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*J Clin Invest.* 1989;**83**(3):764-770. <https://doi.org/10.1172/JCI113955>.

### **Research Article**

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# Transforming Growth Factor Beta Regulates Thyroid Growth

## Role in the Pathogenesis of Nontoxic Goiter

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### Abstract

The production and growth regulatory activity of transforming growth factor  $\beta$  were studied in human thyroid tissue. As estimated by its mRNA expression in fresh tissue samples, transforming growth factor  $\beta$  was produced in normal and in diseased thyroid glands. Transforming growth factor  $\beta$  mRNA was mainly produced by thyroid follicular cells and in lesser quantities by thyroid infiltrating mononuclear cells. The concentrations of transforming growth factor  $\beta$  mRNA were lower in iodine-deficient nontoxic goiter than in Graves' disease and normal thyroid tissue. Transforming growth factor  $\beta$  protein secretion by cultured thyroid follicular cells was also low in nontoxic goiter, but could be increased by addition of sodium iodide (10  $\mu$ M) to the culture medium. Recombinant transforming growth factor  $\beta$  did not affect basal tritiated thymidine incorporation in cultured thyroid follicular cells, but inhibited, at a concentration of 10 ng/ml, the growth stimulatory influence of insulin-like growth factor I, epidermal growth factor, transforming growth factor  $\alpha$ , TSH, and partly that of normal human serum on cultured thyroid follicular cells. This inhibition was greater in Graves' disease than in nontoxic goiter. These results suggest that transforming growth factor  $\beta$  may act as an autocrine growth inhibitor on thyroid follicular cells. Decreased transforming growth factor  $\beta$  production and decreased responsiveness to transforming growth factor  $\beta$  may be cofactors in the pathogenesis of iodine-deficient nontoxic goiter.

### Introduction

Locally produced growth factors such as epidermal growth factor (EGF)<sup>1</sup> and insulin-like growth factors I and II (IGFI and IGFI) have been recognized as potent stimulators of thy-

roid cell growth (1–4). Overproduction and/or increased responsiveness of thyroid cells to these factors have been suggested to be involved in the pathogenesis of benign proliferative thyroid disorders such as nontoxic goiter (NTG; 5, 6). However, little is known about the role of growth inhibitory proteins in thyroid health and disease. Transforming growth factor  $\beta$  (TGF $\beta$ ), a 25-kD homodimeric protein (7–9), can be produced by transformed cells as well as by normal tissues (10, 11) and binds to three structurally distinct, specific receptors present on numerous cells of both mesenchymal and epithelial origin (12–14). Although TGF $\beta$  has been shown to have growth-enhancing properties in some systems, it is mostly growth inhibitory (for review see references 15 and 16).

The aim of the present study was to examine whether TGF $\beta$  was a growth inhibitor in the thyroid gland and whether abnormalities of such regulatory mechanisms might lead to the development of thyroid hyperplasia.

We show that TGF $\beta$  is expressed and secreted by thyroid follicular cells (TFC) and that it may act as a potent inhibitor of thyroid cell growth. We further demonstrate that TGF $\beta$  production is lower in NTG than in normal or Graves' disease (GD) thyroid tissue and that TFC from NTG respond less readily to the growth inhibitory effect of TGF $\beta$ , suggesting that TGF $\beta$  may be potentially important in the prevention of thyroid hyperplasia.

### Methods

**Tissue specimens and preparation of thyroid tissue.** Surgically removed thyroid tissue from ten patients with long-standing multinodular NTG (nine females, one male, age 59 $\pm$ 10 yr, range 44–72 yr) was studied. Thyroid hormone medication had been discontinued at least 8 wk before surgery in all patients. Thyroid tissue from seven patients with GD (five females, two males, age 42 $\pm$ 12, range 26–56 yr) who had small, diffuse goiters and were euthyroid at the time of surgery under low-dose therapy with methimazole or prothiucil, and four normal thyroid tissue samples served as controls. Three of the normal tissue samples were byproducts of parathyroidectomy (two females and one male patient, age 42, 60, and 56 yr, respectively) and one derived from the contralateral lobe from a 54-yr-old female patient operated on for thyroid carcinoma. Parathyroid tissue from four patients with parathyroid adenoma (three males, one female, age 45 $\pm$ 4 yr, range 40–49 yr) and three with secondary parathyroid hyperplasia (all females, age 45 $\pm$ 7 yr, range 39–59 yr) were used as additional controls. All patients lived in Austria, where iodine deficiency is still endemic (17).

Tissue samples were dispersed with 5 mg/ml collagenase (type IV; Cooper Biomedical, Malvern, PA), in RPMI 1640 medium (Gibco Laboratories, Paisley, Scotland) containing 15% FCS (Gibco Laboratories) for 3 h and pipetted through a 200- $\mu$ m mesh. The red blood cells were lysed with an ammonium chloride buffer. When sufficiently high cell numbers were available, cells were separated into follicular and thyroid infiltrating mononuclear cells (TMNC). TFC were purified as

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Received for publication 7 March 1988 and in revised form 18 October 1988.

