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Cell Growth Effects of Triiodothyronine and Expression of Thyroid Hormone Receptor in Prostate Carcinoma Cells

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ABSTRACT: Thiiodothyronine (T3) plays an important role in the regulation of cell growth and differentiation. In this study, we show the different effects of T3 on cell growth response and expression of the thyroid hormone receptor in human prostate cell lines from normal to hormonal refractory metastatic cancer cells. Although the thyroid hormone receptor (TRβ1) ubiquitously express in human prostatic epithelium cell lines (PZ-HPV-7, CA-HPV-10, LNCaP, DU145, PC-3), T3 did not show any effect on the cell proliferation of prostatic cell lines except LNCaP cells in vitro. Immunoblot assay revealed that PZ-HPV-7 and CA-HPV-10 cells express 5–10-fold of TRβ1 more than LNCaP cells; however, the immunocytochemical staining and immunoblot assay of cellular fractions suggested the TRβ1 is located on the cell nuclear membrane of PZ-HPV-7 and CA-HPV-10 cells. Our results suggested that T3 upregulates cellular proliferation on LNCaP cells but not other prostatic carcinoma cells and PZ-HPV-7 and CA-HPV-10 cells express the novel TRβ1, which locates at cell nuclear membrane.

Key words: Cell proliferation, LNCaP, PC-3, PZ-HPV-7, CA-HPV-10, DU145.

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Although a number of clinical and experimental studies have suggested the involvement of thyroid hormones and cognate nuclear receptors in cell growth and differentiation of many cell types (Nogurira and Brentai, 1996; Bassett and Williams, 2003; Jones et al, 2003), the effect of thyroid hormones on prostate cells still is not well understood. Recent studies indicated that triiodothyronine (T3), the active thyroid hormone, may be used as a biomarker in prostate pathology because men with benign prostatic hyperplasia (BPH) or prostate cancer demonstrate significantly elevated serum T3 levels in comparison with normal men (Lehrer et al, 2002, 2003). Although early studies have reported strong expression of thyroid hormone receptor mRNAs in human prostate tissues (Sakurai et al, 1989), only a few studies concerning the functional effects of T3 on prostate cells have been reported. Hedlund et al (1994) suggested that T3 is one of the most critical components for supporting the growth of the prostatic carcinoma cell line, LNCaP. Other studies also indicated that T3 has an important role in the regulation of prostate cell growth and differentiation and can modulate the androgen-mediated transcription of the PSA gene in LNCaP cells (Zhang et al, 1999; Zhu and Young, 2001). Questions arise as to how the thyroid hormones affect other prostate cells, including androgen independent cell lines, and how the thyroid hormonal receptors in these cells are expressed. It seems worthwhile to investigate whether they play an important role in the course of prostate cancer progression.

In this study, we used several human prostate cell lines, from normal cells to hormonal refractory cells, to study the effects of T3 on cell proliferation. Additionally, we studied the expression and localization of thyroid hormone receptors (TRβ1) in those cell lines for elucidating the possible roles of thyroid hormone and thyroid-hormone receptors in the progression of prostate cancer.

Materials and Methods

Cell Culture and Chemicals

The PZ-HPV-7, CA-HPV-10, LNCaP, PC-3, and DU145 cell lines were obtained from the American-Type culture collection (Rockville, Md). The PZ-HPV-7 cells were derived from epithelial cells cultured from the normal tissue of prostatic peripheral zone, and the CA-HPV-10 cells were derived from cells of a localized prostatic adenocarcinoma with Gleason grade 4/4. Both cells were immortalized by transforming the human papillomavirus 18 (Weijerman et al, 1994). The LNCaP cells were from a unique tumor-cell line derived from a supraclavicular lymph node metastasis of human prostate carcinoma, which exhibits cell proliferation in response to androgens in vitro (Horoszeqicz et al, 1983). The PC-3 cells were isolated from bone metastasis of a 62-year-old stage-IV prostatic cancer patient (Kaighn et al,

