TGF β_1 effects on functional activity of porcine thyroid cells cultured in suspension

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Abstract

Thyrotropin (TSH) and transforming growth factor β_1 (TGF β_1) have major roles in the regulation of folliculogenesis and differentiation in thyroid cells. Isolated porcine thyroid cells cultured in the presence of TSH on a plastic surface recover a follicular architecture and exhibit normal functional properties. The addition of TGF β_1 to the culture medium induces important morphological changes and extracellular matrix remodelling. Similarly, thyroid cells lose their ability to organify iodine and their responsiveness to adenylate cyclase. The aim of this study was to analyse the influence of TGF β_1 on the functional

activity of thyrocytes in suspension culture, independent of follicle disruption. In this system, we demonstrate that TGF β_1 inhibits expression of thyroperoxidase, NADPH oxidase activity, iodine uptake and, consequently, iodine organification. Moreover, TGF β_1 decreases basal and TSH-stimulated cAMP production and TSH receptor expression. Taken together, these data converge to demonstrate an essential role of TGF β_1 in the regulation of the thyroid cell function.

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Introduction

Transforming growth factor β_1 (TGF β_1) is a 25 000 Da disulphide-linked homodimer which regulates cell growth and differentiation. This polypeptide belongs to a family of molecules characterized by a considerable complexity. The range of TGF β_1 -mediated effects has been widely described, leading to abundant literature.

TGF β_1 generally exerts a growth-inhibitory effect for most cell types, including epithelial cells (Tucker *et al.* 1984, Masui *et al.* 1986), whereas it stimulates the proliferation of mesenchymally derived cells in monolayer (Roberts *et al.* 1985). Results obtained in cultured rabbit articular chondrocytes demonstrated that, according to culture conditions, TGF β_1 is able either to inhibit or to stimulate cell proliferation, and this could reconcile apparent discrepancies between findings previously reported in the literature (Vivien *et al.* 1993). The inhibition of proliferation described under TGF β_1 treatment can be related to the promotion of differentiation in various

models, including keratinocytes (Reiss & Sartorelli 1987), intestinal epithelial cells (Kurokawa *et al.* 1987) or human leukaemia cells (Bombara & Ignatz 1992). In thyroid cells, numerous studies have shown that TGF β_1 inhibited cell growth in human (Tsushima *et al.* 1988) and bovine and rat (Morris *et al.* 1988) thyroid cells. In this last case, TGF β_1 seemed to control the growth response to thyroid-stimulating hormone (TSH) by a dose-dependent down-regulation of the TSH receptor. This was corroborated by the secretion of TGF β_1 by thyroid cells, suggesting a possible autocrine role for this cytokine.

The action of TGF β_1 on thyroid cell function remains a much debated question. For instance, TSH-stimulated iodine capture can be either enhanced (Morris *et al.* 1988) or decreased (Colletta *et al.* 1989, Pang *et al.* 1992) by TGF β_1 . The effects of TGF β_1 on the accumulation of cAMP in response to TSH are also controversial (Morris *et al.* 1988, Tsushima *et al.* 1988, Taton *et al.* 1993). Even though Taton *et al.* (1993) reported that the responses to TSH mediated by the cAMP signalling pathway were

inhibited, it seems that the effects of TGF β ₁ on cellular differentiation depend on the species or marker of differentiation studied.

Another important role of TGF β ₁ concerns its ability to increase the expression of certain genes encoding cytoskeletal/matrix-associated proteins (Igotz & Massague 1986, Leof *et al.* 1986, Igotz *et al.* 1987). This is assessed by the stimulation of both collagen and proteoglycan synthesis in rabbit articular chondrocytes (Redini *et al.* 1988). The adhesion to extracellular matrix proteins exerts a profound influence upon cell function and behaviour. For example, TGF β ₁ promotes epidermal growth factor (EGF)-induced thyroid cell migration and follicle neoformation in collagen gel separable from cell proliferation (Nilsson *et al.* 1995). These data demonstrated the fundamental role of TGF β ₁ in cellular organization and cell–extracellular matrix relationships. TGF β ₁ up-regulates the expression of several integrins or matrix receptors, enabling cells to adhere more tightly to the substratum. In this context, we studied the relationship between TGF β ₁ and thrombospondin 1 (TSP-1) (Bellon *et al.* 1994, Claisse *et al.* 1999).