1. Abbreviations used in this paper: EGF, epidermal growth factor; GD, Graves' disease; IGFI, insulin-like growth factor I; NTG, nontoxic goiter; TFC, thyroid follicular cells; TGF $\alpha$ , transforming growth factor  $\alpha$ ; TGF $\beta$ , transforming growth factor  $\beta$ ; TMNC, thyroid infiltrating mononuclear cells.

J. Clin. Invest.

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0021-9738/89/03/0764/07 \$2.00

Volume 83, March 1989, 764–770

previously described (18, 19). In brief, TFC were purified on the basis of their strong adherence onto plastic. Cells were incubated in RPMI supplemented with 10% FCS at 37° in 95% air-5% CO<sub>2</sub> and 95% humidity. After 16 h nonadherent and loosely adherent cells were removed by vigorous washing, and the remaining adherent cells were removed from their plastic support with trypsin (0.25% solution, Gibco Laboratories). This cell population consisted of > 95% TFC, as estimated by staining with serum containing a high titer of microsomal antibodies from a patient with GD as previously described (18, 19). The remaining contaminating cells were analyzed by immunofluorescence staining as described (19) using the monoclonal antibodies anti-Leu-M3 (Becton-Dickinson, Oxnard, CA) for tissue macrophages, anti-Leu-1 (Becton-Dickinson) for T lymphocytes, EN 4 (donated by M. Tai, St. George's Hospital, London, UK) for endothelial cells, and F15-42-1 (donated by J. Fabre, Blond McIndoe Centre for Medical Research, East Grinstead, Sussex, UK) for fibroblasts. The proportion of positive cells detected with each antibody ranged from 0.5 to 2%.

TMNC were purified from the nonadherent cell population by centrifugation over a Ficoll-Hypaque gradient (Lymphoprep; Nycomed, Torshov, Norway).

**Preparation of cells and supernatants for TGF $\beta$  analysis.** The *in vivo* production of TGF $\beta$  in the thyroid/parathyroid gland was assessed indirectly by the determination of the respective mRNA as described below. When > 10<sup>7</sup> cells were available/sample Northern analyses were performed; otherwise, slot blotting was used. Unpurified thyroid and parathyroid cells were analyzed after collagenase digestion as above. Purified TFC and TMNC were analyzed immediately after purification.

The active secretion of TGF $\beta$  by TFC was studied in four patients with NTG, in three with GD, and in two normal controls, by analyzing conditioned supernatants for the presence of TGF $\beta$  bioactivity. TFC in subconfluent monolayer were extensively washed and cultured in serum-free RPMI for 3 d. Parallel experiments, in which the culture medium was supplemented either with sodium iodide (10  $\mu$ M) or TSH (100  $\mu$ U/ml), were performed in most patients. In one patient of each group three different doses of iodide (1, 10, and 100  $\mu$ M) were compared. After 3 d supernatants were collected and acid activated as described earlier (20) with minor modifications. Briefly, supernatants were acidified with 1 M HCl to a pH of 3.2 and left for 1 h at 4°C. They were then reneutralized with 1 M NaOH, dialyzed against PBS for 12 h, against ammonium bicarbonate for 24 h, and finally lyophilized. The lyophilized samples were reconstituted in 1 ml of DME.

**RNA isolation and Northern blot analysis.** Total cytoplasmic RNA was isolated by the NP-40 lysis method, denatured, fractionated on a 1% agarose formaldehyde gel, and transferred to nitrocellulose filters as described (21).

**Slot blot analysis.** Total cytoplasmic RNA was extracted from cells essentially as described (22). Briefly, 0.5–3  $\times$  10<sup>6</sup> cells were lysed with 1% NP-40 in 10 mM Tris-HCl, pH 7, 1 mM EDTA, the nuclei separated by centrifugation, and the lysate extracted with phenol/chloroform before precipitation of RNA with ethanol. The RNA was pelleted, dried, and redissolved in 50  $\mu$ l of 10 mM Tris, 1 mM EDTA. RNA was denatured by heating at 60°C for 15 min after addition of 50  $\mu$ l of a 3:2 mixture of 20 $\times$  standard saline citrate (SSC):formaldehyde. The denatured RNA was then applied onto a nitrocellulose filter in doubling dilutions using a minifold (Schleicher & Schuell, Inc., Keene, NH) apparatus. The filters were baked at 80° for 2 h.

**Nucleic acid probes and hybridization.** TGF $\beta$  mRNA was detected with a 1,050-bp Eco RI cDNA insert from the clone  $\lambda$   $\beta$ C1, which codes for most of the human TGF $\beta$  precursor (11) and does not cross-react at high stringency with related homologous substances such as TGF $\beta$ 2 or inhibin. The control probe 7B6, a 708-bp Pst I-Dra I fragment from p7B6, detects a ubiquitous species of mRNA that does not fluctuate during the cell cycle or in response to cell activation (23).