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1979). The DU145 cells were isolated from a lesion in the brain of a patient with metastasis prostate carcinoma (Stone et al, 1978). The PZ-HPV-7 and CA-HPV-10 cell lines were maintained by keratinocyte serum-free medium with EGF and bovine pituitary extract supplement (Life Technologies, Rockville, Md). The T3 (3.3′.5-triiodo-L-thyronine sodium salt), 5α-Adrostain-17β-ol-3one (DHT), charcoal (dextran-coated) and general chemicals in this study were purchased from Sigma (St Louis, Mo). The BCA protein concentration assay kit was obtained from Pierce (Rockford, Ill). The fetal bovine serum (FBS) was purchased from HyClone (Logan, Ut) and RPMI 1640 medium and RPMI 1640 phenol red free (RPMI-PRF) medium were purchased from Life Technologies (Rockville, Md). The LNCaP, PC-3, and DU145 cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS) and the medium was changed twice a week. The FBS, which was treated with charcoal (1 g/500 mL FBS) for 24 hours to remove the steroids, was regarded as the charcoal-dextran-treated FBS (CD-FBS) in this study.

Cell Proliferation Assays

Cell proliferation in response to T3 and DHT was measured with a nonradioactive assay as described before (Juang, 2004). All reagents were purchased from Promega Biosciences (San Luis Obispo, Calif). Cells (5000 cell/well) were grown in 100 μL RPMI-PRF medium with 2% CD-FBS for 2 days in the absence of androgen. After another 2 days, cells were incubated with 100 μL of 0, 0.1, 1, 10, 100 nM T3 in the same medium for 6 days. Cells were than incubated with freshly prepared, combined (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfop heny)-2H-tetrazolium, inner salt) (MTS)/phenazine methosulfate (ratio 1:1 by volume) solution for 3 hours at 37°C in a humidified 5% CO_2 atmosphere.

Cell Immunocytochemical Staining

PZ-HPV-7 and CA-HPV-10 cells were grown on the surface of a cover slide for 48 hours and were immunocytochemically stained for thyroid receptors and androgen receptors as described before (Juang et al, 2004). Briefly, cells were washed with phosphate buffer solution and then fixed using cold aceton for 15 minutes. Cells were immunocytochemically stained using a VECTASTAIN ABC kit, according to the manufacturer's instructions (Vector, Burlingame, Calif). Cells were detected using 1:500 of rabbit anti-human androgen receptor N-terminal (N-20) polyclonal antibody or 1:200 mouse anti-human thyroid hormone (J52) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif).

Immunoblot of Thyroid Hormone Receptor

Cells were lysed using a lysing buffer (62.5 mM Tris [pH 6.8], 2% sodium dodecyl sulfate (SDS), 10% glycerol, 5% β-mercaptoethanol, and 7 M urea, 5 μg/mL leupeptin, and 1 mM phenylmethylsulfony fluoride). The concentrations of protein in the aliquot samples were measured using the BCA protein assay kit. To isolate the nuclear extract and nuclear membrane, cell pellets were resuspended in buffer A (100 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 minutes thereafter. Cell pellets were resuspended in buffer A containing 0.05% Nonidet P-40 to release the nuclei after centrifugation. Successful release of nuclei was checked by phase-contrast microscopy. After centrifugation, the supernatant was stored and represented as the cytoplasm of the cell. The nuclei pellet was resuspended in buffer C (5 mM HEPES, 26% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 300 mM NaCl) and incubated on ice for at least 30 minutes. The successful release of nuclear extract was checked by phase-contrast microscopy. The high-salt solution was centrifuged at 24 000 × *g* for 20 minutes at 4°C. The supernatant was regarded as the nuclear extract and the pellet was represented as the nuclear membrane. The concentrations of proteins in the aliquot samples were precipitated by TCA and measured using the BCA protein assay kit. Equal amounts of protein were analyzed by the ECL detection system, as described by the manufacturer (Amersham Biosciences, New Territories, Hong Kong). The proteins were probed using 1:200 mouse anti-human thyroid hormone (J52) monoclonal antibody (Santa Cruz Biotechnology).

Results

The effect of T3 treatment on cell proliferation was examined in PZ-HPV-7, CA-HPV-10, LNCaP, PC-3, and DU145 cells. Using the MTS tetrazolium salt reduction method, we found T3 increased twofold optimal proliferative response of LNCaP cells (Figure 1C) but not in other cell lines (Figure 1A through E). DHT did not enhance T3 effect on the cell proliferation in the LNCaP cells after incubation for 8 days (Figure 2A). To determine whether T3 effect on cell proliferation is due to the presence of endogenous androgen receptor in the LNCaP cells, we treated the full-length androgen receptor stable-transfected PC-3 cells (PCAR) and mock vector-transfected PC-3 cells (PCDNA), which had been cloned in our laboratory (Juang et al, 2004) with different concentrations of T3. The results indicated that T3 also did not affect the cell proliferation of either PCAR cells or PCDNA cells after incubation for 6 days (Figure 2B and C). The finding suggested that the cell proliferation effect of LNCaP cells by T3 is not modulated by androgen receptors.