In previous reports, we demonstrated that TGF β ₁ induced the disruption of thyroid follicles and stimulated the expression of TSP-1 in a concentration-dependent manner. The effect of TGF β ₁ on cell adhesion and spreading was partly reproduced by TSP-1 and the functional activity of thyroid cells was deeply affected. These data can be related to the findings of other studies that have shown a mesenchyme-like cell shape and modulation of the formation of the luminal structure under TGF β ₁ stimulation (Toda *et al.* 1997). Under these conditions, the loss of the functional capacity of thyroid cells could be attributed to the spatial modification of the thyroid follicle architecture.

In this study, our purpose was to investigate the effects of TGF β ₁ on porcine thyroid cell metabolism, using a biological system in which thyrocytes were cultured in suspension. In this model, the addition of TSH promotes the formation of reconstituted follicles, which present a correct polarization after a 2-day period of culture (Mauchamp *et al.* 1979). A 48-h TGF β ₁ treatment allowed us to define the role of this cytokine in thyroid cell metabolism, independently of TGF β ₁-mediated cell migration and spread.

Material and Methods

Reagents

Unless otherwise stated, all reagents were from Prolabo (Briare, France). Trypsin was purchased from Gibco (Cergy Pontoise, France), fetal calf serum from Dutscher (Brumath, France) and Eagle's minimum essential medium (MEM) from Mérieux (Lyon, France). Bovine TSH (30 UI/mg, NIADDK, bTSH II) was from

Calbiochem (Meudon, France). Forskolin, HEPES and isobutyl methyl xanthine (IBMX) were from Sigma Chemical Company (St Louis, MO, USA). α -[³²P]dCTP was obtained from NEN Life Science Products (Paris, France). Na[¹²⁵I] was purchased from Cis Bio International (Gif sur Yvette, France). Hyperfilms-MP were from Amersham (Les Ulis, France).

Isolation and culture of thyroid cells

Porcine thyroid glands were obtained from Sobevir (Rethel, France). Thyroid cells were dissociated by a discontinuous trypsinization treatment in the presence of EGTA (Sigma) according to the method of Fayet *et al.* (1971) modified by Mauchamp *et al.* (1979). Freshly isolated cells suspended at 2×10^6 cells/ml in Eagle's minimal medium (pH 7.4) containing 10% (v/v) fetal calf serum, penicillin (200 U/ml) and streptomycin sulphate (0.05 mg/ml) were incubated at 37 °C in a 95% air–5% CO₂ atmosphere in Petri dishes untreated for tissue culture. This prevented adhesion of the cells to the surface of the culture vessel.

Cells were cultured in medium containing TSH in a concentration of 0.1 mU/ml. The cells reassociated into follicular structures after 2 days in culture. Reconstituted thyroid follicles were washed twice by centrifugation (200 g) and resuspended in Earle's solution buffered with 20 mM HEPES to pH 7.2. Under chronic treatment, cells were continuously cultured in the presence of TSH (0.1 mU/ml) and various concentrations of TGF β ₁ were added for the last 48 h of culture. Under acute treatment, cells cultured for 4 days in the presence of TSH (0.1 mU/ml) were washed and incubated with various concentrations of TGF β ₁ in the presence or absence of TSH (10 mU/ml).

Measurement of adenylate cyclase activity

Aliquots of washed thyroid cells (10^6 cells/assay) were incubated in the absence or presence of TSH (10 mU/ml) at 37 °C for 5 min in 350 μ l Earle's–HEPES (pH 7.2) containing 10^{-3} M IBMX and TGF β ₁ at various concentrations. The incubation was stopped by adding 39 μ l perchloric acid (final concentration 1 M) and cooling the test-tubes in crushed ice. Cells were homogenized and the cAMP concentrations were measured by radioimmunoassay according to the procedure of Cailla *et al.* (1973), except that bound and free ligand were separated by precipitating the bound ligand with a mixture of γ -globulins (2.5 mg/ml) in citrate buffer (pH 6.2) and polyethylene glycol 6000 (20 g/100 ml water).

Iodination of thyroid protein

The cells were washed and incubated at 37 °C for 45 min in 350 μ l Earle's–HEPES solution (pH 7.2) containing

1 μCi Na ^{125}I . At the end of the incubation, 650 μl Earle's-HEPES buffer containing potassium iodide (10^{-4} M) and bovine serum albumin (fraction V, 5 mg/ml) were added, followed by 2 ml 20% (v/v) cold trichloroacetic acid (TCA). The mixture was centrifuged at 500 g for 5 min and the resulting pellet was resuspended, washed twice with 10% (v/v) cold TCA and the radioactivity counted as protein-bound iodine. Protein iodination is accepted to be a marker of thyrocyte differentiation (Bjorkman & Ekholm 1988).