The inserts were labeled with <sup>32</sup>P using the random oligo-priming method (24) to a specific activity of about 10<sup>9</sup> cpm/ $\mu$ g DNA. The filters were prehybridized for 4–6 h at 42°C in a solution containing 50% formamide, 5 $\times$  SSC (750 mM NaCl, 75 mM sodium citrate), 50 mM

phosphate, pH 6.6, 125  $\mu$ g/ml salmon sperm DNA, 2 $\times$  DH (0.04% ficoll, 0.04% polyvinylpyrrolidone, and 0.04% BSA), and 0.1% SDS. After prehybridization the denatured labeled probe was added to the prehybridization solution and hybridized for 16–24 h. The filters were washed to a stringency of 0.1 $\times$  SSC at 50°C for 30 min and exposed to Fuji x-ray film at –70°C with intensifying screens. The blots were then stripped of the previous probe by washing in 5 mM Tris, pH 8, 0.2 mM EDTA, 0.05% sodium pyrophosphate, and 0.1 $\times$  Denhardt's solution at 65°C for 1–2 h. The blots were then rehybridized with the 7B6 probe.

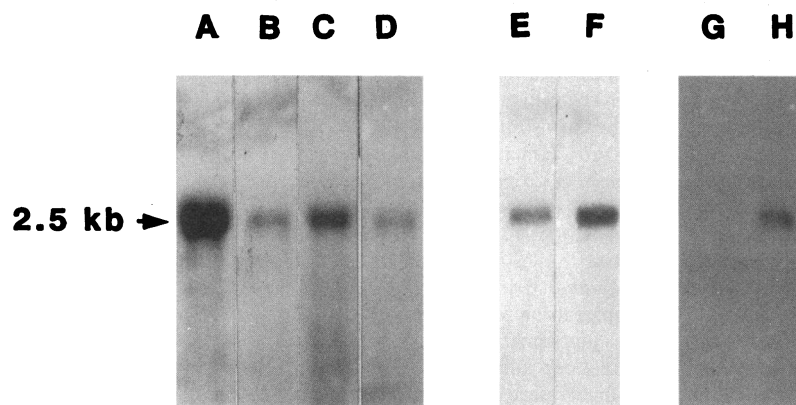
**Quantitative analysis of slot blots.** To avoid interassay variation all samples to be finally compared were hybridized in one experiment. The autoradiographs were scanned on a densitometer (Chromoscan 3; Joyce, Loeb and Co. Ltd., Gateshead, UK) and the integral values from the linear range of the scan (scanning units) used to define the amount of TGF $\beta$ /sample. The values were adjusted for the amount of mRNA/sample detected by the probe 7B6.

**TGF $\beta$  bioassay.** TGF $\beta$  bioactivity was determined by measurement of its inhibitory effect on [<sup>3</sup>H]thymidine incorporation into DNA in monolayers of Mv1Lu cells (obtained from American Type Culture Collection, Rockville, MD [ATCC]), according to a slightly modified version of a published method (14). In brief, three dilutions (1:2, 1:20, and 1:200) of the supernatants from the TFC were added in triplicate to monolayers of Mv1Lu cells (2  $\times$  10<sup>4</sup> cells/well) in the presence of 5% FCS. After 20 h 1  $\mu$ Ci of [<sup>3</sup>H]thymidine (Amersham International, Amersham, UK) was added to each well. After another 4 h cells were harvested. To guarantee full detachment of cells, plates were quickly frozen and defrosted before harvesting. Macromolecular material was harvested onto glass fiber filters using a semiautomatic cell harvester (Titertek; Flow Laboratories, Irvine, UK), and the amount of [<sup>3</sup>H]-thymidine incorporated into DNA/culture was assessed by liquid scintillation counting. A TGF $\beta$  standard curve was constructed using serial dilutions of a known amount of rTGF $\beta$ . Each sample was additionally assayed after preincubation (1 h at room temperature) with 50  $\mu$ g of a neutralizing polyclonal antibody against TGF $\beta$ . Non-antibody-blockable activity was considered to be nonspecific background and hence was subtracted from the final TGF $\beta$  concentration.

**Assessment of the effects of rTGF $\beta$  on the [<sup>3</sup>H]thymidine incorporation in cultured TFC.** Purified TFC were cultured at 10<sup>4</sup> cells/well in 96-well flat bottom plates in RPMI containing 10% FCS for 3 d until they had formed a subconfluent monolayer. They were then washed three times and growth arrested by another 3-d culture in serum-free RPMI medium. rTGF $\beta$  was then added in serum-free medium either alone or in combination with known stimulators of TFC growth such as EGF, transforming growth factor  $\alpha$  (TGF $\alpha$ ), IGF1, TSH, or normal human serum. Each stimulator was studied in at least five different patients with NTG, as well as in three with GD, using triplicate cultures. After a 48-h incubation cultures were pulsed with 1  $\mu$ Ci [<sup>3</sup>H]-thymidine and harvested as described above after 12 h. [<sup>3</sup>H]Thymidine incorporation by TFC after stimulation was expressed as percentage of basal [<sup>3</sup>H]thymidine incorporation (cpm) by TFC incubated in serum-free medium only, which was considered as 100%.

