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ARTICLES

Effect of TSH in Human Thyroid Cells: Evidence for Both Mitogenic and Antimitogenic Effects

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Abstract The well-known mitogenic effects of TSH observed in vivo on the thyroid are not always reproducible on human thyroid cells in vitro where conflicting results have been obtained. In order to clarify this issue, we have used primary cultures of human thyroid cells obtained from normal tissue and maintained in serum-free medium for several days. In this in vitro model we have studied the effect of TSH on growth by measuring three different parameters: [^{3}H]-thymidine incorporation, cell counts, and DNA measurement. Monolayer cultures were plated at both low and high cell density (2×10^4 and 8×10^4 cells/25 mm well, respectively). Although at either cell density cultures were equally able to functionally respond to TSH in terms of cAMP accumulation a significant growth response to TSH was observed only in low density cultures. In high density cultures TSH had an antimitogenic effect. Moreover, TSH potentiated the mitogenic effect of insulin only in low density cultures. In contrast to TSH, FCS induced a similar proliferative response at both high and low cell density. Following TSH stimulation, cAMP content was always increased, paralleling the effect of growth in low density but not in high density cultures. The cAMP analogues dibutyryl-cAMP and 8-bromo-cAMP, as well as cholera toxin and forskolin, did not mimic the mitogenic effect of TSH but had an antiproliferative effect. In addition, these agents blunted the proliferative effect of insulin.

These data suggest that in thyroid cells TSH is able to elicit both a mitogenic and an antimitogenic effect depending on the environmental conditions such as cell density. Moreover, they confirm the existence of cAMP independent pathways for thyroid cell growth. They also provide an explanation for the equivocal effects observed in vitro on human thyroid cell growth after stimulation with TSH.

Key words: TSH, thyroid cells, cAMP, cell growth, mitogenic effect, antimitogenic effect

In vivo, thyrotropin (TSH) is the main regulator of both function and growth of the thyroid gland. In vitro, in thyroid cell cultures, other growth factors (including insulin, IGF1, and EGF) are involved in the regulation of thyrocyte growth in addition to TSH. TSH potentiates the effect of these factors (Roger and Dumont, 1984; Tramontano et al., 1986; Westermark et al., 1983; Roger et al., 1987). When TSH is used alone, however, conflicting data have been obtained on its mitogenic effect in a variety of cell culture systems. Both a mitogenic (Roger et al., 1988; Roger et al., 1983) and an antimitogenic (Westermark et al., 1979; Gartner et al., 1985) effect of TSH, as well as lack of effect on growth (Eggo et al., 1984), have been reported in primary cultures of either human and non-human thyroid cells maintained in a chemically defined medium, a mitogenic effect of TSH being usually observed when insulin is present (Roger et al., 1988; Roger et al., 1983). In a differentiated rat thyroid cell line in permanent culture ($FRTL_5$) both the presence (Dere and Rapoport, 1986; Tramontano et al., 1988) and the absence of a TSH mitogenic effect (Santisteban et al., 1987; Zakarija and McKenzie, 1989) has been reported when cells are cultured in serum-free medium and in the absence of other growth factors.

Another unsolved question is the mechanism of action of TSH in affecting thyroid cell growth; whether this effect of TSH is mediated, totally or in part, by its effect on the intracellular concentrations of cAMP, the main signalling mediator of TSH action, is also unclear. Among the variables that may affect the growth response to TSH and cAMP is cell density which is known to influence gene expression and a variety of biological responses in tissue culture systems (Beale and Schaefer, 1991; Ben-Ze'ev et

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al., 1988; Scott and Baxter, 1987; Nakamura et al., 1983; Filetti et al., 1981). This variable has not been investigated in detail in previous work.

We have studied the mitogenic action of TSH and cAMP analogues using human thyroid cells in primary cultures in serum-free conditions. The thyroid cells were obtained from normal human thyroid tissue. Moreover, we explored the possibility that cell density may also affect the thyroid cell growth response to TSH. Indeed, our data provide evidence that TSH may have both stimulatory and inhibitory growth activity on human thyroid cells, depending on the cell density. Moreover, this complex effect of TSH cannot be explained only by the cAMP production pathway suggesting, therefore, the presence of other intracellular mechanisms.