Uptake of iodine-125

Cells were incubated in 350 μl Earle's-HEPES buffer (pH 7.2) containing 1 μCi Na ^{125}I for 45 min at 37 °C. The reaction was stopped with 2 ml cold Earle's-HEPES. The suspension was centrifuged (500 g, 5 min) and the pellet resuspended and washed twice with cold Earle's-HEPES, then counted.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blotting

Suspension cultures of porcine thyroid follicle cells were homogenized, then centrifuged. Cell pellets obtained from chronic treatments were resuspended in 100 μl Tris HCl, then 50 μl Laemmli buffer was added and samples were boiled for 5 min. Electrophoresis was performed on a 7.5% (v/v) homogeneous polyacrylamide gel in 0.025 M Tris, 0.192 M glycine, 0.1% SDS (w/v) pH 8.3, according to Laemmli (1970); prestained molecular weight markers were used as standards. Proteins were then transferred to a nitrocellulose membrane (2 h, 50 V). The membrane was incubated with a polyclonal rabbit anti-porcine thyroperoxidase (TPO) antibody (dilution 1/2000). Rabbit immunization was achieved with porcine TPO obtained from a mild trypsinization procedure as described by Virion *et al.* (1985). The amount of the antibody was estimated by the ratio of absorbances: 410 nm/280 nm. This reaction with a primary antibody was followed by incubation with anti-rabbit IgG as secondary antibody. The immunoblot detection was by the enhanced chemiluminescence technique (Mattson & Bellehumeur 1996).

Measurement of H₂O₂ production by the thyroid Ca²⁺/NADPH-dependent H₂O₂-generating system

Preparation of membranes Thyroid cells (20×10^6) were washed with 5 ml Earle's-HEPES and centrifuged for 5 min at 200 g. The washing procedure was repeated twice, then the cell pellet was resuspended in 50 mM sodium phosphate buffer pH 7.4, 1 mM EGTA, 0.25 M sucrose and homogenized. The homogenates were centrifuged at 3000 g, 4 °C for 20 min and the pellets

were washed twice in 1 ml homogenizing buffer. The particulate fractions were suspended in 1 ml buffer, frozen immediately and stored at -20 °C.

Measurement of thyroid NADPH oxidase activity

Each determination was made with the particulate fraction from cells (about 20×10^6 thyroid cells). H₂O₂ production was determined as described by Deme *et al.* (1985). Samples of porcine thyroid cell particulate fraction were incubated at 30 °C in 1 ml 150 mM sodium phosphate buffer pH 7.2 containing 1 mM sodium azide, 1.2 mM EGTA and 1.5 mM CaCl₂. The reaction was started by the addition of 0.1 mM NADPH; aliquots (100 μl) were taken at regular intervals and mixed with 10 μl 2.4 M HCl to stop the reaction and destroy the remaining NADPH.

H₂O₂ formation was measured at pH 7.2 by following the decrease in scopoletin fluorescence in a Perkin-Elmer MPF 43A spectrofluorimeter: 1 mol H₂O₂ oxidizes 1 mol scopoletin. The excitation and emission wavelengths were 360 nm and 460 nm respectively.

If F₀ represents 100% of scopoletin fluorescence for $t=0$ min, ΔF (slope of the cross-section)/F₀ represents the fraction of scopoletin disappearing every 1 min.

Using determination of protein content, enzymatic activity was then calculated and expressed as nmol H₂O₂ formed/h per mg protein in the particulate fraction.

Analysis of TSH receptor mRNA levels by RT-PCR

Total RNA was extracted from thyrocytes using the RNeasy minikit from Qiagen (Courtaboeuf, France). RNA content was evaluated by measurement of absorbance at 260 nm, and its integrity checked by 1.5% agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μg purified total cellular RNA by reverse transcription at 42 °C for 60 min, using avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA). A 20 μl reaction volume contained 15 U/ μg AMV reverse transcriptase, 5 mM MgCl₂, 1 U/ μl RNasin, 1 mM dATP, dCTP, dGTP and dTTP, 0.5 μg oligo-(dT)₁₅ primers and 50 mM KCl and 0.1% Triton X-100 in 10 mM Tris HCl buffer, pH 9. Products were amplified by PCR using synthetic gene primers specific for human TSH receptor and rat S26 ribosomal protein produced from the reported sequences. S26 ribosomal protein was used as invariant control for gene regulation (Vincent *et al.* 1993). A 1- μl aliquot of the reverse transcriptase reaction product was amplified with 0.625 U Taq DNA polymerase (Promega) and 10 nM oligonucleotide primers (forward and reverse). The reaction was performed in a HyBaid omnigene temperature cycler, for 30 cycles.