**Reagents and cell lines.** The TGF $\beta$  probe, human rTGF $\beta$ , and human TGF $\alpha$  were generous gifts from Genentech Inc. (San Francisco, CA), the 7B6 probe was a kind gift from Dr. U. Torelli (Modena, Italy), human rIGF1 was donated by Ciba Geigy (Basel, Switzerland), purified murine EGF was purchased from Gibco Laboratories, and bovine TSH from Sigma Chemical Co. (St. Louis, MO). The normal human serum was pooled from healthy blood donors. The neutralizing anti-TGF $\beta$  antibody was purchased from R&D Systems Inc. (Minneapolis, MN). HepG2, a human hepatoma line, HL60, a human promyelocytic leukemia line, and U937, a human histiocytic lymphoma line, were obtained from ATCC. Jurkat subclone J6 was donated by M. Owen (Imperial Cancer Research Fund, London, UK).

**Statistical analysis.** Data in text and figures are presented as medians and range or, when normally distributed, as mean $\pm$ SEM. Wilcoxon's rank sum tests on unpaired data, *t* tests, and regression analysis were used for statistical evaluation.



**Figure 1.** Northern analysis of TGF $\beta$  mRNA in thyroid tissue and control cell lines. Analysis of unpurified cells (lanes A and B) and of purified TFC (lanes C and D) from two patients with GD (lanes A and C) and two with NTG (lanes B and D). Lane E, Jurkat subclone J6 (human T cell lymphoma); lane F, Hep G2 (human hepatoma); lane G, HL60 (human promyelocytic leukemia); lane H, U937 (human histiocytic lymphoma). The samples in lanes A–D, in E and F, and in G and H were analyzed in three different experiments. In each 25  $\mu$ g of total cytoplasmatic RNA was loaded per track, fractionated, and hybridized with the  $^{32}$ P-labeled TGF $\beta$  probe as described in Methods.

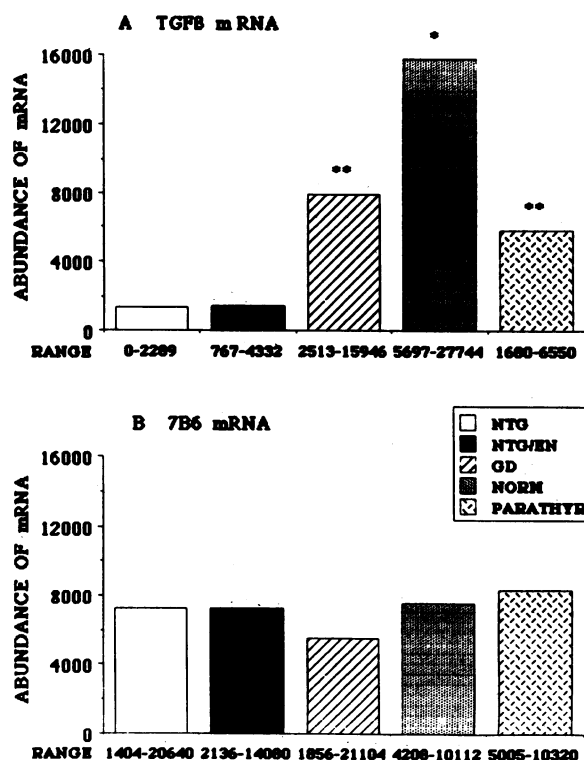
## Results

**In vivo production of TGF $\beta$  mRNA.** The specificity of the TGF $\beta$  cDNA probe and the size of the transcript were demonstrated by Northern blot analysis. TGF $\beta$  transcripts of previously reported size (2.5 kb) were found in freshly dispersed thyroid tissue (Fig. 1, lanes A and B) as well as in purified TFC (lanes C and D). The Jurkat subclone J6 (lane E), the hepatoma line HepG2 (lane F), and the histiocytic lymphoma line U937 (lane H) served as positive controls, whereas the promyelocytic leukemia line HL60 (lane G) did not produce TGF $\beta$  message. Samples from NTG (lanes B and D) showed weaker bands than samples from GD thyroids (lanes A and C), suggesting that intrathyroidal TGF $\beta$  production might be lower in NTG than in GD. Thus it was of interest to examine this possibility on a larger number of samples. In view of the low cell numbers usually available from pathological specimens and the lack of nonspecific binding in the Northern blot, slot blot analysis was used for TGF $\beta$  mRNA analysis in all subsequent experiments.

