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TGFβ inhibits rat thyroid cell proliferation without alterations in the expression of TSH-induced cell cycle-related genes

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Transforming growth factor β (TGFβ) is a secreted polypeptide factor that is thought to play a major role in the regulation of proliferation of many cell types and various differentiation processes. TGFβ acts on thyroid cells by inhibiting cell proliferation and expression of differentiation markers, such as thyroglobulin production and iodide uptake. Exponentially growing thyroid cells cultures accumulate mostly in G0\G1 after exposure to TGFβ for 48 hours. TGFβ inhibits the TSH induced transition of quiescent thyroid cell from the G0 to the S phase. These effects on the thyroid cell growth, however, are not mediated by changes in the TSH-induced cell cycle-related genes expression; both immediate early and progression genes expression is unaffected by the TGFβ treatment.

Transforming growth factor β is a growth and differentiation factor that is secreted by a large variety of cells and modulates the growth of many cell types. While TGFβ stimulates the proliferation of selected cell types, mostly of mesenchymal origin, it inhibits the proliferation of many cell types (1,2). The growth of lung epithelial cells and keratinocytes is virtually arrested by TGFβ, while osteoblasts and fibroblasts are stimulated under certain conditions (3,4,5). The mechanisms of growth inhibition by TGFβ are still unknown. TGFβ does not appear, in general, to inhibit cell growth by inactivating receptors for growth promoting factors (6). A number of studies have shown that TGFβ does not affect early G1 events induced by growth factors: for instance, the antiproliferative effect of TGFβ does not seem to be a consequence of phosphoinositide breakdown, ribosomal S6 protein phosphorylation, or Na+\H+ antiport (6,7). Recently it has been proposed that a protein kinase activity may be involved in mediating TGFβ effects (8). In lung cells and in keratinocytes TGFβ inhibition of proliferation

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involves events that occur late in G1, possibly, at the G1-S interphase. This inhibition is linked to suppression of retinoblastoma protein (rb) and p34 ^{cdc2} phosphorylation (3,4,9).

TGFβ inhibits rat epithelial thyroid cell growth, and it is able to antagonize the mitogenic effect of thyrotropic hormone (TSH) on the thyroid cells (10,11), but the nature of this inhibitory effect is unclear at the present.

In this work the influence of TGFβ on the TSH-regulated rat thyroid cell cycle was investigated. Similarly to the action produced in other, different cell systems (3,4,12), the TGFβ treatment of proliferating thyroid cells increases the percentage of cells in the G1 phase. Addition of TGFβ on quiescent thyroid cells limits the mitogenic action of TSH. Entry of rat thyroid cells into S phase can be prevented by addition of TGFβ during early or middle G1, but not during the late stages of G1. However the TGFβ treatment does not modify the TSH-induced immediate early events, such as induction of expression of transcription factors. Moreover the TSH-mediated induction of progression genes that happens in the early G1, is only slightly modified by TGFβ.

Materials and methods

Cell culture

The FRTL-5 (13) rat thyroid cells are derived from 3 week old normal Fisher rats, passaged and grown in Coon's modified Ham's F-12 medium (W\O) supplemented with 5% heat inactivated mycoplasma-free calf serum and a six hormone (6H) mixture containing the following: TSH, 1 x 10^{-10} M; insulin 10mg\ml; hydrocortisone 1 x 10^{-8} M; human transferin 5mg\ml; somatostatin 10ng\ml; and glycyl-L-histidyl-L-lysine acetate 10ng\ml. TGFβ used is porcine $TGF\beta_1$ isoform from R & D System (Minneapolis, Mn).

DNA synthesis assay

DNA synthesis was measured by incorporation of ^{3}H-thymidine (1mCi\ml, 40 Ci\mmol, Amersham, United Kindom) into trichloroacetic acid-insoluble material as described previously (14). Each point was assayed in duplicate. Autoradiography was performed on glass coverslips as described (15). At least 200 cells were counted per point.

DNA content analysis by PI staining

FRTL-5 were fixed in 70% ethanol and stored at 4° until the analysis. One ml of a PI (propidium iodide) staining solution (50 mg\ml of PI in Ca++\Mg++ free PBS, pH 7.4, plus RNAase 0.5mg\ml) was added to 2 x 10^{6} cells previously washed in Ca++\Mg++ free PBS. After 30 min at room temperature, the DNA content was analysed by a FACScan flow cytometer (Becton Dickinson, Mountain View, Ca, USA) coupled with a Hewlett-Packard computer. Cell cycle data analysis was performed using standard procedure (16). For each sample 20.000 events were stored in list mode.