For elucidating the reason why the T3 effect on prostate cells is cell-type specific only in the LNCaP cell, we further studied the expression of thyroid receptors in different prostate cell lines. Immunoblot assays indicated higher concentrations of TRβ1 in the PZ-HPV-7 and CA-HPV-10 cells. The levels of TRβ1 expression are PZ-HPV-7 ≅ CA-HPV-10 > DU145 ≅ PC-3 > LNCap (Figure 3A through B). Moreover, our result also revealed that PC-3 and DU145 cells express other TRβ1 isoforms because the major expression has lower molecular weight than the native form in immunoblotting assays (Figure 3A). Immunocytochemical staining assay indicated AR

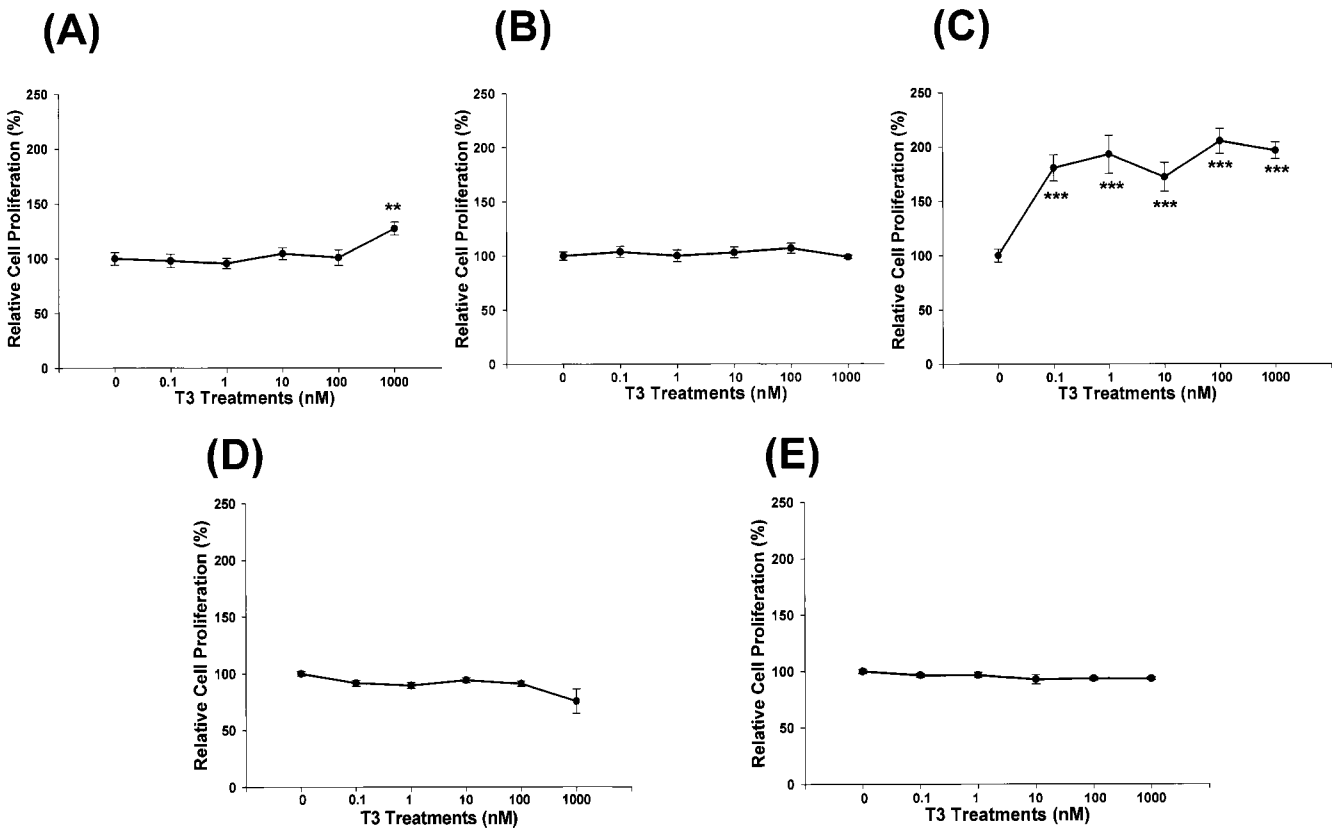

Figure 1. Modulation of T3 on cell proliferation of human prostatic cells. Cells (5000 cell/well) were incubated at each well of a 96-well plate. At day 2, cells were treated with different concentrations of T3 (0–1000 nM) in 100 μL RPMI-PRF medium with 2% charcoal-dextran-treated FBS. The MTS assay was performed at day 6 after incubation. (**A**) PZ-HPV-7, (**B**) CA-HPV-10, (**C**) LNCaP, (**D**) PC-3, and (**E**) DU145 cells. Bars indicate standard error.

expression in the nucleus; however, the expression of TRβ1 in PZ-HPV-7 and CA-HPV-10 cells is mainly on the nuclear membrane (Figure 4). To prove these immunocytochemical staining results, we isolated the cell nuclei by treating cells with Nonidet P-40 and separating the nuclear extracts and nuclear membrane by adding 300 mM NaCl to the cell nuclei thereafter. The results from immunoblot assay (Figure 3C) are in agreement with the results of cell immunocytochemical staining, which demonstrated that the location of TRβ1 is in the cell nuclear membrane of normal human prostatic epithelium cell line, PZ-HPV-7.