Analysis of unpurified cells showed that TGF $\beta$  transcripts were present in 21 of 23 samples (16 thyroids and 7 parathyroids). The two samples in which no TGF $\beta$  message could be detected were thyroid tissue from NTG patients. In the remaining NTG samples TGF $\beta$  mRNA was expressed at relatively low levels (200–2,289 scanning units; Fig. 2 A). TGF $\beta$  mRNA concentrations were lower in thyroid tissue from patients with NTG than in GD ( $P < 0.01$ ) and normal control thyroids ( $P < 0.05$ ), and lower than in a control group of parathyroid tissue samples derived from patients with hyperparathyroidism due to either parathyroid adenoma or hyperplasia ( $P < 0.01$ ). TGF $\beta$  message was as low in selectively prepared extranodular tissue as in samples prepared normally from both intra- and extranodular tissue, excluding the possibility that low TGF $\beta$  transcription was a phenomenon restricted to nodular, potentially transformed areas of the gland. Two of the three normal thyroids showed higher TGF $\beta$  mRNA expression (27,744 scanning units and 15,765 scanning units, respectively) than any of the other samples investigated.

In contrast, 7B6 control mRNA did not differ among groups (Fig. 2 B). Although 7B6 values were generally homogenous, considerable variations were observed in some of the samples. This was presumably the result of variability in RNA loading due to low cell numbers in some of the samples and to

difficulties in making exact counts of cells in follicular clusters. Corrected TGF $\beta$  and 7B6 mRNA values did not, however, show an inverse relationship ( $r = 0.1359$ ,  $n = 23$ , NS), excluding the possibility that the TGF $\beta$  mRNA data had been influenced by excessively high or low correction factors.

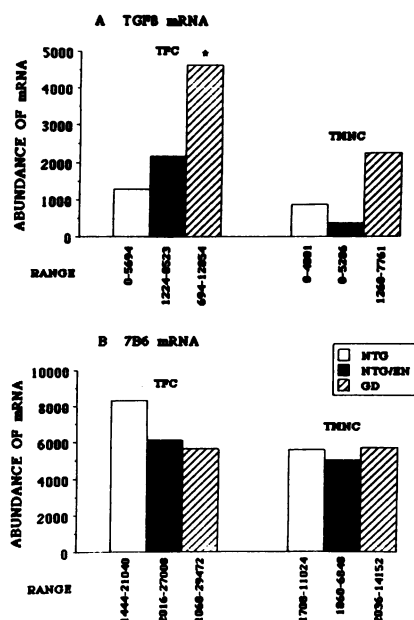


**Figure 2.** Slot blot analysis of TGF $\beta$  (A) and 7B6 (B) mRNA in thyroid and parathyroid tissue. Analysis of unpurified cells from NTG ( $n = 7$ ), extranodular NTG tissue (NTG/EN;  $n = 4$ ), GD ( $n = 6$ ), and normal thyroids (NORM,  $n = 3$ ), as well as from parathyroid adenomas and hyperplasias (PARATHYR,  $n = 7$ ; adenoma,  $n = 4$ ; hyperplasia,  $n = 3$ ). Values correspond to densitometer scanning units (abundance of mRNA) and are expressed as median and range. TGF $\beta$  values were adjusted to the total amount of RNA/sample, assessed by reprobing of the filters with the control probe 7B6 (data shown in B). Wilcoxon's rank sum tests for unpaired data were used for statistical evaluation. \* $P < 0.05$ ; \*\* $P < 0.01$ ;  $P$  vs. NTG, all other differences were not statistically significant.

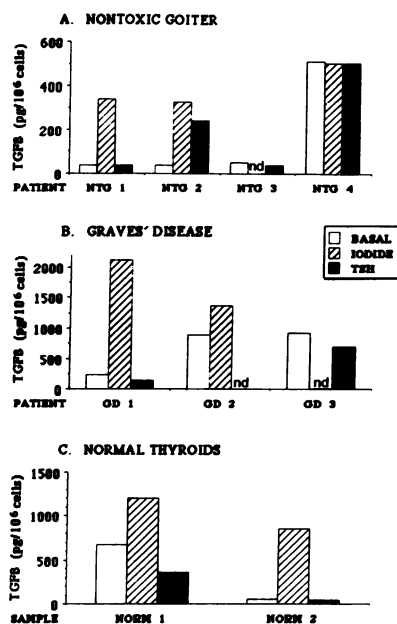
In six of the NTG and six of the GD thyroids there were sufficient cells for purification of TFC and TMNC. TGF $\beta$ , but not 7B6 mRNA production, was higher in TFC than in TMNC ( $P < 0.05$  in GD, NS in NTG; Fig. 3), suggesting that although TMNC are also capable of transcribing TGF $\beta$ , TFC seem to be the major source of TGF $\beta$  production in the thyroid gland. In individual patients from both groups TGF $\beta$  mRNA was higher in purified TFC and TMNC than in the unpurified population, suggesting that FCS may induce TGF mRNA during the 16-h incubation period necessary for purification. Due to the resulting variability in the data in each group, differences between NTG and GD purified cells were not significant.