MATERIALS AND METHODS

In order to better understand the effect and the mechanism of TSH action on human thyroid cell proliferation, we first established a human thyroid cell model and then analysed the effect of various agents and hormones in this model measuring three different parameters of cell growth (i.e., [^{3}H]-thymidine incorporation, DNA content, and cell number). Primary thyroid cultures were established from normal human thyroid tissue by seeding dissociated follicles in medium containing 3% FCS. Cell monolayers obtained after 2 days of culture (Rapoport et al., 1983) were then maintained in serum free medium to avoid the interference of serum factors.

Thyroid Cell Primary Cultures

Thyroid tissue specimens (normal thyroid tissue adjacent to benign nonfunctional thyroid nodules) were obtained at surgery. The tissue was fragmented with a scalpel, suspended in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (CMF-PBS), and digested with a solution of type V collagenase (1 mg/ml) (Sigma Chemical Co., St. Louis, MO) in a 37°C shaking bath for 90 min. Large fragments were allowed to sediment and the supernatant was decanted and centrifuged (400g for 10 min). The cellular pellet, consisting mainly of fragmented and intact follicles, was suspended in culture medium, seeded in 90 mm Petri dishes, and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The following culture medium was used: Dulbecco's modification of Eagles medium (Gibco, Grand Island, NY)/Ham's F12 medium/MCDB 104 medium (2:1:1) supplemented with 2 mM glutamine, 5 μg/ml bovine insulin (Sigma Chemical Co., St. Louis, MO), 200 μU/ml bovine TSH (Thytropar, Armour, Phoenix, AZ), 5 μg/ml transferrin (Sigma Chemical Co., St. Louis, MO), antibiotics (neomycin sulfate 50 μg/ml, and amphotericin B 2.5 μg/ml), and 3% fetal calf serum (FSC, Gibco, Grand Island, NY). Under these conditions follicles formed a monolayer after 1–2 days.

To score the degree of non-epithelial cells present in the cultures, epithelial thyroid cells were identified by indirect immunofluorescence (IFL) staining with a monoclonal antibody against cytokeratin. Cell monolayers were grown on glass coverslips and stained after fixation with 50% v/v methanol/acetone for 5′ for the detection of cytoplasmic cytokeratin network. The stained cultures were mounted on buffered glycerol and inspected under a photomicroscope equipped with an epifluorescence. Cytokeratin network was stained by the mouse MoAbs LE-61 against cytokeratin 18 (kindly provided by B. Lane, ICRF, London) (Lane, 1982). The second layer was provided by fluorescein isothiocyanate conjugate (FITC) rabbit anti-mouse antibody (Dakopatts, Glostrup, Denmark). In these conditions, less than 5% cells were cytokeratin-negative, indicating that contamination with non epithelial cells was rare.

Cell Growth Assays

For growth evaluation studies primary cultures of thyroid cells were detached by treating with a trypsin/EDTA solution (Gibco, Grand Island, NY). Cells were seeded in 25 mm Falcon's multiwell plates both at low density (2×10^4 cells/well) and at high density (8×10^4 cells/well) in the same culture medium. After 48 h the cell monolayers were washed 3 times with PBS and incubated in the same culture medium in which FCS was replaced by 0.1% BSA, for 72 h. The medium was then changed and cells incubated in either the presence or the absence of stimulators for further 48 h. At the end of this period, cells were first incubated with [^{3}H]thymidine, 1 μCi/well for 6 h, and then washed once with ice-cold PBS and once with ice-cold 5% TCA for 5 min. Cells were then solubilized with 0.1 N NaOH, and the radioactivity counted in a liquid scintillation counter.

For DNA content studies, cells were seeded both at high and at low density and processed as described. The exposure to stimulators was prolonged to 6 days with a medium change on the

third day. Monolayers were then scraped in 0.5 ml of NaCl salt solution (2 M) using a rubber policeman, sonicated, and the DNA content determined by the fluorimetric method of Labarca and Paigen (Labarca and Paigen, 1980), using calf thymus DNA as a standard.