Primers used were: TSH receptor forward 21-mer, 5'-ACACGGGCTGACCTTTCTTAC-3'; TSH receptor reverse 21-mer, 5'-TTGTAGTGGCTGGTGAGGAGA-3'; S26 forward 20-mer, 5'-GTGCGTGCCCAA GGATAAGG-3'; S26 reverse 20-mer, 5'-ATGGGC

TTTGGTGGAGGTCG-3'. PCR amplification was performed according to the following schedule: denaturation at 95 °C for 1 min, annealing at 48 °C (TSH receptor) or 50 °C (S26) for 1 min and elongation at 72 °C for 1 min for 25 cycles (S26) or 30 cycles (TSH receptor). PCR products were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. All PCR reactions resulted in the amplification of a single product of the predicted size (TSH receptor: 518 bp; S26: 261 bp). The identity of the amplification products was verified by sequencing (data not shown).

Analysis of TSP-1 mRNA levels by RT-PCR

RNA extraction was achieved as described above. cDNA probes for porcine TSP-1 were prepared in the laboratory by RT-PCR as follows. cDNA was prepared from 1 µg total cellular RNA by reverse transcription at 57 °C for 45 min. The reaction product was amplified in a 50 µl PCR mixture containing 0.2 µM sense and 0.2 µM antisense primers, 200 µM each deoxyribonucleotides triphosphate, 2 U recombinant Taq DNA polymerase, 2 mM MgCl₂ and 50 mM KCl in the above buffer. The TSP-1 (sense) 5' primer used was 5'-CTCAGGAACAAA GGCTGCTC-3' and the TSP-1 (antisense) 3' primer was 5'-ACTCCTGAATGTGGCAGGTC-3', defining a 663-base target sequence of human TSP-1 cDNA covering amino acid residues 284 to 504. Amplification was controlled by ethidium bromide staining and 1.5% (w/v) agarose gel electrophoresis. Markers of molecular size were used to verify the length of the fragments. The amplification products were excised from the gel and purified using Gene Clean II kit (Bio 101, La Jolla, CA, USA).

Electron microscopy

Thyroid cells were cultured for 4 days as described above, in the presence of TSH 0.1 mU/ml with or without TGFβ₁ (2 ng/ml) during the last 48 h of culture.

For transmission electron microscopy, cells were fixed for 1 h at room temperature in a solution of 2% (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.24). Each sample was rinsed three times for 5 min in the 0.1 M phosphate buffer (pH 7.24). The samples were post-fixed for 1 h in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer (pH 7.24), dehydrated in an alcohol series, transferred to acetone and embedded in Araldite. Ultra-thin sections were harvested on 150-mesh copper grids with a diamond knife on a Reichert U2 ultratome, stained with uranyl acetate/lead citrate and examined on a Jeol JSM 201 electron microscope at 60 or 80 kV.

Statistical evaluation

Mean values of triplicate determinations are given, with standard errors of the mean (S.E.M.). The statistical

significance of differences was calculated using Student's *t*-test. *P* values with respect to the corresponding controls are indicated in the legend of each Figure.

Results

Morphological analysis

Isolated thyroid cells cultured in suspension in the presence of low concentrations of TSH (0.1 mU/ml) reconstitute after 48 h into thyroid follicles similar to those found *in vivo* (Fig. 1A). The polarity and the tightness of thyrocytes reorganized in follicular structures have been described elsewhere (Munari-Silem *et al.* 1990). Nevertheless, we observed that clusters of cells delimited a cavity corresponding to an intrafollicular lumen. Cells facing the culture medium were devoid of microvilli (Fig. 1C), whereas cells bordering the internal cavity were covered with numerous microvilli (Fig. 1E). It can be seen that intercellular spaces contain villi and remnants of membrane.

After cells had been cultured for 48 h in the presence of TSH, the addition of TGFβ₁ (2 ng/ml) did not affect the intercellular junctional complexes (Fig. 1B). In fact, we did not observe any disruption of the three-dimensional cellular structure or the appearance of isolated cells, even when intercellular spaces were widened as a result of the presence of the observed cellular clusters. However, the cell aggregates adopted an organization in which the polarity of the thyroid cells would be inverted in comparison with that of TSH-treated cells. Thyroid cells in contact with the culture medium exhibited on their external membrane microvilli that were heterogeneous in size and shape (Fig. 1D). In contrast, cells in clusters bordering the internal cavity exhibited an absence of microvilli (Fig. 1F).

These structures, and the inversion of cellular polarity, were observed in the absence of an interaction with the culture substratum, as the thyroid cells were floating in the medium. This model of culture allowed us to define the exact role of TGFβ₁ in thyroid cell metabolism, independently of TGFβ₁-mediated cell migration and spread.