**Production of TGF $\beta$  by cultured TFC.** TGF $\beta$  bioactivity was found in supernatants conditioned by TFC from patients of all groups (Fig. 4). Although TGF $\beta$  activity was detectable in all nine samples analyzed, concentrations were very low in three of four NTG samples (note the different scale in Fig. 4A) and in one normal control. Addition of sodium iodide (10  $\mu$ M) to the culture medium induced an increase in the production of TGF $\beta$  bioactivity in all but one sample. This sample (NTG 4) also contained more basal TGF $\beta$  bioactivity than any of the other NTG samples. It derived from a patient with extremely large and longstanding goiter, in whom thyroid scintigraphy under thyroxine suppression had shown multicentric autonomy. A relatively high basal TGF $\beta$  production combined with a decreased stimulatory effect of iodide might thus indicate loss of regulatory control and autonomous TGF $\beta$  production as possible mechanisms in patients with longstanding autonomously functioning goiters.

TSH, at a concentration of 100  $\mu$ U/ml, did not affect TGF $\beta$  production in four of eight samples. It induced an increase in the secretion of TGF $\beta$  bioactivity in one NTG sample (NTG 2) and had a minor inhibitory effect in two GD and one normal sample. As demonstrated by limited dose-response curves performed in one patient of each group, iodide exerted



**Figure 3.** Slot blot analysis of TGF $\beta$  (A) and 7B6 (B) mRNA in purified TFC and in TMNC from NTG ( $n = 6$ ), NTG extranodular tissue (NTG/EN;  $n = 4$ ) and GD ( $n = 6$ ). mRNA concentrations are expressed as described in Fig. 2. Wilcoxon's rank sum tests for unpaired data were used for statistical evaluation. TGF $\beta$  mRNA was significantly higher in TFC than in TMNC in GD, but not in NTG ( $*P < 0.05$ , comparison of cell types). 7B6 mRNA was not significantly different between TFC and TMNC. Differences between disease groups were not statistically significant.



**Figure 4.** TGF $\beta$  bioactivity in supernatants conditioned by TFC from patients with NTG (A), GD (B), and from normal thyroids (C), cultured in serum-free medium with and without addition of sodium iodide (10  $\mu$ M) or TSH (10  $\mu$ U/ml). TGF $\beta$  bioactivity was assessed by its inhibitory effect on [ $^3$ H]thymidine incorporation in Mv1Lu cells in response to stimulation with FCS as described in Methods. Values are expressed as pg/10<sup>6</sup> cells, cell numbers referring to the end of the culture period.

its maximal stimulatory effect on the secretion of TGF $\beta$  bioactivity at a concentration of 10  $\mu$ M, after which the curves tended to plateau (Table I).

**Regulatory influence of exogenous TGF $\beta$  on [ $^3$ H]thymidine incorporation by TFC.** To assess whether TGF $\beta$  produced by TFC was of significance as a regulator of thyroid cell growth, the effect of rTGF $\beta$  on [ $^3$ H]thymidine incorporation by TFC was studied in NTG and GD.

rTGF $\beta$  at 10 ng/ml did not affect [ $^3$ H]thymidine uptake by unstimulated TFC from either NTG or GD patients (data not shown), but suppressed [ $^3$ H]thymidine incorporation by TFC when cultured with known stimulators of thyroid cell growth, such as IGFI, EGF, and TGF $\alpha$  (1–4; Fig. 5). In NTG (Fig. 5, left) suppression was not as complete as in GD (Fig. 5, right, comparison of the maximal TGF $\beta$  effect between disease groups:  $P < 0.05$  for IGFI and EGF, NS for TGF $\alpha$ ). Normal human serum was the strongest stimulator of [ $^3$ H]thymidine incorporation. The effect was only partly suppressible with TGF $\beta$  in each of the two groups, suggesting the inactivation of rTGF $\beta$  by human serum (Fig. 6). Due to the great variability in the responses to human serum, changes were not significant. TSH was not a reliable stimulator of [ $^3$ H]thymidine incorporation in different samples (Fig. 6). It induced suppression of basal [ $^3$ H]thymidine incorporation in one of three patients with GD and in two of eight with NTG. In the remaining patients stimulating responses varying in magnitude were ob-

**Table I.** Effect of Sodium Iodide on the Secretion of TGF $\beta$  Bioactivity by Purified TFC

	Patient No.	NTG 1	GD 2	Normal thyroid
TGF bioactivity	BASAL	<40	888	672
(picograms/10 <sup>6</sup> cells)	NaI 1 $\mu$ M	<40	960	960
	NaI 10 $\mu$ M	324	1,368	1,200
	NaI 100 $\mu$ M	300	1,200	1,200

TGF $\beta$  was assessed as described in Fig. 4.