RNA extraction and hybridization

RNA was purified from cultured cells by a modification of the guanidine hydrochloride extraction method. Total RNA, 10μg\lane, was fractionated on a 1% formaldehyde agarose gel, transferred to nytran membranes (Schleicher and Schuell) and hybridized following standard procedure. ^{32}P nick-translated gel purified fragments of DNA (2 x 10^8 cpm\$\mu$g) were used for hybridization. The probes used were:c-jun cDNA (p-hcJ-1) (17), the mouse Xrox20 cDNA (AC16 plasmid) (18), the human ODC cDNA (pODC934) (19), the human Ts11 cDNA (pcD-ts11-5A) (20), the human 2-F1 cDNA (hp2F) (21). The size of transcripts were indicated relative to 18S and 28S which were assumed to be 1.8 and 5.4 kilobases, respectively.

Results

It has been previously shown that TGFβ is able to inhibit thyroid cell growth: a concentration of 10ng\ml of TGFβ is sufficient for a clear reduction in the proliferation rate of thyroid FRTL-5 cells (11). Following a slight increase ten hours after TGFβ treatment (10ng\ml), a 75% reduction in thymidine uptake is shown by thyroid proliferating cells after 24 hours (fig.1). The level of ^{35}S methionine incorporation in cells treated with TGFβ in parallel was similar to that of untreated proliferating cells, indicating that TGFβ does not modulate the overall protein synthesis. To analyse in which phase of the cell cycle the thyroid cells were arrested by the TGFβ, flow cytometry assays were performed. Data presented in Table 1 show that exponentially growing FRTL-5 cultures, which have a 32 hours

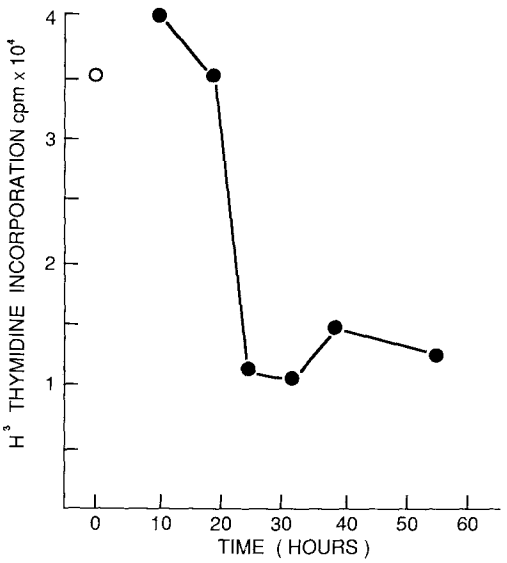

Figure.1. TGFβ inhibition of thyroid cell proliferation.

Proliferating cells, i.e. cells growing in the presence of 5% calf serum and 6H mixture (o), have been treated with TGFβ (10ng\ml). After the times indicated from TGFβ treatment, cells are labeled with 1mCi\ml of ^3H-thymidine for two hours (●). The points represent the mean of duplicate determinations.

Table 1

Cell cycle distribution of growing FRTL-5 cells in presence of TGFβ

	Cell cycle distribution %		
	G0\G1	S	G2\M
24h Control	74.8	9.8	15.3
24h + TGFβ	82.2	6.1	11.6
48h Control	76	10	13
48h + TGFβ	86.5	6.5	7

Exponentially growing FRTL-5 cells in presence of 6H mixture were incubated with TGFβ (10ng\ml) for 24 and 48 hours. Data represent average of three different experiments. The cell cycle distribution was analysed by flow cytometry.

doubling time, accumulate mostly in G0\G1 after exposure to TGFβ for 24\48 hours.

To further investigate the mechanism of TGFβ action on the thyroid cell cycle, the kinetics of TSH-induced DNA synthesis was analysed. It has been previously shown that TSH is the major factor controlling the cell growth in this system of differentiated rat thyroid cells in culture (13). In fact, TSH alone in a serum free system is able to stimulate DNA synthesis of rat thyroid cells (14). FRTL-5 cells can be rendered quiescent when grown in W\O medium plus 0,025 % of BSA (Bovine Serum Albumin) for two days. Upon TSH addition, the quiescent thyroid cells are stimulated and enter into S phase 12 hours later, with a peak in DNA synthesis at 18-20 hours. Fig. 2A shows the synthesis of DNA, as evaluated by incorporation of labeled thymidine, in TGFβ treated and untreated thyroid cells. The results indicate that treatment of quiescent cells with 10 ng\ml of TGFβ efficently reduces the TSH-induced synthesis of DNA. Similar results have been obtained by autoradiography of labeled nuclei (results not shown).

