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Thyroid Hormone Inhibits Androgen-enhanced DNA Synthesis in Shionogi Carcinoma 115 Cells Without Affecting Autocrine Growth Factor mRNA Expression

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The growth of the mouse mammary Shionogi carcinoma 115 (SC 115)-derived cell line (SC-3) is markedly stimulated by androgen through induction of a heparin-binding growth factor termed androgen-induced growth factor (AIGF). This androgen-induced growth is partly blocked by thyroid hormone(s) such as triiodothyronine (T_3). Transforming growth factor β1 (TGFβ1) also inhibits the growth of SC-3 cells. Thus, we have investigated the possibility that T_3 exerts its inhibitory effects on SC-3 cell growth through an alteration in AIGF or TGFβ1 mRNA expression. Unexpectedly, T_3 failed to modulate the expression of AIGF mRNA. T_3 was also unable to significantly affect TGFβ1 mRNA levels in androgen-stimulated SC-3 cells. More importantly, a neutralizing antibody against TGFβ1 could not block T_3-dependent inhibition of androgen-induced SC-3 cell growth. However, the inhibitory ability of T_3 was potentiated by TGFβ1. In addition, T_3 treatment resulted in a significant inhibition of AIGF-induced DNA synthesis. Thus, T_3 treatment renders SC-3 cells somehow refractory to AIGF. The signal pathway for T_3 to reduce AIGF responsiveness may be shared by TGFβ1.

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INTRODUCTION

The growth of Shionogi carcinoma 115 (SC 115), mouse mammary carcinoma, is stimulated by androgen *in vivo* [1]. A cell line (SC-3) cloned from an SC 115 tumor is also growth-stimulated by androgen in serum-free culture [2]. Androgen-induced growth of the SC-3 cells is mediated through induction of a heparin-binding growth factor. This growth factor has recently been purified and cloned [3]. The amino acid sequence deduced from its cDNA clone reveals that this growth factor, tentatively termed androgen-induced growth factor (AIGF), is composed of 215 amino acids with a putative signal sequence and *N*-glycosylation site and has approx. 20% homology with basic fibroblast growth factor (bFGF). AIGF exerts its mitogenic action in an autocrine fashion through binding to the FGF receptor [4]. In line with these observations, only FGFs among putative growth factors can stimulate the growth of SC-3 cells [5].

Recently, evidence has emerged that growth-inhibitory signal pathways and their disruption play a pivotal role in the growth of cancer cells [6]. Among the humoral factors examined, thyroid hormone as well as transforming growth factor (TGF) β1 have been shown to inhibit the androgen-enhanced growth of SC-3 cells [7, 8]. Although the growth inhibitory potency of TGFβ1 on most transformed epithelial cells is widely accepted [9], the growth inhibition of the SC-3 cells by thyroid hormone seems to be unique. In addition, thyroid hormone can inhibit androgen-induced, but not bFGF-induced, DNA synthesis of the SC-3 cells through a thyroid hormone receptor-dependent mechanism [7].

Several possible mechanisms may explain the effects of thyroid hormone on androgen- or bFGF-stimulated SC-3 cells: (i) thyroid hormone could inhibit AIGF expression; (ii) thyroid hormone could reduce the

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sensitivity of the cells to AIGF, but not to bFGF; (iii) thyroid hormone could induce a growth inhibitor such as TGFβ1 which inhibits AIGF-induced synthesis; or (iv) androgen could activate an additional AIGF-independent pathway which is blocked by thyroid hormone. To differentiate between these possibilities, it is a prerequisite to quantify the AIGF mRNA expression level.

Since we succeeded in cloning cDNA encoding AIGF [3], it is now possible to determine whether or not thyroid hormone influences AIGF mRNA expression. In addition, the present experiments were also designed to address the possible involvement of TGFβ1 in the process of thyroid hormone-dependent inhibition of androgen-stimulated SC-3 cells.