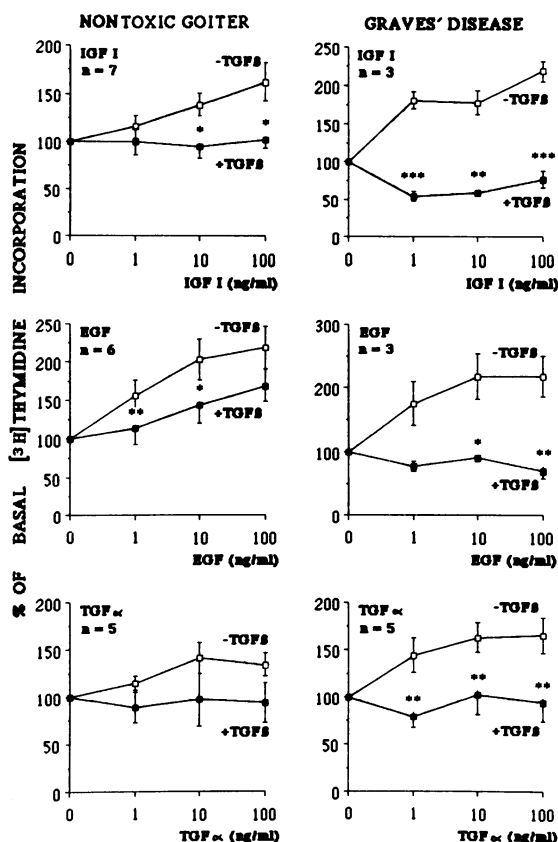


Figure 5. Effect of rTGFβ (10 ng/ml) on [<sup>3</sup>H]thymidine incorporation by purified TFC in monolayer culture, stimulated with rIGFI, purified EGF, and rTGFα. Comparison of TGFβ effects on TFC from NTG (left) and GD (right). Incorporation values ( $\bar{x} \pm \text{SEM}$ ) are expressed as percentage of basal [<sup>3</sup>H]thymidine incorporation by TFC incubated in serum-free medium only, which was considered as 100%. *t* Tests were used for statistical evaluation. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.005, *P* vs. stimulation values in the absence of TGFβ. The maximal suppressive effect of TGFβ was additionally compared between disease groups by *t* test. The difference was *P* < 0.05 for IGFI and EGF and NS for TGFα.

served. In the samples in which TSH stimulated [<sup>3</sup>H]thymidine incorporation, responses were fully suppressed by TGFβ. Due to the high deviation among responses, TGFβ suppression was not significant in either of the two groups.

## Discussion

NTG is a benign thyroid disorder that is characterized by excessive proliferation of TFC (25). Although the importance of iodine deficiency for the development of the disease is generally recognized (25), low iodine intake does not necessarily result in goitrogenesis, and NTG may occur in spite of a sufficient iodine supply (17, 26). Iodine deficiency is thus permissive for the development of NTG, but the initiating pathogenetic factor is still unknown (25).

Recent analyses of the mechanisms of cell growth have revealed that stimulatory mechanisms are counteracted by negative regulators (15), and that normal cell growth seems to depend on the balance between these conflicting influences

(15, 27). It was therefore of interest to determine whether NTG may be brought about by a disequilibrium in thyroid cell homeostasis due to a lack of negative growth control. We chose to examine the role of TGFβ as a potential inhibitor of TFC growth.

In this study we demonstrated that TFC produce TGFβ (Figs. 1–4) and that the growth of TFC in response to a number of stimuli (IGFI, EGF, TGFα, and TSH) is inhibited by TGFβ (Fig. 5). These results thus lead to the suggestion that TGFβ may act as an autocrine growth regulator in thyroid tissue. Preliminary reports on the growth inhibitory effect of TGFβ on cultured rat or porcine TFC suggest that the growth regulatory function of TGFβ extends to other species (28, 29).

We also demonstrate two abnormalities of the negative growth control of TGFβ on TFC in NTG. First there is decreased production of TGFβ, which was evaluated in most of the samples at the mRNA level. In view of the limited human thyroid tissue available, this had to be done by slot blot rather than by Northern analysis. As quantitative interpretation of slot blots may be complicated by a slightly different binding pattern of the probe to unfractionated RNA, message results were confirmed whenever possible at the protein level. The lower production of TGFβ detected reflects the *in vivo* situation, as the various experimental manipulations performed in thyroid tissue, including the use of FCS, stimulate TGFβ production *in vitro*. TGFβ production is low in the nodular as well as in the extranodular NTG tissue, but is normal in GD, which is accompanied by diffuse thyroid hyperplasia, as well as in parathyroid adenoma and nodular parathyroid hyperplasia. These findings demonstrate that reduced TGFβ production is not a general consequence of excessive proliferation of endo-