It has been shown in keratinocytes, as well as in lung cells, that TGFβ effects can be elicited in late G1 (3,4). To determine when in the FRTL-5 cell cycle TGFβ is required for inhibition of DNA synthesis, a stimulation assay was performed in which TGFβ was added at various times during G1. In the experiment described in fig.2 B, FRTL-5 cells were arrested in their growth by hormones deprivation for two days, and then stimulated with TSH. Entry into S phase was determined by thymidine uptake in a 2 hours pulse from 22-24 hours following stimulation. TGFβ was added at different time following TSH stimulus. When TGFβ was added after 1,2,4,6,8, hours after TSH stimulation, the TSH induced cell entry into the S phase was inhibited. However, when TGFβ was added after entry into S phase no inhibition of DNA synthesis was observed (fig.2 B). These results

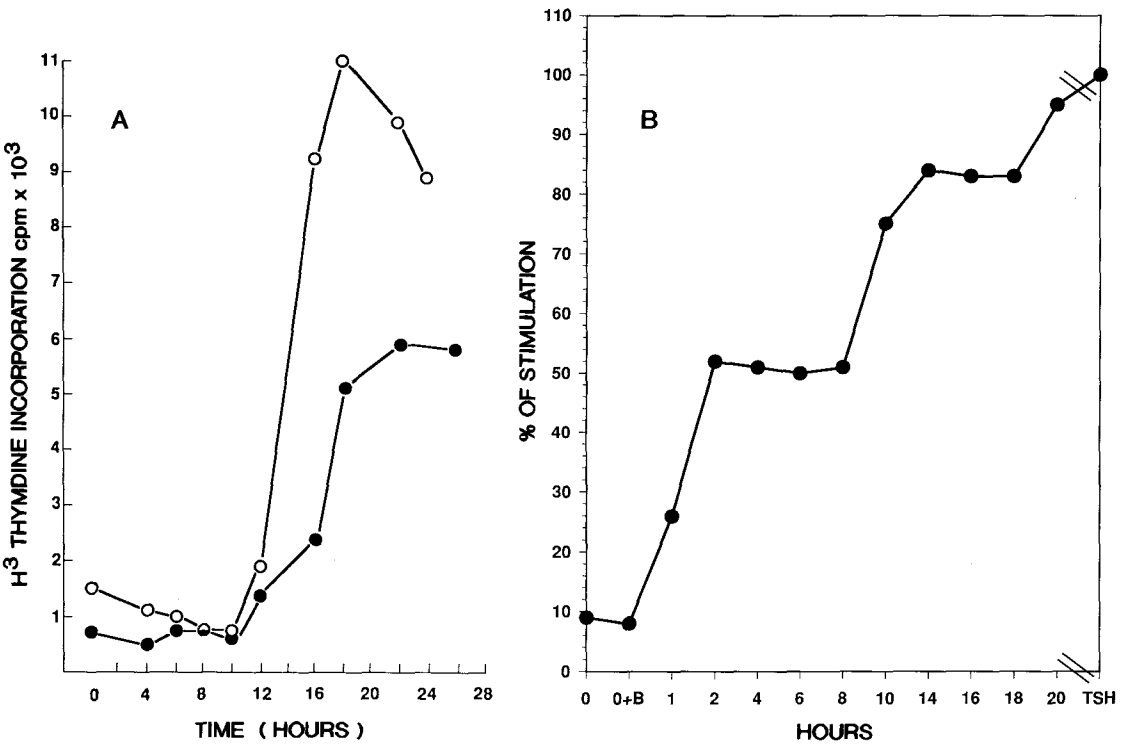

Figure 2.A) Inhibition of S phase entry by TGFβ.

Quiescent cells, i.e. cells in presence of medium plus 0.025% BSA, pretreated or not for 24 hours with TGFβ (10ng\ml) were stimulated with TSH 10mU\ml. Cells were pulse labeled with 1 mCi\ml of ^{3}H-thymidine for 1 hour at the indicated hours after stimulation. TGFβ treated cells (●---●), untreated (o---o).

B) Kinetics of TGFβ growth inhibition.