MATERIALS AND METHODS

Materials

Eagle's minimum essential medium (MEM) and Ham's F-12 were obtained from Nissui Pharmaceutical Co., Ltd (Tokyo, Japan). Fetal calf serum (FCS) was from Cell Culture Labs (Cleveland, OH). Testosterone (T), triiodothyronine (T_3), Norit A and bovine serum albumin (BSA) (essentially fatty acid free) were from Sigma Chemical Co. (St Louis, MO). Human TGFβ1 and anti-TGFβ1 neutralizing antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). [Methyl-^{3}H]thymidine (sp. act. 70–90 Ci/mmol), [^{125}I]TGFβ1 (sp. act. 2000–4500 Ci/mmol) and [^{32}P]dCTP (3000 Ci/mmol) were obtained from Amersham Japan (Tokyo, Japan). [^{125}I]bFGF (870 Ci/mmol) was purchased from New England Nuclear (Boston, MA). The restriction enzymes were from Toyobo Co., Ltd (Tokyo, Japan). AIGF cDNA was cloned as described previously [3]. The cDNAs for human TGFβ1 and TGFβ2 were kindly provided by Drs R. Derynck and R. de Martin, respectively.

Cell culture and DNA synthesis

The SC-3 cells were maintained in MEM supplemented with 10^{-8} M T and 2% (w/v) dextran-coated charcoal (DCC)-treated FCS as described previously [7, 10]. To examine the DNA synthesis, the SC-3 cells were plated on a 96-well plate at a cell density of 10^4/well in 0.15 ml of MEM supplemented with 2% DCC-treated FCS. On the following day, the cells were washed with and cultured in serum-free medium [Ham's F-12–MEM (1:1; v/v) containing 0.1% (w/v) BSA; HMB] without any stimulant for 24 h, and then exposed to various test compounds. After 24-h treatment, the cells were pulsed with [^{3}H]thymidine (0.3 μCi/well) for 2 h. Radiaoctivity incorporated into the cells was measured [7]. During these procedures, the culture conditions were maintained at 37°C in 95% air:5% CO_2.

Partial purification of AIGF from conditioned medium

AIGF was partially purified from the conditioned medium as described previously [11]. Briefly, the SC-3 cells were plated at a cell density of 10^6/100-mm dish. After being washed, the cells were stimulated with 10^{-8} M T for 3–4 days and their conditioned medium was collected. The conditioned medium was applied to a heparin Sepharose affinity column, from which AIGF was eluted with a linear gradient of NaCl.

Northern blot analysis

The SC-3 cells were plated at an initial cell density of 10^6/100-mm dish and treated as described above. After treatment, the cells were lysed in 5.5 M guanidium isothiocyanate. The total RNA was precipitated through 5.7 M CsCl by ultracentrifugation and electrophoresed (20 μg/lane) in 1% agarose gel containing 0.66 M formaldehyde. After being transferred onto a nitrocellulose filter, hybridization was carried out in a stringent condition [12]. The probes used here were human TGFβ1 (0.5 kg EcoRI fragment), human TGFβ2 (1.5 kb EcoRI fragment) or mouse AIGF (1.0 kb BstXI fragment) cDNA. These probes were labeled with [^{32}P]dCTP by a random priming method. After hybridization, the filter was washed three times with 0.2 × SSC, 0.1% (w/v) SDS at 55°C for 20 min, and autoradiographed at −70°C for appropriate periods (2–5 days) of time. The band intensities were semiquantified by a densitometer scanning method [12]. The filter was always rehybridized with human β-actin (0.4 kb HinfI fragment) cDNA.

Binding assays for TGFβ1 and bFGF

The TGFβ1 binding assay was carried out by the method of Massague *et al.* [13]. Briefly, the SC-3 cells were plated at an initial cell density of 10^5/well (24-well plate) and treated with 10^{-8} M T or 10^{-8} M T plus 10^{-7} M T_3 for 24 h as described above. After being washed four times with PBS containing 0.1% (w/v) BSA, the cell monolayers were incubated at 20°C for 2 h with various concentrations (6–100 pM) of [^{125}I]TGFβ1 in the absence (total binding) or presence (nonspecific binding) of unlabeled TGFβ1 (10 nM), and then washed four times with PBS containing 0.1% (w/v) BSA. These treated monolayers were solubilized in the solubilization buffer [0.02 M Hepes, pH 7.4 at 20°C, containing 10% (v/v) glycerol and 1% (v/v) Triton X-100]. The radioactivity in the cell lysates was counted by a Packard gamma counter. The specific binding was considered to be the total minus nonspecific binding. The binding parameters were calculated by a method of Scatchard [14].