Discussion

It is well known that thyroid hormone is a major physiological regulator of mammalian development through specific effects on the rate of cell differentiation and gene expression (Bernal and Nunez, 1995; Bassett and Williams 2003; Jones et al, 2003). A prostate-thyroid axis has been found that exists between rat ventral prostate and thyroid gland (Bilek et al, 1992). Although it is also well known that thyroid hormone modulates thyrotrophin-releasing hormone (TRH) and TRH-Gly levels in the male reproduction system, including prostate, the direct effect of thyroid hormone on prostate is still unknown (Mani Maran et al, 2001). In animal models, the levels of thyroid hormone determine the size of the prostate (Howland and Ibrahim, 1974). In fact, thyroid hormone has been regarded as a factor in the regulation of the benign prostatic hyperplasia and prostate cancer (Dunzendorfer et al, 1981; Lehrer et al, 2002). Recently, studies have shown that T3 can modulate cell growth and potentiates the androgen-mediated transcription of PSA gene in LNCaP cells (Esquenet et al, 1995; Zhang et al, 1999; Zhu and Zhang, 2001). Although previous studies showed T3 alone could achieve more pronounced growth enhancement than in the presence of androgens (Zhang et al, 1999), our results indicated that 10 nM DHT did not enhance T3 on cellular proliferation in LNCaP cells. Moreover, T3 have no effect on the cell proliferation in androgen refractory PC-3 cells even after stable transfected with androgen receptor.

The present study demonstrated that T3 can enhance cell proliferation in LNCaP cells; however, the effect is