Quiescent thyroid cells were stimulated with TSH (10mU\ml), at the times indicated TGFβ was directly added to a final concentration of 10ng\ml. The culture was pulsed with 1 mCi\ml of ^{3}H-thymidine for two hours, 22-24 hours following initial TSH stimulation. Results are expressed as % of stimulation of the thymidine uptake of control TSH-treated cells, each point was assayed in duplicate. 0 = quiescent cells, 0+B = quiescent cells plus TGFβ, TSH = control cells TSH-treated.

suggest that TGFβ is able to prevent DNA synthesis when thyroid cells are in G1; once they have progressed further into G1\S they become insensitive to TGFβ growth inhibition.

Since TGFβ could prevent the TSH-stimulated thyroid cells to enter the S phase, experiments were performed to verify whether TGFβ modulate events in early G0\G1. It has been shown that TSH is able to induce expression of genes coding for the API complex : c-fos and c-jun, 40' from stimulation (22). A gene coding for a finger protein Xrox 20 is also induced by TSH (22). To analyse whether TGFβ treatment of thyroid cells alters the expression of these immediate early genes, total RNA was extracted from quiescent thyroid cells, treated and untreated with TGFβ (10ng\ml), stimulated by TSH in serum free medium. As

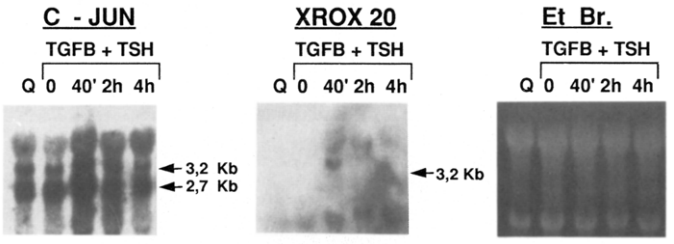

Figure 3. C-jun and Xrox20 induction in TGFβ-pretreated thyroid cells.
Northern blots of total RNA extracted from TGFβ (10ng\ml) pretreated cells and then stimulated with TSH (10mU\ml) at the times indicated. Quiescent cells (Q), quiescent cells after TGFβ and TSH treatment harvested at the indicated times. The probes used for hybridization are indicated.

shown in fig.3 TGFβ does not affect induction of c-jun and Xrox20 genes in thyroid cells. TPA (50ng\ml) and forskolin (0.5 mM), both induce c-jun, and Xrox20 gene expression in quiescent thyroid cells (22). TGFβ pretreatment of thyroid cells does not interfere with the TPA or forskolin induction of early genes (results not shown). These data indicate that TGFβ inhibition of thyroid progression in the cell cycle is not due to alteration in the biochemical pathways used by the TSH or by these drugs in inducing immediated early genes expression.

Since the previous results suggested that the primary mechanism by which TGFβ inhibits thyroid cell proliferation is not by modulating immediate early events, later events were considered. After 4-5 hours from TSH addition to the quiescent thyroid cells, induction of progression genes has been reported (22). To analyse whether TGFβ treatment of quiescent thyroid cells modifies the pattern of progression genes induced by TSH, northern blots experiments were performed. The level of mRNA of genes as ODC, coding for ornitine decarboxylase; 2F-1, coding for a protein with homology to a mitochondrial ADP\ATP carrier; and TS11, coding for the asparagine synthetase; were analysed in TGFβ treated and untreated cells. Northens blots, in fig. 4, show that the steady-state levels of these mRNA increase about 3-4 folds in the TGFβ treated cells after TSH stimulation. Quantitative densitometric analysis of hybridization experiments are also reported: they show slight differences in the pattern of TSH-induced progression genes expression, between TGFβ-treated and untreated cells.

Discussion

TGFβ action on rat thyroid cells involves modulation of differentiation properties, as well as modifications of cytoskeleton and extracellular matrix (10,11,23). TSH-controlled thyroid cell proliferation is also affected by TGFβ treatment.

