it. The experimental data indicate that T_3 only significantly suppresses the growth of HepG2-TR over-expressing cells. However, this T_3 -repressed effect was not observed in the control cell line (HepG2-Neo) that did not express detectable TR. The study does not contradict the results of *in vivo* studies reported by Ledda-Columbano *et al.* (2000). Their results demonstrated that T_3 supplemented

an increase in the BrdUrd-labeling index in the carcinogen-induced rat hepatocellular carcinoma (HCC). However, the TR expressing level in rat HCC is unknown (Ledda-Columbano *et al.* 2000). Actually, their data indicated that T_3 administration, despite stimulating hepatocyte proliferation, resulted in a 70% reduction in the number of glutathione S-transferase (GST)-positive lesions, the marker enzyme used to identify pre-neoplastic lesion, with no increase in the size of the remaining nodules. In addition, repeated exposure of nodule-bearing rats to T_3 caused a 50% reduction in the incidence of HCCs and 100% inhibition of lung metastasis. Their data also support the concept that T_3 -induced cell proliferation might not necessarily represent a promoting condition for putative pre-neoplastic lesions and demonstrates an anticarcinogenic effect of T_3 . To confirm that the suppressive effect of T_3 treatments on hepatoma cell proliferation did not simply result from the toxic effects of this hormone, this study examined the expression of a number of factors that are known to be significantly involved in the cell cycle, for example cdk2, Rb, p21 and cyclin E. Additionally, this work used the GC cell line, which is known to proliferate when stimulated by T_3 (Barrera-Hernandez *et al.* 1999). This study found that T_3 repressed hepatoma cell growth by lengthening the G1 phase of the cell cycle, concomitantly decreasing the expression of cdk2 and cyclin E. To study the expression kinetics of the cell cycle, several regulatory factors were assayed, including proliferation cell nuclear antigen (PCNA), which represents an endogenous protein of the DNA polymerase δ . PCNA is an essential auxiliary protein of DNA polymerase δ , and is synthesized in the early G1 and S phases for processing of DNA replication. PCNA mRNA was repressed following T_3 treatment in HepG2-TR stable lines (data not shown). This provides further support for the notion that T_3 can suppress proliferation in cells that express TR.

cdk2 has been demonstrated to play a pivotal role in regulating cell cycle progression, is regulated by phosphorylation and can associate with cyclins A, E, D1, and D3. This investigation found that the inhibitory effect of T_3 on cell proliferation occurs during the G0/G1 phase of the cell cycle. Interestingly, the

Figure 5 Induction of TGF- β by T_3 in HepG2-TR α 1 cells. (A) HepG2-TR α 1 cells were incubated using T_3 -depleted medium with or without 10 nM T_3 for 12 to 48 h, after which total RNA was isolated and subjected to Q-RT-PCR for TGF- β expression as described in the Materials and methods section. Results are expressed as fold induction by T_3 . Data are means $\pm$ S.E. of values from three independent experiments. * $P < 0.05$, ** $P < 0.01$, T_3 -treated vs Td-treated (Student's *t*-test). (B) Immunoblot analysis of TGF- β in HepG2-TR α 1#1 cell lines. Lysates (100 μ g protein) from HepG2-TR α 1 cells were subjected to immunoblot analysis with monoclonal antibody to TGF- β as described in the Materials and methods section. The position of the 12-kDa TGF- β is indicated. Actin served as an internal control. (C) T_3 -dependent trans-activity of TR α 1 and Neo control cells in TGF- β promoter. Cells were transfected using a luciferase reporter plasmid containing the TGF- β 5'-flanking region encompassing nucleotides -1362/+101 (Kim *et al.* 1989a) and with a β -galactosidase plasmid to control for transfection efficiency. Subsequent procedures are described in the Materials and methods section. Data were obtained from three independent experiments, each performed in duplicate. The ordinate indicates 'Fold induction' and non-induced=1. **, $P < 0.01$, T_3 -treated vs Td-treated (Student's *t*-test). Open bars, 0 nM T_3 ; solid bars, 10 nM T_3 ; hatched bars, 100 nM T_3 .

cdk2–cyclinE complex is active in the G1 and S phases and is important for the progression from G1 to S (Harwell *et al.* 2004, Lents & Baldassare 2004). Additionally, cyclin E is pivotal in regulating the restriction point transition in the cell cycle. The role of cyclin E as a cdk2 activator in controlling restriction point transition in the cell cycle makes cyclin E an excellent candidate as a factor for involvement in tumor development. The hepatoma cell system presented here demonstrated reduced expression of both cyclin E and cdk2 following T_3 application, although further study is required to determine whether this phenomenon is because of a direct or an indirect effect. Aberrant regulation of cyclin E is a common phenomenon seen in tumor cells and has been reported in tumor tissues isolated from breast cancer patients (Akli *et al.* 2004, Harwell *et al.* 2004). An interesting study by Pibiri *et al.* (2001) demonstrated that, in Wistar rats, hepatocyte proliferation induced by T_3 occurred in the absence of AP-1, nuclear factor- κ B, and STAT3 activation or any change in the mRNA levels of the immediate early genes *c-fos*, *c-jun*, and *c-myc*. However, this study found that T_3 treatment increased cyclin D1 mRNA and protein levels, and moreover this increase occurred much more rapidly than liver regeneration following a two-thirds partial hepatectomy. Regrettably, the expression level of TGF- β was not examined. In contrast, in this investigation, we did not observe any significant change in the level of cyclin D1 expression following T_3 treatment (data not shown). It is possible that T_3 influences the late G1 but not the early G1 phase. The cyclin D1–cdk4, cdk-6 complexes are activated in early G1, whereas cyclin E–cdk2 is activated in late G1 (Sherr 1996, Martin-Castellanos & Moreno 1997). Therefore, our observations regarding hepatoma cells differ from those for normal regenerating rat liver cells. Summarizing the results of this and previous studies, T_3 appears both to induce proliferation of normal hepatocytes and to suppress the proliferation of hepatoma cells with TR expression.