The SC-3 cells were also plated and treated as described above to perform the bFGF binding assay. The cells were washed twice with 2 M NaCl in 20 mM sodium acetate (pH 4.0) so as to remove FGF-like growth factor bound to FGF receptors. The cell mono-

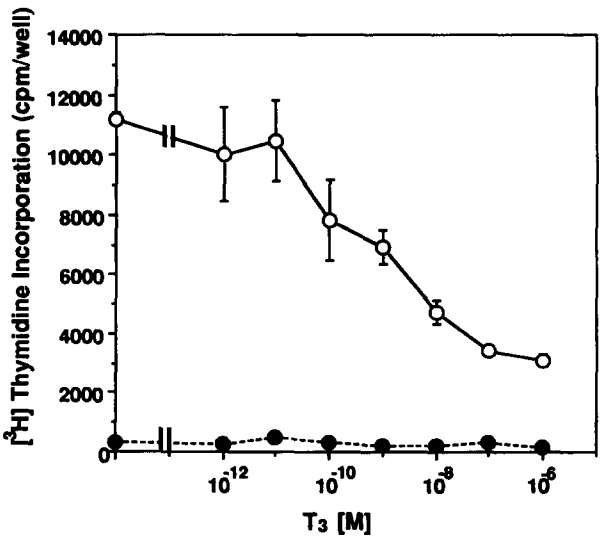

Fig. 1. T_3-dependent inhibition of DNA synthesis in androgen-stimulated SC-3 cells. The SC-3 cells were unstimulated (●---●) or stimulated (○——○) with 10^{-8} M T in the presence of various concentrations of T_3. After 24-h treatments, the DNA synthesis was measured. The representative data obtained from the triplicate assay are depicted. An additional four experiments gave similar results.

layers were incubated at 4°C for 3 h with various concentrations (2.5–500 pM) of [^{125}I]bFGF in the presence or absence of unlabeled bFGF (10 nM) in MEM containing 20 mM Hepes (pH 7.4 at 20°C) and 0.15% gelatin. After the cells were washed twice with 2 M NaCl in 20 mM Hepes to remove [^{125}I]bFGF bound to low affinity binding sites, [^{125}I]bFGF bound to FGF receptors was extracted with 2 M NaCl in 20 mM sodium acetate (pH 4.0 at 20°C) [15].

Statistical analysis

The data presented here are expressed as mean ± SE. The paired Student's t-test is used to discuss the significant ($P < 0.05$) difference.

RESULTS

Effects of T_3 on the DNA synthesis and AIGF mRNA expression of SC-3 cells

The SC-3 cells were incubated with various concentrations of T_3 alone or in combination with 10^{-8} M T. The DNA synthesis of androgen-stimulated cells was inhibited in a dose-dependent manner by T_3 while that of androgen-unstimulated cells was not affected by T_3 (Fig. 1). These results indicate that T_3 can partly block androgen-enhanced DNA synthesis.

Next, we examined the effect of T_3 on AIGF mRNA expression. Northern blot analyses revealed that T markedly induced AIGF mRNA expression but T_3 failed to significantly modulate this androgen-induced expression (Fig. 2). These results eliminate the possibility that T_3 exerts its inhibitory action via the suppression of AIGF mRNA expression. Thus, we examined the effects of T_3 and/or T on the TGFβ mRNA expression.