TSH is the main factor regulating the thyroid cell cycle. TSH acts on thyroid cells through a well defined receptor, similar to the G protein coupled

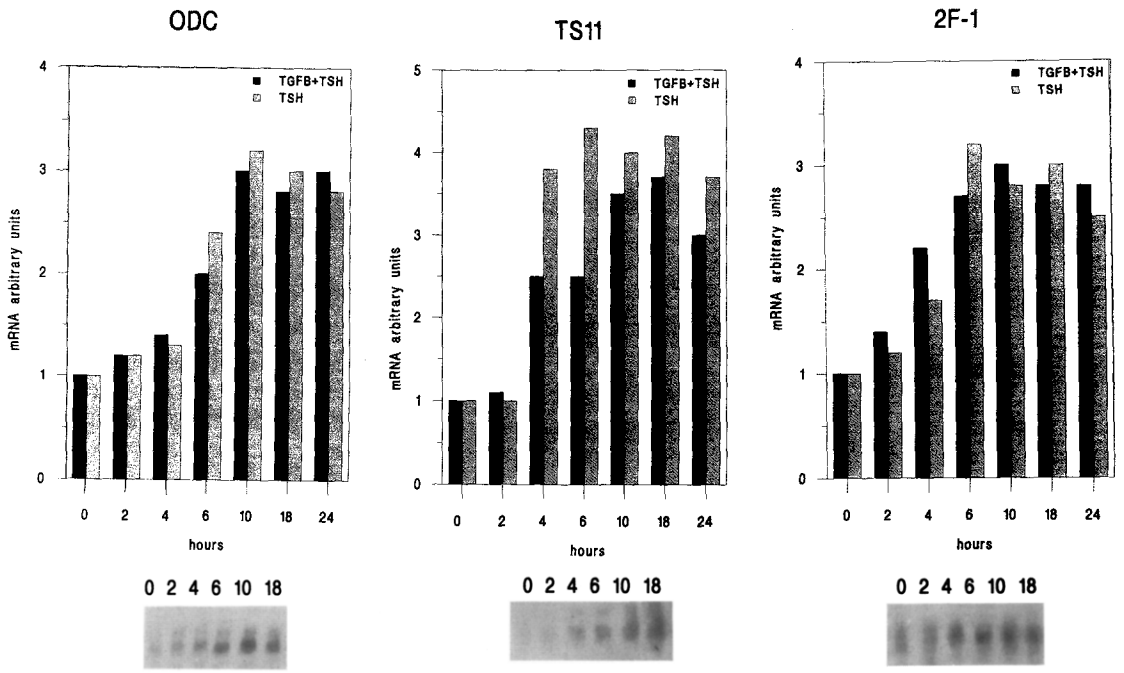

Figure 4. Progression genes expression in TGFβ-pretreated thyroid cells.
Northern blots of total RNA from TGFβ (10ng\ml) pretreated cells and then stimulated with TSH (10mU\ml) extracted at the times indicated ; the probes used for hybridization are indicated. Relative amounts of hybridized RNAs were determined by quantitative densitometric scanning of the autoradiograms, and the value expressed as the mean of more than one experiments. TSH = control TSH-treated cells, TGFB+TSH = TGFβ pretreated cells and then stimulated with TSH.

receptors (24). The action of TSH on its receptor generates a cAMP postreceptor signal that contributes to the mitogenic stimulus (25). The TSH stimulus induces quiescent thyroid cells to enter into the S phase. This event is preceeded by the induction of two sets of genes: immediate early genes, such as c-fos,Xrox20,c-jun, and progression genes, as TS11,ODC,2F-1.

In analogy with different epithelial cell systems we show here that TGFβ can inhibit the growth of thyroid cells by arresting their proliferative cycle in late G1 phase. An increase in percentage of cells in G1 is shown after 24-48 hours from TGFβ treatment of exponentially growing thyroid cells. Moreover, entry of FRTL-5 cells into S phase can be prevented by addition of TGFβ during early or middle G1, but not by adding TGFβ during the late stages of G1, i.e. after 12-14 hours from TSH stimulus. These data suggest that this factor can inhibit DNA replication by interfering with some events in the late G1 phase. In fact, early events induced by TSH as cAMP production (11) or immediate early gene expression are not modified by TGFβ treatment. The TSH mediated induction of transcriptions factors such as Xrox20 or the API complex is not affected by TGFβ, indicating that the postreceptor signals generated by this factor do not interfere with the TSH-induced pathways that bring to the expression of such genes.

Since the inhibition of cell proliferation caused by this growth factor does not alter also the TSH-mediated induction of progression genes, like ODC, that happens in the early G1, (4-5 hours after TSH addition), in this system the effects of TGFβ should involve following events in late G1, probably at the G1-S boundary. The nature of this action is still unknown at present in thyroid cells. In conclusion TGFβ is able to inhibit thyroid cell growth acting probably in late G1, but it does not interfere with the TSH-induction of cell cycle related genes: immediate early and progression genes. It remains to be demonstrated that the negative control exterted by TGFβ on thyroid cell system is mediated by the same mechanisms operating on nuclear proteins in other epithelial cells.

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