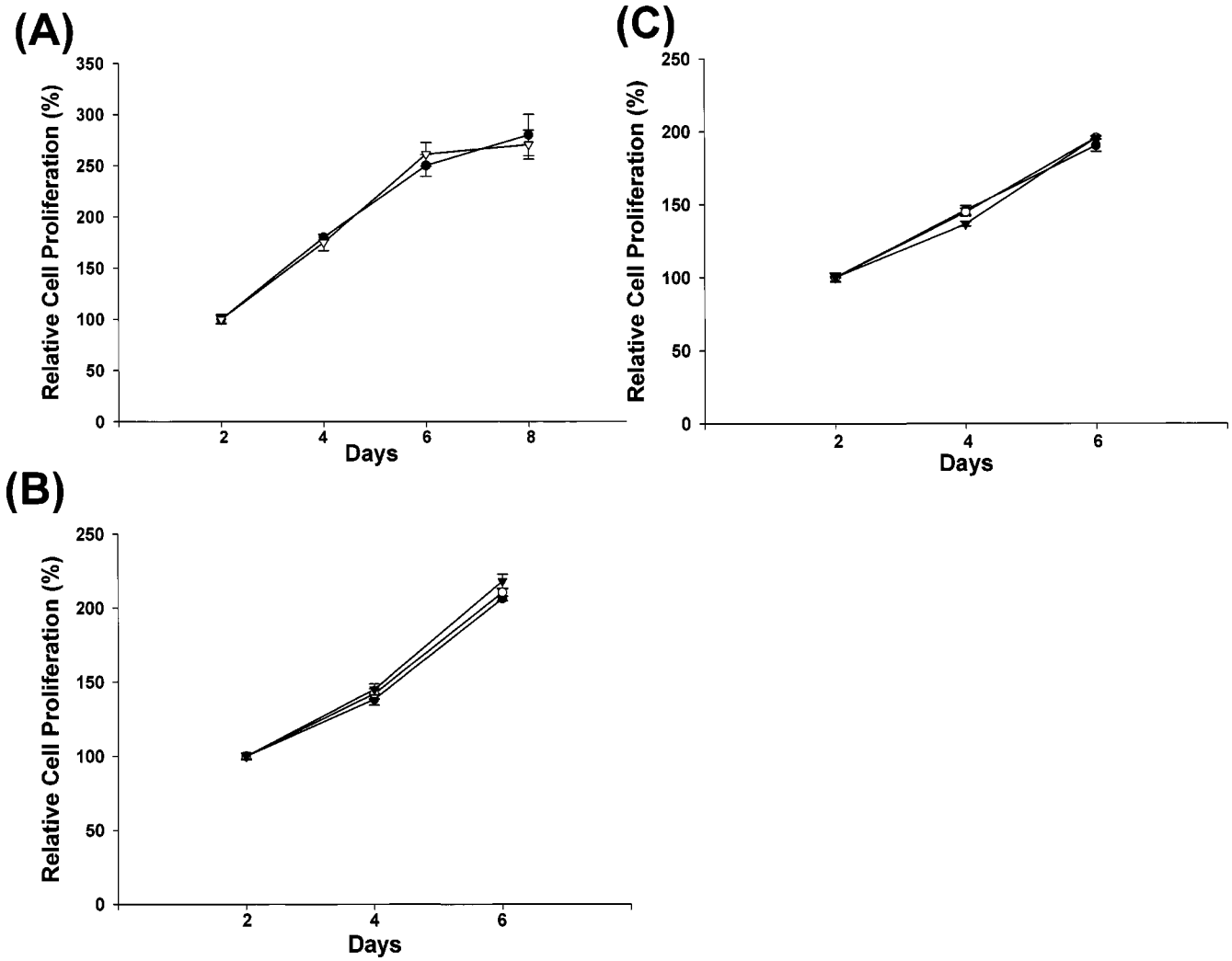

Figure 2. Interactive effects of T3 and androgen in LNCaP cell growth. (**A**) Cell density was measured by MTS assay after LNCaP cells were incubated in T3 (1 nM) treated medium with (solid triangle) or without (open triangle) 10 nM DHT for 8 days. The androgen receptor stable-transfected PC-3 cells (**B**) or mock-vector-transfected PC-3 cells (**C**) were incubated with 0 (solid circle), 1 (open circle), and 10 (solid triangle) nM T3 for 6 days. The cell density was measured by MTS assay at the day as indicated. Bars indicate standard error.

cell-specific. T3 did not affect the cell proliferation in PZ-HVP-7, CA-HPV-10, PC-3, and DU145 cells. The LNCaP cell line, derived from metastatic lymph nodes of a prostate cancer patient, is a well-differentiated, androgen-responsive cell line (Horoszeqicz et al, 1983). The androgen receptor of LNCaP cells is a mutant form that could be inappropriately activated by progestins, estrogens, adrenal androgens, and the antiandrogen hydroxyflutamide (Veldscholte et al, 1990). Whether the mutant receptors of LNCaP cells play an important role in T3 proliferative effect remains to be determined.

It is puzzling why T3, in the absence of androgens, could mediate positive growth response in LNCaP cells, yet have no effect on PZ-HPV-7 or PC-3 cells. Although previous study has shown that the concentrations of thyroid hormone receptor in PC-3 and DU145 cells are about 186 ± 6 and 199 ± 13 fmol/mg DNA, respectively (Esquenet et al, 1995), there was no report indicating the effect of T3 on cellular response in PC-3 or D145 cells. Other studies from different independent laboratories suggested that response of LNCaP cells to T3 stimulation seems to be related to thyroid hormone receptors (Esquenet et al, 1995; Zhang et al, 1999; Zhu and Young, 2001). In fact, there are 3 forms of thyroid hormone receptors in human tissue and thyroid hormone receptors strongly expresses in brain and prostate predominantly as a 10-kilobase mRNA (Sakurai et al, 1989). However, the thyroid hormone receptors in several carcinoma cells may be regarded not as thyroid hormone receptors when they contain a negative domain (Lin et al, 1994; Nomura et al, 1996; Yamiya et al, 2002). Our results also indicated that PC-3 and DU145 cells express a TRβ1 isoform that has