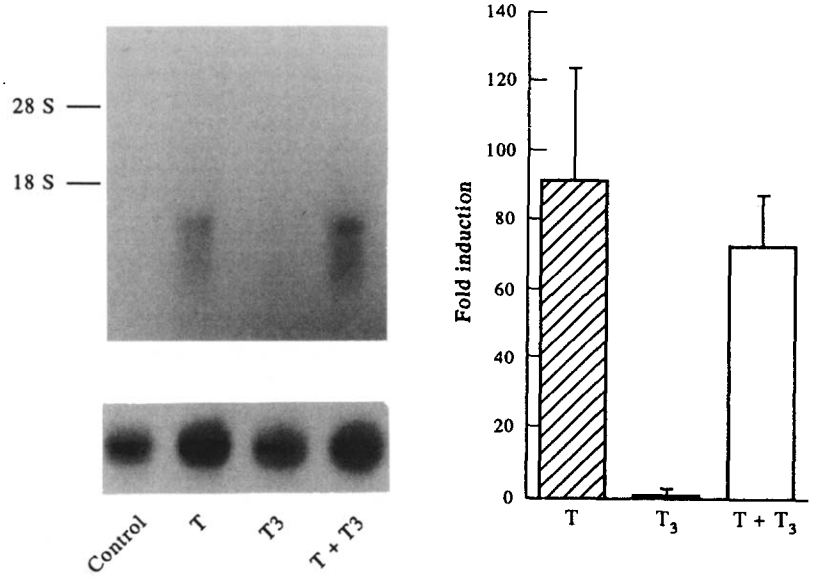

Fig. 2. Effects of T and T_3 on AIFG mRNA expression. The SC-3 cells were stimulated with 10^{-8} M T, 10^{-7} M T_3 or 10^{-8} M T plus 10^{-7} M T_3 for 24 h. As a control, the unstimulated cells were also included. The total RNAs extracted from these cells were subjected to Northern blot analysis using AIGF cDNA as a probe (left upper panel). The filter was rehybridized with β-actin cDNA (left lower panel). The intensity of the AIGF band was divided by that of the β-actin band. These values were used to calculate the hormone-induced fold induction of AIGF mRNA expression (right panel). These values were obtained by three separate experiments.

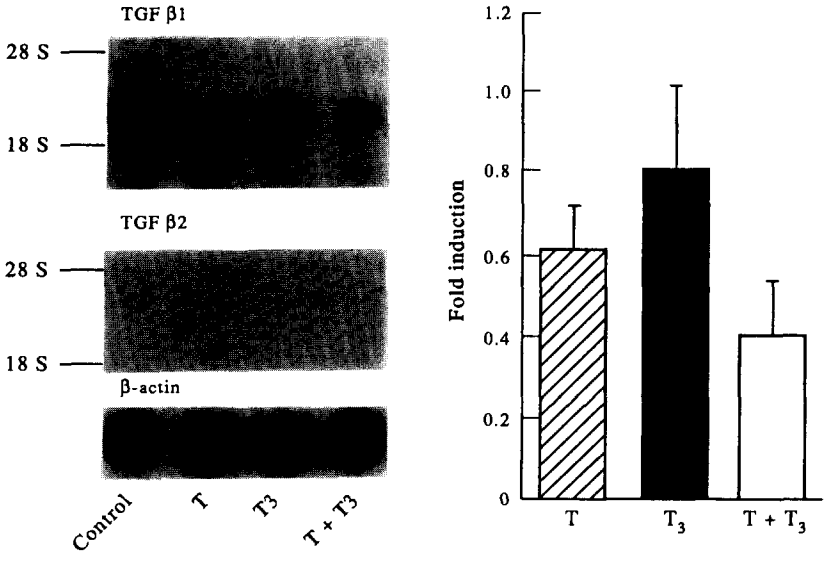

Fig. 3. Effect of T and T_3 on TGFβ1 and TGFβ2 mRNA expression. The SC-3 cells were treated and analyzed as described in the legend of Fig. 2 except that TGFβ1 or TGFβ2 cDNA were used instead of AIGF cDNA (left panel). The induction of TGFβ1 mRNA was also calculated as described in the legend of Fig. 2 (right panel).

TGFβ1 mRNA expression and its receptor of T_3-treated SC-3 cells

SC-3 cells were treated with T, T_3 or T plus T_3 for 24 h, and their total RNA was isolated to examine whether or not the mRNA expression of TGFβ, known as a growth inhibitor against androgen-stimulated SC-3 cell growth [8], is modulated in response to these treatments. As shown in Fig. 3, stimulation with T alone resulted in some reduction of the TGFβ1 mRNA level when compared with that in the untreated cells. When TGFβ2 cDNA was used as a probe, no activity was detected. Exposure of the cells to both T and T_3 appeared to further decrease rather than increase TGFβ1 mRNA levels. Although densitometric analysis failed to show a significant difference in mRNA level between T and T plus T_3 treatments, these results suggest that TGFβ1 does not play a role in T_3-dependent inhibition of the DNA synthesis in the SC-3 cells.