In parallel studies cells were washed twice with CMF-PBS, detached with a 0.2% EDTA solution, and a single cell suspension obtained by repeated pipetting. Cell number was counted in a haemochromocytometer.

cAMP Assay

The cellular cAMP content was measured in thyroid cells seeded in 12-well plates and cultured in serum-free medium as described above for growth studies. After 72 h in serum-free culture medium, cells were exposed to various TSH concentrations for 2 h at 37°C in the presence of 0.5 mM 3-isobutyl-1-methyl-xanthine. The reaction was terminated by medium aspiration, the addition of 400 µl cold absolute ethanol and freezing over-night at −80°C. The cells were then scraped and transferred to 1.5 ml microfuge tubes. The supernatants were dried and resuspended with 0.05 M sodium acetate, pH 6.2, and cAMP was measured using a RIA method. Results were expressed as picomoles of cAMP/10^6 cells. Each experimental point was always obtained from triplicate values.

Statistical Analysis

Statistical significance was calculated on raw data by the Anova test and by the paired Student's test when appropriate.

RESULTS

Influence of Cell Density on the Proliferative Effect of TSH in Thyroid Cultures

The effect of TSH on [^{3}H]-thymidine incorporation in thyroid cell cultures was clearly influenced by cell density. In the absence of serum, as shown in Figure 1, TSH had a stimulatory effect on cells plated at low density. TSH significantly increased [^{3}H]-thymidine incorporation at 0.1 U/L, and its effect further increased at 1 and 10 U/L. In contrast, in high-density cultures, TSH inhibited [^{3}H]-thymidine incorporation. The inhibitory effect was already seen at 0.1 U/L TSH and it increased in a dose-dependent manner. A similar TSH effect on [^{3}H]-thymidine incorporation in relation to cell density was observed in parallel experiments carried out in medium containing 1% FCS (data not shown). When thyroid cells were incubated for 6 days in the presence of TSH and cell number was determined, TSH significantly increased cell number in low density but not in high density cultures (Table I), confirming the data on cell growth obtained with [^{3}H]-thymidine incorporation experiments. In the period when cells were maintained in serum-free medium, no morphologic changes and no detachment were observed.

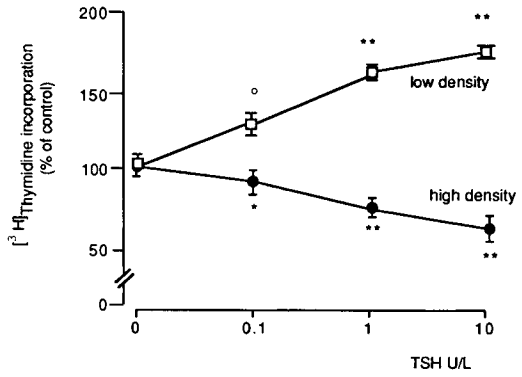

Fig. 1. Effect of TSH on [^{3}H]-thymidine incorporation in human thyroid cultures. Cells were seeded in 12 multiwell plates at low density (2×10^4 cells/well) and at high density (8×10^4 cells/well). Cells were then incubated in serum-free medium supplemented with 0.1% BSA with or without TSH for 48 h. Thymidine incorporation was determined during the last 6 h of incubation as described in Methods. Each experiment was performed in triplicate. Each point represents the mean ± SD of six experiments. $^*P < 0.05$, $^\circ P < 0.02$, $^{**}P < 0.001$.

At variance with TSH, when 1 to 10% FCS was added, the [^{3}H]-thymidine incorporation response was not affected by cell density. In the presence of 1% FCS, [^{3}H]-thymidine incorporation was 338% ± 14 vs. 327% ± 43 over control in low density and in high density cultures, respectively (mean ± SD of three experiments). These results indicate that cells plated at high density retain their proliferation potential. The basal [^{3}H]-thymidine incorporation in cells incubated in the absence of growth factors was 400 ± 53 and 1200 ± 155 (mean ± SD) cpm/well in low and high density cultures, respectively.