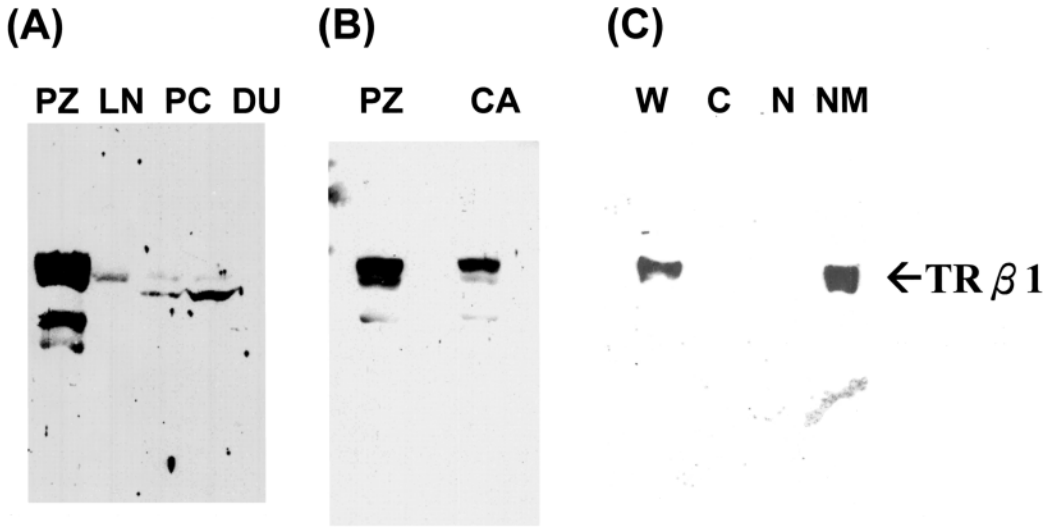

Figure 3. Immunoblot analysis of thyroid hormone receptor in human prostatic cells. Proteins extracted from whole cell were separated by SDS-PAGE (7.5% polyacrylamide) and blotted onto nitrocellulose. The blots were incubated with 1:200 monoclonal mouse anti-human thyroid hormone (J52). Secondary antibody was horseradish peroxidase-linked anti-rabbit IgG or anti-mouse IgG, and detection was done by enhanced chemiluminescence. Total protein for each sample is 60 μg (**A**) and 20 μg (**B**), respectively. PZ indicates PZ-HPV-7; LN, LNCaP; PC, PC-3; DU, DU145. (**C**) Localization of thyroid hormone receptors in PZ-HPV-10 cells. Proteins extracted from different compartments of PZ-HPV-10 cells were analyzed for thyroid receptor expression as in above procedures. The protein concentration for each sample is 10 μg. W indicates whole cells; C, cytoplasma; N, nucleus; and NM, nuclear membrane.