Analysis of TSP-1 mRNA expression

In a first approach, we have verified that TGFβ₁ was able to stimulate the expression of the mRNAs encoding TSP-1, as had been demonstrated using adherent thyroid cells (Claisse *et al.* 1999): a 48-h treatment of reconstituted thyroid follicles with TGFβ₁ revealed an up-regulation of TSP-1 expression, as shown in Fig. 2. This finding confirmed that TGFβ₁ acts specifically on the expression of extracellular matrix proteins, even though this effect was not associated with thyroid cell adhesion and spread.

Figure 1 Electron microscopic examination of 4-day-cultured porcine thyroid cells. Two-days reconstituted thyroid follicles were treated for 48 h in the presence of TSH (0.1 mU/ml) alone (A, C, E) or in the presence of TSH (0.1 mU/ml) and TGF β_1 (2 ng/ml) (B, D, F). (A) Thyroid cells after 4 days of culture in the presence of TSH ($\times 2000$). (C, E) Ultrastructural features ($\times 8000$): note the absence of microvilli on the surface of cells bordering the culture medium (C), whereas cells bordering follicular lumina present numerous microvilli (E). (B) Effects of 48 h of TGF β_1 treatment ($\times 2000$). Thyroid cells remain associated into clusters. (D, F) Ultrastructural features ($\times 8000$). The situation is the converse of the observations reported in the presence of TSH alone: external cells present microvilli in contact with the culture medium (D), and cells bordering the internal cavity are devoid of villi (F).

Basal cAMP and sensitivity of cells to acute TSH stimulation

We next investigated the effects of TGF β_1 on adenylate cyclase sensitivity and iodine organification – the main functional parameters related to TSH action. TGF β_1 , added as an acute treatment to thyroid cells treated with TSH for 4 days, was unable to modulate the basal concentration of cAMP. Moreover, the ability of reconstituted thyroid follicles to produce cAMP in response to TSH (10 mU/ml) was not altered in the presence of various concentrations of TGF β_1 (data not shown). In a similar way, we tested the capacity of TGF β_1 to affect protein iodination. After a 45-min period of treatment with concentrations of TGF β_1 in the range 0–4 ng/ml, we established that TGF β_1 did not present any effect on

iodine organification (data not shown). In accordance with these data, we analysed the long-term effects of TGF β_1 . In this set of experiments, TGF β_1 was added to TSH-treated thyroid cells for the last 48 h of culture. First, we were interested in the regulation of the adenylate cyclase system under these conditions. The kinetics of cAMP production were performed over 5 min in the presence or absence of TSH (10 mU/ml) in the incubation medium. Inhibition of phosphodiesterase activity by the methylxanthine derivative increased the amount of cAMP produced by thyroid cells. Figure 3A shows that, with a 48-h treatment, the basal rate of production of cAMP was inhibited by concentrations of TGF β_1 as low as 1 ng/ml, and that there was a marked diminution of cAMP production (around 70%) with 4 ng/ml TGF β_1 . Figure 3B shows

Figure 2 Analysis of TSP-1 mRNA by semi-quantitative RT-PCR. Cells were treated with agonists for 48 h. Total RNA was extracted using the RNeasy minikit from Qiagen. The reverse transcription was performed with 1 µg total RNA and oligo(dT) at 57 °C. cDNA was amplified by PCR using synthetic gene primers for human TSP-1 and rat S26 ribosomal protein. S26 was used as invariant control for gene regulation.

the long-term effects of TGFβ₁ on the TSH-stimulated accumulation of cAMP: 1 ng/ml TGFβ₁ inhibited by 50% the accumulation of cAMP stimulated by TSH, and this inhibition reached 80% with 4 ng/ml TGFβ₁.

Using forskolin as a direct agonist of the adenylate cyclase system, we confirmed the data on cAMP production presented in Fig. 3. Forskolin-treated cells incubated for 48 h with TGFβ₁ showed a progressive inhibition of the basal rate of production of cAMP, up to $75.1 \pm 3\%$ with 4 ng/ml of the cytokine. In a similar way, the forskolin-stimulated production of cAMP was strongly diminished in the presence of 4 ng/ml TGFβ₁ ($85.6 \pm 2\%$).

TSH receptor mRNA analysis

Taken together, these results suggested that TGFβ₁ could counteract the regulation of the thyroid cell function by TSH. To investigate this hypothesis further, we examined the amount of TSH receptor mRNA expression in reconstituted thyroid follicles after treatment with TGFβ₁ for various times. TSH receptor mRNA was detected by semi-quantitative RT-PCR analysis. The expected PCR product of 518 bp was obtained in stable amounts in untreated reconstituted thyroid follicles (Fig. 4). The TSH receptor mRNA rapidly decreased up to 4 h in response to incubation with 2 ng/ml TGFβ₁. After 24 h of treatment, TSH receptor expression was totally abolished. This observation can explain the inhibition of TSH-stimulated production of cAMP presented in Fig. 3.