Effect of Cell Density on cAMP Production

Since cAMP stimulation is a major effect of TSH on thyroid cells, we assessed whether cAMP production paralleled the different [^{3}H]-thymidine incorporation induced by TSH in thyroid cell cultures at different cell density. Cells were seeded at low and at high density and stimulated with increasing TSH concentrations. cAMP production and [^{3}H]-thymidine incorporation were

TABLE I. Influence of Cell Density on the Proliferative Effect of TSH and $(bu)_2$cAMP in Human Thyroid Cells†

Treatment	Low density cultures		High density cultures	
	[^{3}H-thymidine incorporation (% of control)	Cell number (% of control)	[^{3}H]-thymidine incorporation (% of control)	Cell number (% of control)
Control	100	100	100	100
TSH, 0.1 U/L	125.0 ± 6.2*	138.5 ± 18.5*	92.0 ± 4.5	94.0 ± 13.3
TSH, 1.0 U/L	152.0 ± 3.7*	152.0 ± 15.0*	78.0 ± 4.7**	86.0 ± 12.5***
$(bu)_2$cAMP, 0.1 mM	94.0 ± 5.0	104.0 ± 12.8	68.0 ± 5.0***	86.0 ± 15.0***
$(bu)_2$cAMP, 0.5 mM	80.0 ± 6.0**	99.0 ± 2.0	52.0 ± 5.2***	80.0 ± 12.0**

†Data are expressed as mean ± SD. Data on [^{3}H]-thymidine incorporation were obtained from six experiments and data on cell counts were obtained from three experiments. Each experiment was performed in triplicate culture wells.
Significance in respect to control: $*P < 0.01$, $**P < 0.02$, $***P < 0.05$.

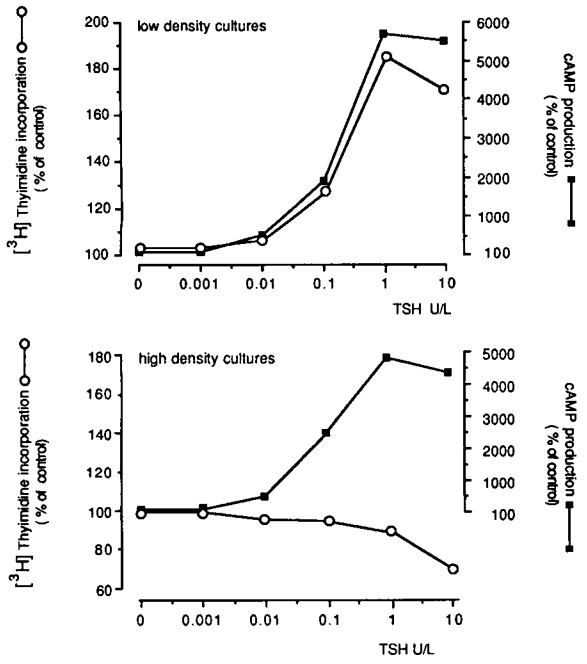

Fig. 2. Relationship of [^{3}H]-thymidine incorporation and cAMP production in human thyroid cell cultures. Cells were seeded at low density (upper panel) and at high density (lower panel). Cells were incubated with or without TSH in serum-free medium and [^{3}H]-thymidine incorporation and intracellular cAMP production determined as described in Methods. Figure shows one representative of two experiments. Basal intracellular cAMP levels were 22.0 pmol/10^6 cells vs. 16 pmol/10^6 cells in low and high density cultures, respectively.

measured in parallel experiments. Both basal concentration of intracellular cAMP (22 pmol/10^6 cells vs. 16 pmol/10^6 cells in low and high density cultures, respectively) and cAMP increment after TSH stimulation were similar in both conditions whereas [^{3}H]-thymidine incorporation followed the pattern already described (Fig. 2). Therefore, no direct relationship between TSH effect on cellular cAMP levels and [^{3}H]-thymidine incorporation was observed in high density cultures.

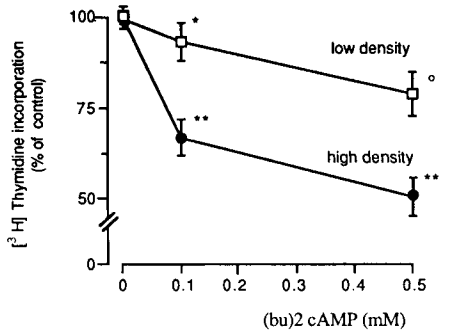

Fig. 3. Effect of $(bu)_2$cAMP on [^{3}H]-thymidine incorporation in human thyroid cell cultures seeded at low and at high density, 2×10^4 cells/well and 8×10^4 cells/well, respectively. Each experiment was performed in triplicate. Each point represents the mean ± SD of six experiments. $*P < 0.05$, $°P < 0.02$, $**P < 0.001$.