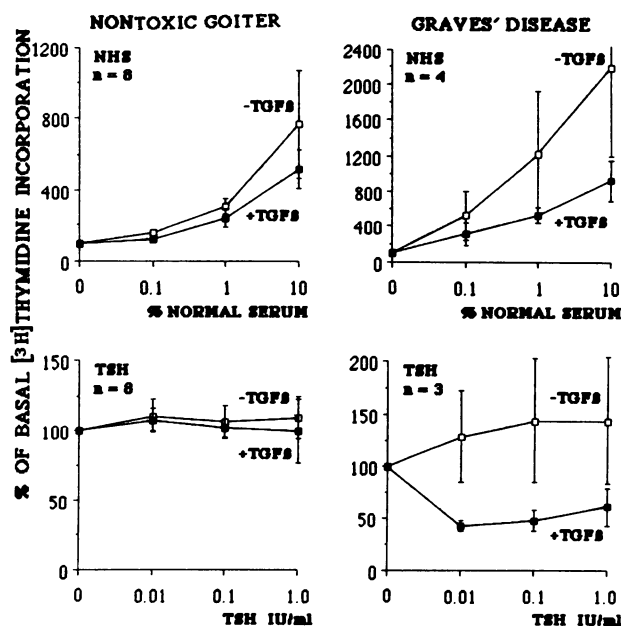


Figure 6. Effect of rTGFβ (10 ng/ml) on [<sup>3</sup>H]thymidine incorporation by purified TFC in monolayer culture, stimulated with human serum or bovine TSH. Comparison of TGFβ effects on TFC from NTG (left) and from GD (right). Incorporation values ( $\bar{x} \pm \text{SEM}$ ) are expressed as described in Fig. 5. There was no statistically significant difference in [<sup>3</sup>H]thymidine incorporation between TGFβ-untreated and TGFβ-treated samples.

crine cells, but is relatively specific to NTG and thus of potential pathogenetic significance.

The increase in the production of TGF $\beta$  by TFC in response to the addition of iodide, but not of TSH, to the culture medium (Fig. 4), suggests that TGF $\beta$  synthesis in TFC is dependent on a sufficient supply of iodine. It is thus tempting to speculate that iodine deficiency might exert its permissive influence in goitrogenesis, directly or indirectly, via decreased TGF $\beta$  production. Whether the reduced TGF $\beta$  production observed in NTG results from reduced in vivo stimulation, from an intrinsic abnormality of the cells, or from a combination of both factors is not known at present. Future work on a larger number and different types of samples, including more extensive dose-response curves and time courses, will be necessary to prove the validity of these possibilities. In situ hybridization for TGF $\beta$  mRNA will be useful in localizing and defining cells producing TGF $\beta$  in various thyroid diseases, and should also permit mRNA analyses in smaller samples.

The second abnormality in NTG is the reduced sensitivity of TFC to the inhibitory influence of TGF $\beta$ . The mechanism of this impairment is of interest. It is known that some ligands regulate the expression of their own receptors. Interleukin 2, for example, stimulates the production of interleukin 2 receptors in T cells (30). The reduced response to TGF $\beta$  in NTG may be due to reduced expression of receptors after reduced exposure to TGF $\beta$ . This concept avoids the need to propose two entirely discrete abnormalities of the TGF $\beta$  pathway in NTG, since a lack of TGF $\beta$  would consequently lead to a lack of receptors and hence reduced responsiveness. This possibility is under investigation, but faces problems due to the limited human thyroid tissue available.

It is of interest that TGF $\beta$  is only partly able to suppress the stimulatory effect of normal human serum on TFC. It is known that serum contains factors such as alpha 2 macroglobulin, which can neutralize TGF $\beta$  activity (31). This suggests that the function of TGF $\beta$  would be strictly localized and subject to functioning activation/lack of inactivation mechanisms. Due to the limited amount of human thyroid tissue available, only total (acid-activated) TGF $\beta$  could be determined in this study. Thus, no information can be provided at present on the ratio of inactive vs. active TGF $\beta$  in thyroid tissue.

Our considerations are obviously dependent on the purity of the TFC. The proportion of contaminating cells was low and thus it seems unlikely that a contaminating population would, at a frequency of < 2%, significantly affect the total TGF $\beta$  mRNA level or the responsiveness of TFC to the growth regulatory effects of exogenous TGF $\beta$ .

The results reported here suggest that TGF $\beta$  may be an important autocrine regulator of thyroid growth and indicate that abnormalities of this pathway are demonstrable in NTG. Recent work has shown that TGF $\beta$  is part of a family of related peptides, with platelets also containing a second form of TGF $\beta$  (TGF $\beta$ -2; 14) and glioblastoma another (32). These various peptides have related but partly different functions (14, 16). Future work will need to document their production and role in thyroid tissue. It is of interest that other endocrine regulators such as inhibins and activins are also related to TGF $\beta$  (16). The profound effects of TGF $\beta$  in the thyroid seem to be part of the regulatory effects of this family of molecules in the endocrine system.

## Acknowledgments

We thank the scientists and companies referred to in the text for the generous gifts of reagents. We are specially grateful to R. Derynck for the TGF $\beta$  probe.

This work was supported by the "Fonds zur Förderung der wissenschaftlichen Forschung," Austria (Erwin Schrödinger Scholarship), the Wellcome Trust, the Juvenile Diabetes Foundation International, the Arthritis and Rheumatism Council, and ICI public limited company.

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