p21 can bind and inhibit each member of the cdk family. It also directly binds to PCNA and thus inhibits DNA replication. The present work showed that the expression of p21 was stimulated markedly by T_3 at both the mRNA and protein levels, and may be, at least partially, responsible for blocking cell proliferation.

TGF- β is a pleiotropic cytokine that elicits a broad range of cellular responses, including cell growth, differentiation, and apoptosis. One of the biological effects of TGF- β is to inhibit epithelial cell proliferation by inducing cell cycle arrest (Sporn & Roberts 1992, Massague 1998). The effectors of TGF- β -induced cell growth inhibition are cyclin-dependent kinase inhibitors; among these inhibitors, p21^{Cip1} plays a major role in numerous biological contexts (Li *et al.* 1995). Additionally, Buzzard *et al.* (2003) demonstrated that T_3 and other hormones induced the progressive accumulation of the cell cycle inhibitors p27^{Kip1} and p21^{Cip1} in Sertoli cells. However, the underlying mechanisms responsible for suppressing proliferation remain largely unknown (Gong *et al.* 2003). This study found that T_3 acted on the TGF- β promoter to activate transcription. However, further work is required to determine whether this activation is due of a direct or an indirect effect. Therefore, in these hepatoma cells, the two signaling pathways are linked and TGF- β works downstream of the T_3 pathway.

Rb is a nuclear phosphoprotein that undergoes differential phosphorylation during the cell cycle. Hypophosphorylated Rb protein complexes with E2F to inhibit its trans-activity on target genes and thus halts cell cycle progression. Barrera-Hernandez *et al.* (1999) reported that T_3 increased the phosphorylation of Rb protein and thus stimulated GC cell line proliferation, similar to the results obtained here using these cells. By contrast, in this study T_3 caused the accumulation of hypophosphorylated Rb protein, thus suppressing cell cycle progression in hepatoma cells overexpressing TR proteins.

Shimizu *et al.* (2004) reported that OSI-461 (a potent protein kinase G activator) enhanced the G0/G1 arrest resulting from acyclic retinoid (belonging to the thyroid/steroid super-family), and a combination of these agents synergistically decreased expression of cyclin D1 protein and mRNA, inhibited cyclin D1 promoter activity, reduced the level of hyperphosphorylated forms of the Rb protein and induced cellular levels of the p21^{Cip1} protein and mRNA in HepG2 cells. These observations mirror some of the results reported here.

Sumitani *et al.* (1994) reported that androgen significantly stimulates growth of the mouse mammary

Figure 6 T_3 , TGF- β and its neutralizing antibody influence cyclin E, cdk2, and Rb expression. (A) HepG2-TR α 1#1, (B) HepG2-TR α 1#2, (C) HepG2-TR β 1 and (D) HepG2–Neo stable lines were incubated using T_3 -depleted medium with or without 10 nM T_3 for 48 h, after which cell lysates (100 μ g protein) were subjected to immunoblot analysis using antibodies against cyclin E, cdk2, and Rb. In lanes 3 and 4, the final 40 min before the cells were harvested, 5 nM TGF- β 1 were added. Cells were incubated simultaneously with T_3 and 800 ng of the TGF- β neutralizing antibody (nAb), lane 6, or non-specific control antibody (nsAb), lane 5. Subsequently, cell lysates (100 μ g protein) were subjected to immunoblot analysis. The intensities of the cyclin E (open bars), cdk2 (hatched bars), and ppRb (solid bars) protein bands on the blots were quantified. The data are intensities of lanes 2 to 6 compared with those in lane 1 (Td condition) and were from three independent experiments. Actin was used as an internal control. The ordinate indicates 'intensity' and non-induced (Td)=100. * $P < 0.05$, ** $P < 0.01$, lanes 2 to 6 vs lane 1 (Student's *t*-test).

Shionogi carcinoma SC-3 cells. This androgen-induced growth is partially blocked by T_3 . TGF- β also inhibits SC-3 cell growth. Thus, they investigated whether T_3 exerted its inhibitory effects on SC-3 cell growth through TGF- β mRNA expression. This study showed that T_3 stimulated the expression of TGF- β at the mRNA and protein levels in HepG2-TR stable cells. Meanwhile, TGF- β 1 exerted its inhibitory effects through down-regulation of PCNA, cdk2, and cyclin E. Although this study did not demonstrate the effect of TGF- β neutralizing antibody on the growth of HepG2 cells, it did show that TGF- β antibody neutralizing T_3 repressed cdk2.

In conclusion, this work provides evidence that T_3 and its receptor mediates the suppression of hepatoma cell proliferation by TGF- β . The results presented here raise the possibility that T_3 , via its receptors and TGF- β , helps to regulate hepatocyte tumor growth and development.

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