Organification and uptake of iodide

Porcine thyroid cells cultured in the presence of TSH (0.1 mU/ml) for 4 days transported iodide and incorpo-

ated iodine into thyroglobulin. In these culture conditions, cells treated with TSH for 4 days exhibited a high basal concentration of protein-bound iodine (564 c.p.m./µg protein) compared with that in non-treated cells (143 c.p.m./µg protein). Figure 5 shows that TGFβ₁ (0.1 ng/ml) did not significantly affect the TSH-positive regulation of protein-binding of iodine, whereas we observed a progressive inhibition of binding with greater concentrations of TGFβ₁. The TGFβ₁-induced inhibition of protein-binding of iodine reached a plateau at 2 ng/ml, when it was 80%. In another set of experiments we analysed the effect of TGFβ₁ on iodide uptake in the presence of methyl thiouracil (10^{-4} M) to prevent organification, and we noted time-courses of uptake similar to those for protein-binding of the iodine (Fig. 6).

TPO expression and NADPH oxidase activity

The mechanism of iodine organification involves an oxidation process catalysed by thyroperoxidase (TPO) and a system generating H₂O₂, at the apical membrane of thyrocytes. H₂O₂ generation occurs via a pluri-enzymatic system in which NADPH oxidase plays the main part (MacLachlan & Rapoport 1992). We therefore studied the consequences of a 48-h treatment with TGFβ₁ on TPO expression and NADPH activity. After 4 days of culture (from day 0 to day 4) in the presence of TSH (0.1 mU/ml), the ability of cells to organify iodide was restored. This reflects recovery of the functional properties of the thyroid cells, and is correlated to the organization of cells into follicular structures. The amount of TPO determined by western blot analysis was decreased by TGFβ₁ in a dose-dependent manner (Fig. 7A). Under similar experimental conditions, NADPH oxidase activity, measured as H₂O₂ production, was dramatically reduced by TGFβ₁.

Figure 3 Measurements of adenyl cyclase activity. Follicles treated for 2 days with TSH (0.1 mU/ml) were cultured for a further 2 days in the presence of TGF β_1 at the concentrations indicated. At the end of the culture period, cells were incubated at 37 °C for 5 min in Earle's-HEPES pH 7.2 containing 10^{-3} M IBMX in the absence (A) or presence (B) of 10 mU/ml TSH. Incubation was stopped by the addition of cold HClO₄. Cells were homogenized and cAMP concentrations were measured by radioimmunoassay. Bars represent means $\pm$ S.E.M. of three separate experiments. NS, not significant; *** $P < 0.001$ compared with control.

treatment (Fig. 7B). This inhibition took effect with concentrations as low as 0.1 ng/ml TGF β_1 and reached a maximum at 1 ng/ml. These observations are fully in agreement with the results obtained from protein iodination.

Discussion

It is well established that thyroid function is controlled by a negative feedback mechanism. Any event (iodine deficiency, defects of iodine metabolism, etc.) able to induce a modification of the equilibrium between the concentrations of thyroid hormones and that of TSH may result in the loss or dysfunction of differentiated functions of the gland. One of the major elements controlling thyroid growth and function is the concentration of TSH, but it is no less evident that thyrocytes also respond to factors synthesized and secreted by the thyrocytes. Among these factors, TGF β_1 has been shown to be produced particularly in the FRTL-5 rat thyroid cell line (Morris *et al.* 1988). The ability of TGF β_1 to inhibit thyroid cell growth has been described (Tsushima *et al.* 1988, Grübeck-Loebenstein *et al.* 1989, Cirafici *et al.* 1992). The mechanism of action of TGF β_1 could consist in the mediation of a negative autocrine loop limiting normal cell proliferation (Roberts & Sporn 1990).

Independent of its action on cell growth, TGF β_1 promotes EGF-induced thyroid cell migration (Nilsson *et al.* 1995). In FRTL-5 cells (Garbi *et al.* 1990), TGF β_1 profoundly affects cell morphology and organization of the thyrocyte actin microfilaments. Finally, TGF β_1 also modulates the differentiation of thyroid cells, even though this aspect remains a much debated question.