Growth Effect of cAMP Analogues, Cholera Toxin, and Forskolin

To further study the mechanism of TSH effect on thyroid cell growth, we examined whether cAMP can mimic this effect of TSH. Two cAMP analogues which cross the cell membrane, dibutyryl cAMP [$(bu)_2$cAMP] and 8-bromo cAMP, were used and, in addition, thyroid cell cAMP production was stimulated by cholera toxin or forskolin.

In contrast to TSH, $(bu)_2$cAMP inhibited [^{3}H]-thymidine incorporation in a dose-dependent manner, in both low density and high density thyroid cell cultures, even if its effect was clearly more marked in high density cultures (Fig. 3). The preincubation with 1% FCS or the presence

of 1% FCS in the culture medium throughout all the experiment, did not affect the $(bu)_2$cAMP inhibitory effect on thyroid cell growth (data not shown). This growth response to $(bu)_2$cAMP, at both low and high density conditions, was confirmed when cells were cultured for 6 days and cell number was counted (Table I).

Results similar to those obtained with $(bu)_2$cAMP were observed also with 8-bromo cAMP, cholera toxin and forskolin. A significant inhibition of the [^{3}H]-thymidine incorporation was seen at 1 mM 8-bromo cAMP, 10 μM forskolin, and 10 μM cholera toxin (Fig. 4).

TSH Potentiation of the Mitogenic Effect of Insulin

As already reported (Roger and Dumont, 1984; Bachrach et al., 1985), other factors, including EGF (0.1–10 nM), TPA (0.1–10 nM), and insulin, were also mitogenic for human thyroid cells in our system. In particular, insulin stimulated [^{3}H]-thymidine incorporation in a dose-dependent manner with a maximal stimulation of +240% ± 18.0 at 10 nM (Fig. 5). In low density cultures, TSH plus insulin increased [^{3}H]-thymidine incorporation significantly more than either TSH or insulin alone (Fig. 5). This phenomenon was confirmed when cell growth was measured in terms of cell number or DNA content increase (Table II). By contrast, the addition of 0.1 mM $(bu)_2$cAMP plus 10 nM insulin resulted in a blunted proliferative response in comparison to that elicited by 10 nM insulin alone (Table II).

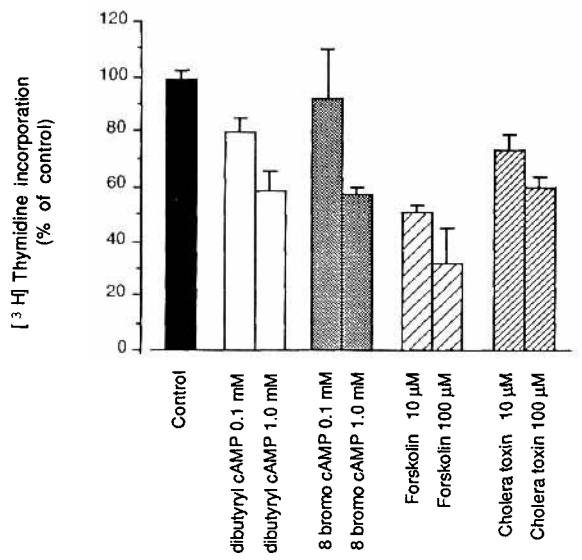

Fig. 4. Effect of cAMP analogues (dibutyryl cAMP, 8 bromo cAMP), forskolin, and cholera toxin on [^{3}H]-thymidine incorporation in human thyroid cell cultures. Each bar represents the mean ± SD of four experiments, each performed in triplicate culture wells. Pooled data from low and high density cultures.