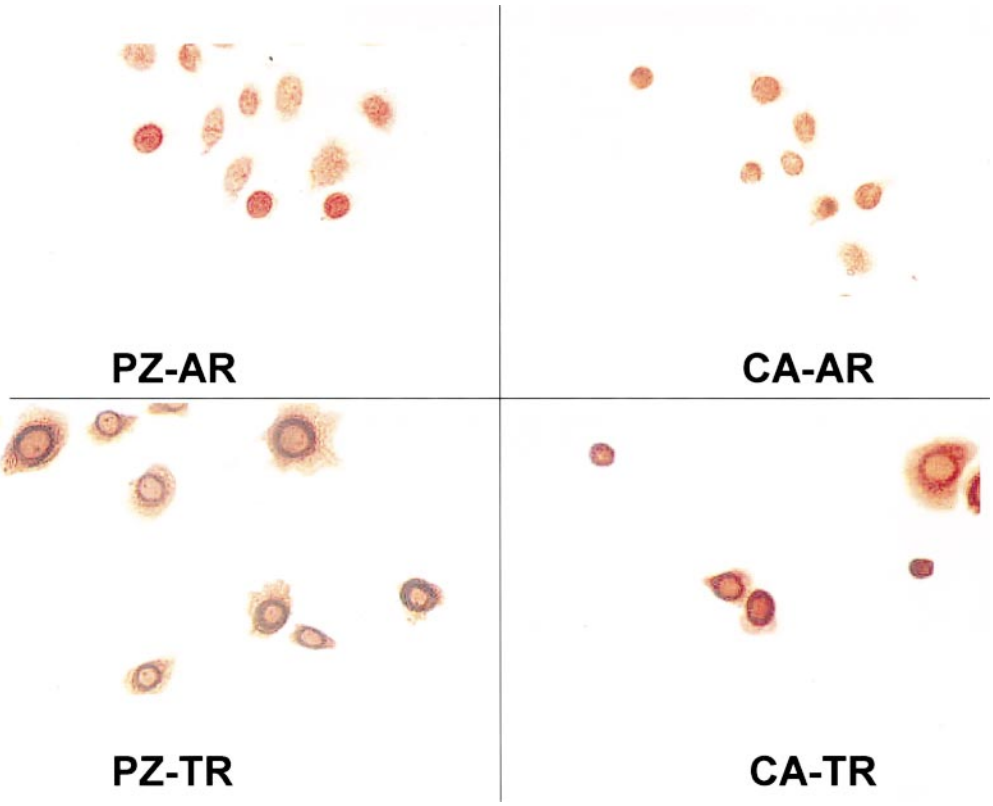

Figure 4. Cell immunocytochemical staining assay for expression of thyroid receptor and androgen receptor in PZ-HPV-7 and CA-HPV-10 cells. Cells were well prepared and then incubated in rabbit anti-human androgen receptor polyclonal antibody (AR-19; 1:500) or monoclonal mouse anti-human thyroid hormone (J52; 1:200), respectively. Biotinylated goat anti-rabbit IgG, avidin-HRP complex, and DAB reaction solution were added to the cells using VECTASTAIN ABC kit.

lower molecular weight than the native form. Whether TRβ1 in prostate carcinoma cells is a non-T3-binding form or a dominant negative form of TR still needs to be further investigated.

Early study has reported strong expression of thyroid hormone receptor mRNAs in human prostate tissues (Sakurai et al, 1989). Results from our immunoblot assay indicated a higher concentration of TRβ1 in the PZ-HPV-7 and CA-HPV-10 cells. However, results from this study indicated that expression of TRβ1 in PZ-HPV-7 and CA-HPV-10 cells is mainly located at the nuclear membrane as determined by cell immunocytochemical staining assay and immunoblot assay. The location of TRβ1 in different tissues or cells is variable. Some studies showed positive nuclear staining of all 3 TR isoforms in human ovary granulosa cells and rat cerebral neuron (Wakim et al, 1993; Puymirat et al, 1992a). However, other studies using immunocytochemical staining found that TRβ1 is located in the perinuclear region and the cytoplasm or in the nuclei of rat cerebral hemisphere astrocytes, Purkinje cells of the adult cerebellum, and human A431 carcinoma cells (Fukada et al, 1988; Puymirat et al, 1992b; Lebel et al, 1993). Our study provides direct evidence that TRβ1 in the PZ-HPV-7 and CA-HPV-10 cell is located at the nuclear membrane. The fact that T3 does not affect the proliferation of PZ-HPV-7 and CA-HPV-10 cells could correlate with the results of the study of rat cerebral hemisphere astrocytes because the location of TRβ1 in those cells is outside of the nucleus. The translocation of the TRβ1 from the cytoplasm to the nucleus independent of the presence of hormone would provide an additional regulatory mechanism for the T3 nuclear receptor to function properly. Other studies also indicated that protein-protein interaction of TRβ1 with various cofactors rather than specific DNA interactions would play the predominant role in determining the intracellular distribution of the receptor (Baumann et al, 2001). Further studies are necessary to identify the factors involved in the transport of the receptor from the cytoplasm to the nucleus.

In summary, our study demonstrates that T3 did not affect the cell proliferation of prostatic cell lines except LNCaP cells in vitro, although the thyroid hormone receptor isoforms (TRβ1) express in human prostatic epithelium cell lines (PZ-HPV-7, CA-HPV-10, DU145, PC-3, LNCaP). Results from immunocytochemical staining and immunoblot assay of cellular fractions suggested that PZ-HPV-7 and CA-HPV-10 cells express the novel TRβ1, which locates at cell nuclear membrane.

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