Study of the effects of TGF β_1 could not be undertaken using the conventional adhesive substratum in which we demonstrated that TGF β_1 was able to promote the disintegration of TSH-induced follicles. The fact that the thyrocyte phenotype shifts to a fibroblastoid type under TGF β_1 treatment is consistent with the loss of cell-cell contact and modification of the cytoskeleton. Information relative to the negative feedback triggered by TGF β_1 on the TSH-dependent control of thyroid function could not be extrapolated from these data, which prompted us to investigate the effects of TGF β_1 in suspension cultures. Thyroid cells cultured in suspension with TSH (0.1 mU/ml) underwent reconstitution of their follicular structures. This morphological effect, originally described by Mauchamp *et al.* (1998), is closely related to the ability of cells to organify iodine and to stimulate adenylate cyclase responsiveness (Haye *et al.* 1985). In this system, the addition of TGF β_1 for 48 h did not lead to follicular disruption and cell scattering, even though thyroid cells largely expressed TSP-1. However, the stimulation of the mRNAs encoding TSP-1 indicates that extracellular matrix proteins are effectively synthesized, but are diluted

Figure 4 Analysis of TSH receptor mRNA expression by semi-quantitative RT-PCR. Total RNA was extracted with the RNeasy minikit (Qiagen). cDNA was amplified by PCR using synthetic gene primers for human TSH receptor (TSHr) and rat S26 ribosomal protein. S26 was used as invariant control for gene regulation.

in the culture medium, so preventing cells adhering to the plastic surface. TGFβ₁ induced a reorientation of the polarity of thyroid cells, but this occurred in the continuous presence of tight junctions and did not require prior dissociation of follicle structure.

The results presented in this study concerning the effects of TGFβ₁ on adenylate cyclase activity are inconsistent with data published by Colletta *et al.* (1989) from FRTL-5 cells, Tsushima *et al.* (1988) in porcine cells and Taton *et al.* (1993) in human thyroid cells. These authors reported that both basal and TSH-stimulated cAMP production remained invariant after pretreatment of thyroid cells with TGFβ₁. This apparent discordance concerning the effects of TGFβ₁ on cAMP production calls for comment. It should be noted that the observations of Taton *et al.* (1993) were made in thyroid cells seeded on an adhesive substratum. In these conditions, TGFβ₁ receptors were probably less accessible than in suspension cultures. In other respects, the same group stated that the data presented were obtained in serum-free conditions. The absence of serum components such as growth factors or differences in cell species could also account for this discrepancy. For our part, we clearly demonstrated a marked inhibitory long-term effect of TGFβ₁ on adenylate cyclase activity, suggesting that TGFβ₁ was able to interfere with the cAMP signalling pathway when this cytokine did not counteract cAMP production in acute conditions. Taken together, these data strongly suggest that TGFβ₁ more probably acts on the expression of adenylate cyclase components than on the enzymatic

activity. Parallel studies performed with forskolin, a classical adenylate cyclase agonist, support this statement. Forskolin acts directly on the catalytic subunit of the cyclase and bypasses the binding of TSH to its receptor. In this set of experiments, we reproduced the effects reported in Fig. 3 over a similar range of concentrations of TGFβ₁.

The explanation for TGFβ₁-mediated inhibition of the TSH-stimulated formation of cAMP can be found in the down-regulation of TSH receptor expression. This observation has been reported recently by Franzen *et al.* (1999) with 10 ng/ml TGFβ₁. In our experiments, 2 ng/ml TGFβ₁ was able to provoke the extinction of TSH receptor mRNA as early as after 8 h of treatment. It is interesting to note that porcine thyroid growth and function seem to be controlled by a fine balance between TSH and TGFβ₁. Indeed, in the presence of TSH, thyroid cells synthesize TGFβ₁, as we observed in our culture conditions using an enzyme-linked immunosorbent assay (40.6 pg/ml TGFβ₁ in medium from TSH-treated cells, compared with 12.8 pg/ml in medium from non-treated cells). The physiological relevance of the synthesis of TGFβ₁ in the thyroid could correspond to an autocrine role of this cytokine on the TSH-controlled signalling pathways that govern thyroid cell function. This situation is apparent in pathological disorders: TGFβ₁ is expressed to a greater extent in thyroid follicular adenoma than in normal tissue (Morosini *et al.* 1994). Similarly, Patel *et al.* (1996) described an increase in TGFβ₁ in the hyperplastic rat thyroid.