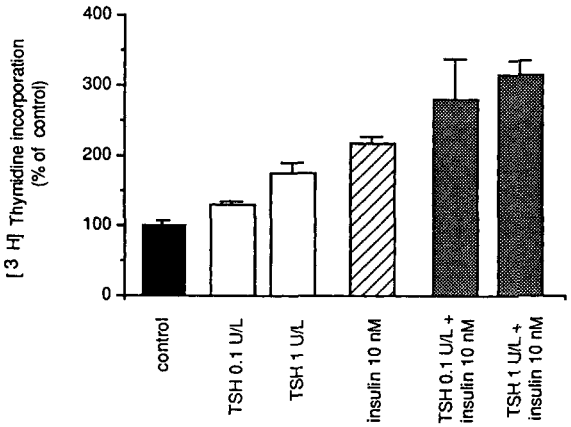

Fig. 5. Potentiation effect of TSH plus insulin on [^{3}H]-thymidine incorporation in human thyroid cell cultures seeded at low density. Each bar represents the mean ± SD of three experiments, each performed in triplicate culture wells.

DISCUSSION

Pituitary TSH not only directly stimulates thyroid function but also exerts a trophic effect on the gland as indicated by both its goitrogenic action and its ability to stimulate thyroid cancer proliferation (Clark, 1981). However, the mechanism by which TSH elicits its trophic effect in vivo is unclear. To address this issue, a variety of thyroid cell cultures has been used, but results are still conflicting. These conflicting results have been explained, at least in part, by the use of non-human vs. human thyroid cells (with the possibility that species specificity may affect the growth response to TSH). The use of abnormal thyroid tissues or the different procedures used to assess cell growth have been reported as possible variables affecting the results. Also, the use of a non completely defined medium in terms of growth factors (i.e., FCS) may have affected the results obtained. Finally, cell density is known to affect a number of biological responses including enzyme mRNA transcription (Beale et al., 1991), cytoskeletal gene expression (Ben-Ze'ev et al., 1988), growth factor receptors (Scott and Baxter, 1987), and response to growth factors (Nakamura et al., 1983).

In the present study we established human thyroid cell cultures from non-pathological thyroid tissue. Cells were allowed to form monolayers and then maintained for several days in serum-free medium to avoid the interference of

TABLE II. Proliferative Effect of TSH or Insulin Alone and of TSH Plus Insulin in Human Thyroid Cells Cultured in Serum-Free Medium and Seeded at Low Density*

Treatment	[^{3}H]-thymidine incorporation (c.p.m.)	DNA (μg/well)	Cell number ($\times 10^4$)
Control	54.7 ± 69.5	0.8 ± 0.01	7.0 ± 0.7
TSH, 1.0 U/L	962.0 ± 76.5	1.7 ± 0.05	13.5 ± 0.5
Insulin, 10 nM	1,186.0 ± 60.0	1.9 ± 0.2	19.2 ± 1.3
$(bu)_2$cAMP, 0.1 mM	498.7 ± 72.8	0.74 ± 0.01	7.2 ± 0.9
TSH, 1.0 U/L + insulin, 10 nM	1,723.0 ± 109.0	2.2 ± 0.03	24.8 ± 1.5
$(bu)_2$cAMP, 0.1 mM + insulin, 10 nM	602.0 ± 71.8	0.9 ± 0.01	8.1 ± 0.7

*Data are expressed as mean ± SD. Data on [^{3}H]-thymidine incorporation were obtained from six experiments and data on cell counts were obtained from three experiments. Each experiment was performed in triplicate culture wells.

serum factors. Growth responses were assessed by three different parameters of cell growth (i.e., [^{3}H]-thymidine incorporation, DNA content, and cell number). In this model system we investigated the effect of cell density on the growth response to TSH and cAMP analogues.

In this human thyroid cell system TSH, in the absence of other growth factors and FCS, had different effects on cell proliferation depending on cell density. In low density monolayer cultures TSH had a stimulatory effect on cell proliferation and this effect was potentiated by the presence of insulin. On the contrary, in high density cultures TSH had an anti-proliferative effect. These high density cultures, however, were fully responsive to the mitogenic effect of FCS.