The presence of cell-surface receptors is obligatory for TGFβ1 to exert its biological action. Thus, the effect of T_3 on TGFβ1 receptor was examined. Scatchard analysis of binding data obtained under equilibrium conditions revealed that the androgen-stimulated SC-cells contain high affinity TGFβ1 binding sites (Fig. 4). T_3 treatment did not affect these binding parameters (Table 1), indicating that the growth inhibitory effect of T_3 could not be explained by effecting TGFβ1 receptors.

Confirmatory results were obtained in experiments using the TGFβ1 antibody. This antibody could partly but significantly block the TGFβ1 (40 pM)-induced inhibition of the DNA synthesis (Table 2). In contrast, T_3-dependent inhibition of the DNA synthesis in androgen-stimulated cells was not reversed by anti-TGFβ1 antibody, indicating that TGFβ1 does not play a role in T_3-dependent inhibition of the DNA synthesis.

Potentiation of growth-inhibitory activity of T_3 by TGFβ1

Although TGFβ1 seemed not to be an autocrine growth inhibitor in the process of T_3-dependent growth inhibition, the possibility was addressed of whether or not exogenously added TGFβ1 could potentiate the inhibitory ability of T_3 (Fig. 5). At low concentrations, TGFβ1 alone did not affect the DNA

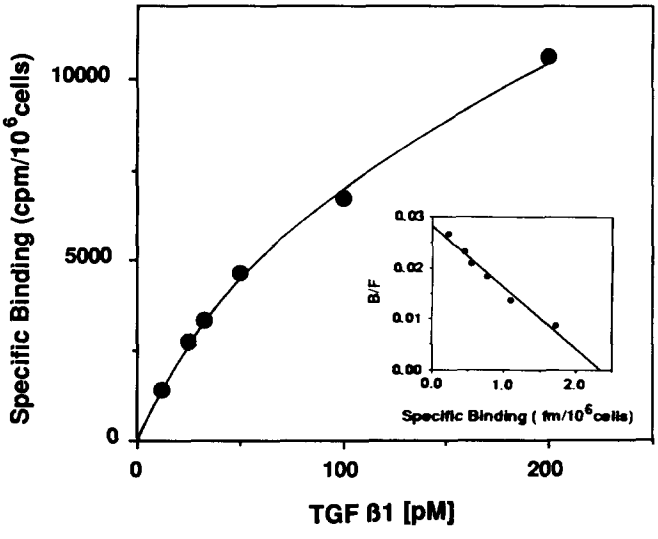

Fig. 4. High affinity binding site for TGFβ1 on androgen-stimulated SC-3 cells. The androgen-stimulated SC-3 cells were incubated with various concentrations of [^{125}I]TGFβ1 in the presence or absence of unlabeled TGFβ1 as described in Materials and Methods. The specific binding data were plotted. The Scatchard analysis on the specific binding data was also depicted in the insert.

Table 1. Lack of effect of T_3 on TGFβ high affinity binding sites on androgen-stimulated SC-3 cells

	TGFβ1 high affinity binding sites	
	Dissociation constant (pM)	Number of maximum binding site (site/cell)
T alone	110 ± 25	1340 ± 210
T and T_3	136 ± 29	1580 ± 500

The SC-3 cells were treated with 10^{-8} M T alone or in combination with 10^{-7} M T_3 and then subjected to the TGFβ1 binding assay as described in Materials and Methods. The data were obtained from 3 separate experiments.

synthesis of androgen-stimulated cells. However, these concentrations of TGFβ1 did significantly potentiate the inhibitory effect of T_3. At high concentrations, TGFβ1 alone inhibited the DNA synthesis. However, it seems to be noteworthy that the degree of the inhibition induced by TGFβ1 plus T_3 was more profound than that by TGFβ1 alone.