Figure 5 Measurement of protein-bound iodine (PBI). Follicles treated with TSH (0.1 mU/ml) for 2 days were cultured for a further 2 days in the presence of TGF β_1 at the indicated concentrations. At the end of the culture, cells were incubated for 45 min at 37 °C in Earle's-HEPES pH 7.2 containing 1 μ Ci Na 125 I. The reaction was stopped by the addition of 10^{-4} M potassium iodide, 0.5 mg/ml bovine serum albumin and 20% (w/v) cold trichloroacetic acid to precipitate proteins. The mixture was centrifuged and the pellet washed twice with 10% (w/v) trichloroacetic acid. The radioactivity associated with proteins was measured with a γ -counter. Bars represent means $\pm$ S.E.M. of three separate experiments, NS, not significant; *** P < 0.001 compared with control.

Figure 6 Concentration-dependent effects of TGF β_1 on the uptake of iodine-125. Conditions were the same as for the measurement of protein-bound iodide, except that, after 45 min of incubation with iodine-125, the reaction was stopped with cold buffer and the thyroid cells were pelleted and washed before counting, as described in Materials and Methods. Graph points represent means $\pm$ S.E.M. of three separate experiments. NS, not significant; ** P < 0.01, *** P < 0.001 compared with control.

It seems, therefore, that the loss of the reciprocal control between TSH and TGF β_1 may result in great modification of the functional properties of thyroid cells. To assess this

Figure 7 (A) NADPH oxidase activity. Samples of porcine thyroid cell particulate fraction were incubated at 30 °C in 1 ml 150 mM sodium phosphate buffer pH 7.2, containing 1 mM Na N_3 , 1.2 mM EGTA and 1.5 mM CaCl $_2$. The reaction was started by the addition of 0.1 mM NADPH, and stopped by the addition of 10 μ l 2.4 M HCl, which destroyed the remaining NADPH. Formation of H_2O_2 was measured at pH 7.2 by following the decrease in scopoletin fluorescence in the presence of horseradish peroxidase, in a Perkin-Elmer MPF 43 A spectrofluorimeter. The excitation and emission wavelengths were 360 nm and 460 nm respectively. Enzymatic activity was calculated from the variation in fluorescence determined from the linear portion of the reaction curve, and is expressed as nmol H_2O_2 formed/h per mg protein in the particulate fraction. Bars represent means $\pm$ S.E.M. of three separate experiments. NS, not significant; *** P < 0.001 compared with control. (B) Thyroperoxidase detection by western blot. Aliquots of cell homogenates were separated by 7.5% (v/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal rabbit anti-porcine TPO antibody, followed by incubation with anti-rabbit IgG as peroxidase-conjugated secondary antibody. The immunoblot was visualized by chemiluminescence. *** P < 0.001 compared with control.

hypothesis, we performed preliminary experiments using neutralizing anti-TGF β_1 antibodies. The abolition of endogenous effects of TGF β_1 seemed to be associated with a moderate decrease in concentrations of protein-bound iodide. These data show that TGF β_1 is essential to the correct expression of thyroid function. However, further studies are necessary to establish the exact role of TGF β_1 in this phenomenon.

The second aspect of our work concerned the regulation of iodine metabolism. Different reports have established that TGF β_1 could either stimulate or inhibit iodine trapping (Bidey *et al.* 1999). We demonstrated here that this effect is directly related to the concentration of TGF β_1 used. The potentializing effect on iodine uptake of TGF β_1 at low concentration appears to corroborate the hypothesis that TGF β_1 and TSH can act synergistically until the ideal combination is obtained. In a similar way, treatments with growth factors such as insulin-like growth factor I increase the ability of TSH to stimulate the accumulation of iodide (Pang *et al.* 1992). The regulation of Na⁺/I⁻ symporter (NIS) activity is complex, but could depend on its pattern of phosphorylation (Riedel *et al.* 2001). After a 48-h period of treatment, TGF β_1 also strongly decreased the amount of protein bound iodine-125, in a dose-dependent manner. In other respects, we were not able to observe any modulation of iodine organification with an acute stimulation by TGF β_1 . The inhibitory effect of TGF β_1 probably results from the reduced NIS mRNA levels (Pekary & Hershman 1998) that are associated with a decrease in synthesis of thyroperoxidase protein and a marked inhibition of NADPH oxidase activity.

Our observations demonstrated that TGF β_1 could reflect a negative feedback of the action of TSH. In addition, the expression of TGF β_1 in response to chronic TSH treatment involves the maintenance of optimal concentrations of adenylate cyclase and optimal iodine organification activity. The participation of TGF β_1 in this regulatory loop is under current investigation in our laboratory. Analysis of the expression of TGF β_1 and its receptors in normal or tumorous cells should provide important information regarding the loss of responsiveness to TGF β_1 that is reported in thyroid pathologies (Blaydes & Wynford-Thomas 1996, Heldin *et al.* 1999) and the possible cross-talk between signalling pathways controlled by TSH and TGF β_1 .

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