In human thyroid cells, Kraiem et al. (1990) have recently observed that TSH has a mitogenic effect during the first 3 days of culture and then has an antimitogenic effect, a finding consistent with our observation of a change of TSH effect depending on cell density. In their study cAMP analogues were also mitogenic during the first 3 days after plating, when the cells were supposed to be at low density, and antimitogenic by day 4. In our model system cAMP analogues were antimitogenic also in low density cultures, although less markedly than in high density cultures. We studied, however, cultures at day 7 after plating. It is likely, therefore, that both cell density and culture stage are independent factors in modulating the effect of cAMP analogues on thyroid cell growth. Furthermore, in Kraiem's study, thyroid cells were cultured in the presence of serum. In our in vitro system, a variety of polypeptide growth factors are able to have, by themselves, a mitogenic effect on human thyroid cells. They can, therefore, variably interfere with the TSH effects. Insulin, for instance, significantly potentiated TSH action on cell growth. These observations support the possibility that studies on TSH action on thyroid cell growth carried out in the presence of FCS may be misinterpreted due to the interference (either positive or negative) of these factors. The present study demonstrates that TSH has different effects on cell growth also in the absence of other interfering growth factors.

In view of these results, some of the discrepancies present in the literature may, therefore, be explained by the different plating cell densities as well as by the different timing in exposing the cultures to TSH.

The molecular pathway through which TSH can differently affect thyroid cell growth depending on cell density is not known. cAMP production is regarded as the main intracellular signalling pathway of TSH response. cAMP may, by itself, induce both mitogenic and antimitogenic effects in dependence of cell type and cell cycle stage (Smets and Van Rooy, 1987). It is, however, unlikely that in our system these TSH effects are mediated by cAMP alone. This conclusion is supported by three independent lines of evidence. First, in high density cultures, TSH stimulated cAMP production but, at the same time, inhibited thyroid cell proliferation. It is noteworthy, at this regard, that high cell density causes an escape from desensitization of the thyroid cell cAMP response to TSH stimulation, thereby causing higher cAMP levels (Filetti et al., 1981). Second, when thyroid cultures were exposed to cAMP analogues or to agents able to stimulate cAMP production like cholera toxin or forskolin, a negative growth response was always observed. Finally, in our system, $(bu)_2$cAMP, forskolin, and coleratoxin clearly blunted the growth response to insulin exposure, whereas TSH potentiated the mitogenic

effect of insulin. Taken together, these data suggest that the growth effect of TSH cannot be explained only on the basis of intracellular cAMP accumulation. Similar conclusions have been reached in the $FRTL_5$ rat thyroid cell line where both cAMP-dependent (Tramontano et al., 1988; Jin et al., 1986) and cAMP-independent (Damante et al., 1990) pathways appear to be involved in TSH mediated growth. Similarly, in a human thyroid cell line immortalized by fusion with myeloma cells, TSH induces growth without affecting cAMP production (Karsenty et al., 1988). One possible explanation for these contrasting growth effects of TSH comes from the observation that TSH induces TGFβ like activity in the rat thyroid cell line FRTL5. This effect increases with the dose of TSH and the cell density (Morris et al., 1988). High density cultures may have, therefore, increased levels of TGFβ and/or other factors with a growth inhibiting activity (Morris et al., 1988; Grubeck-Loebenstein et al., 1989) in response to TSH. Interestingly, TGF-β does not affect the TSH induced cAMP accumulation in thyroid cells while inhibiting TSH mitogenic effect (Colletta et al., 1989).

TSH, therefore, may have a stimulatory effect on both thyroid cell function and growth but, depending on the cell environment conditions, may also have a dissociated effect, stimulating thyroid function but not thyroid growth. This observation is consistent with previous work showing that reconstituted thyroid follicles, obtained from TSH treated rats, fail to respond to TSH in terms of proliferation while maintaining the ability to organify iodine (Stringer et al., 1985) and that a selective loss of the mitogenic effect of TSH is observed in rat thyroid cells after prolonged TSH stimulation (Smith et al., 1987).

In conclusion, our study indicates that TSH must be added to the list of multifunctional agents (Sporn et al., 1988) having both stimulatory and inhibitory effects on cell proliferation as well as effects unrelated to cell growth. The TSH effect on growth is modulated by the cell density, the presence of other signal molecules like growth factors and, possibly, other less known factors. The intracellular cAMP production is not necessarily followed by thyroid cell proliferation, the effect of TSH on cAMP and growth being dissociated under specific conditions.

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