Effects of T_3 on AIGF-induced DNA synthesis and the FGF receptor

AIGF was partially purified from the conditioned medium and its growth-promoting activity was examined using the untreated or T_3-treated SC-3 as a target cell. As shown in Fig. 6, T_3 inhibited the AIGF-induced DNA synthesis in a dose-dependent manner. Since AIGF has been shown to exert its effect via binding to FGF receptors [4], we examined the effect of T_3 on FGF receptor binding in SC-3 cells. Scatchard plot analysis, however, indicated that T_3 did not significantly modulate the binding parameters of bFGF to the FGF receptor (K_d, 77 pM; the binding site, 4000/cell for the cells stimulated with T alone: K_d, 83 pM; the binding site, 5600/cell for the cells stimulated with T and T_3).

Table 2. Effect of anti-TGFβ1 antibody on T_3 or TGFβ1-dependent inhibition of androgen-enhanced DNA synthesis

	DNA synthesis (cpm/well)
No addition	415 ± 100
T (10^{-8} M) alone	4141 ± 314
+ TGFβ1 (40 pM)	846 ± 112
+ TGFβ1 (40 pM) + anti-TGFβ1 antibody (10 μg/ml)	2414 ± 133
+ T_3 (10^{-7} M)	2541 ± 165
+ T_3 (10^{-7} M) + anti-TGFβ1 antibody (10 μg/ml)	3205 ± 650
	3205 ± 650

The DNA synthesis was examined in the presence of various test compounds as described in Materials and Methods. The data were obtained by triplicate assays.

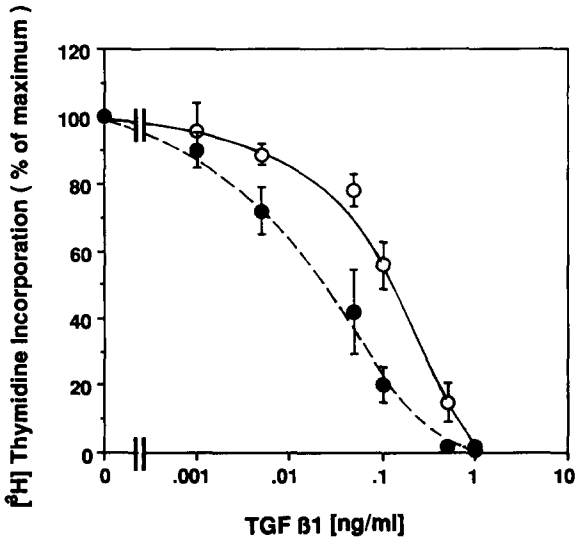

Fig. 5. Effect of TGFβ1 and T_3 on their inhibitory activity against androgen-stimulated DNA synthesis. The androgen-stimulated SC-3 cells were exposed to various concentrations of TGFβ1 alone (○) or in combination with 10^{-7} M T_3 (●) for 24 h, and then their ability to synthesize DNA was measured. The data were obtained from four separate experiments.

DISCUSSION

The present results indicate that T_3 inhibits androgen-enhanced DNA synthesis of the SC-3 cells without affecting AIGF mRNA expression. T_3 also fails to modulate the expression of TGFβ1 and its receptor characteristics, suggesting that TGFβ1 is unable to act

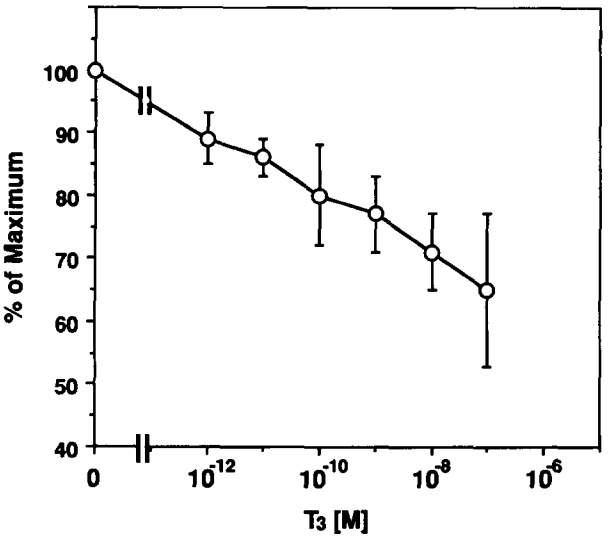

Fig. 6. Inhibition of AIGF-induced DNA synthesis by T_3. The SC-3 cells were exposed to partially purified AIGF with various concentrations of T_3 for 24 h, and their ability to synthesize DNA was measured. AIGF alone enhanced DNA synthesis at levels similar to those induced by 10^{-8} M T (data not shown). The values, obtained by three different AIGF preparations, were expressed as percents, taking the amount of DNA synthesis without T_3 as 100%.

as an autocrine regulator in the process of T_3-dependent growth inhibition. Our previous study showed that bFGF-induced DNA synthesis cannot be blocked by T_3 [7], suggesting that T_3 exerts its inhibitory activity through an inhibition of AIGF mRNA expression. Thus, the present data showing the lack of T_3 effect on AIGF mRNA expression is quite unexpected. Two possibilities are taken into consideration to explain these apparently contradictory observations: (i) T_3 renders the cell refractory to AIGF; and (ii) the signal transduction of AIGF somehow differs from that of bFGF. Treatment of the cells with T_3 was found to result in a reduced response to AIGF partially purified from androgen-stimulated SC-3 cells. Since both AIGF and bFGF are associated with the same receptor, T_3-dependent inhibition of androgen-stimulated DNA synthesis could not be explained at the FGF receptor. In this regard, T_3 did not affect the kinetic parameters of FGF receptors on SC-3 cells.

The detailed molecular mechanism linking androgen and thyroid hormone actions remains to be determined. However, the present data combined with our previous observations may provide some clues. Upon secretion from androgen-stimulated SC-3 cells, the mitogenic activity of AIGF has been found to be extracellularly modulated. For instance, heparin, which potentiates the mitogenic activity of bFGF on the SC-3 cells, can inhibit the AIGF-mediated DNA synthesis of the SC-3 cells [16]. Heparan sulfate localized on the cell-surface plays a more critical role in mediating AIGF biological activity than bFGF [17]. Furthermore, AIGF is much more sensitive to suramin (a known growth factor inhibitor [18]) than bFGF in terms of blocking DNA synthesis [15]. These results suggest that modulation of the extracellular matrix and/or cell-surface proteoglycan results in a marked effect on AIGF-mediated DNA synthesis while bFGF is affected by the extracellular environment to a lesser degree. Thyroid hormone has been shown to regulate components of the extracellular matrix, for instance, fibronectin mRNA expression in the rat liver is regulated by thyroid hormone [19]. Proteoglycan synthesis in fibroblasts has also been known to be modulated by thyroid hormone [20]. TGFβ1 is also known as a regulator of proteoglycan synthesis in a wide variety of cells. The amount of sulfate incorporated into proteoglycans, especially chondroitin sulfate, has been observed to be augmented in the rat hepatic lipocyte by TGFβ1 [21]. This cytokine also enhances the biosynthesis of collagen and proteoglycans in cultured rabbit articular chondrocytes [22]. Thus, both thyroid hormone and TGFβ1 appear to modulate the cell-surface proteoglycan and extracellular matrix components although each molecule belongs to entirely different class of cell function regulators. These events may explain why T_3-dependent inhibition of androgen-stimulated DNA synthesis is potentiated by TGFβ1. However, the detailed mechanism is currently unknown since the nature of the naturally occurring molecule(s) important for "activation" of AIGF or bFGF is not known.

The present study indicates that SC-3 cells are unable to synthesize a sufficient amount of TGFβ1 to modulate DNA synthesis. In *in vivo* conditions, however, TGFβ1 is considered to play some role. TGFβ1 is known to be synthesized by a wide variety of cells. Therefore, it seems possible that the SC-3 cells are exposed to TGFβ1, which is synthesized by adjacent cells, at concentrations sufficient for potentiation of T_3-inhibitory action against the androgen-stimulated DNA synthesis. This interaction is an interesting research target in terms of the signal transduction mechanism of AIGF as well as the *in vivo* tumor growth.

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