Role of non-TSH factors in thyroid cell growth

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Abstract. The effects of insulin, the tumour promotor
tetradecanoyl phorbol acetate (TPA), TSH and combina-
tions of these factors on growth and DNA synthesis
have been examined in the FRTL-5 cell strain and in
sheep thyroid cells. In addition the regulation of the
production by sheep thyroid cells of the insulin-like
growth factors (IGF) by TSH and their possible auto-
crine roles have been investigated. We found that
insulin and the IGF's stimulated DNA synthesis in both
rat FRTL-5 cells and sheep cells. TPA also stimulated
growth in both cell types, and its effects were additive to
those of insulin. In the FRTL-5 cells, TPA was a less
potent stimulator of growth than TSH, but the effects
of TPA and TSH were not additive which may imply
growth stimulation through a common pathway. In
sheep cells TSH was not mitogenic and did not appear
to activate protein kinase C, the receptor for TPA.
Sheep cells, unlike FRTL-5 cells, were found to pro-
duce IGF-I and IGF-II, and their syntheses were regu-
lated by TSH. Sheep cells were also found to produce
IGF-binding proteins which may modulate the biologic
effects of the IGF's. Sheep thyroid IGF binding pro-
teins were found to copurify with urokinase-like plas-
minogen activator on immunoaffinity chromatography.
The production of this serine protease has also been
shown to be regulated by TSH.

In vivo thyroid stimulating hormone (TSH) in-
fluences many aspects of thyroid follicular cell
metabolism and ultimately, the production of
the thyroid hormones. Elevated levels of TSH are also
held to be responsible in part for thyroid growth
(goitre) in cases of iodide deficiency. However,
the putative growth stimulating effects of TSH in
vivo are not always apparent, goitre being seen in
patients with serum TSH levels within the normal
range. The heterogeneous clinical data involving
enlarged thyroid glands would suggest that other
effectors may influence thyroid growth (see Stu-
der (1985) for review). In order to examine fac-
tors capable of stimulating thyroid cell growth we
have developed a primary thyroid cell culture
system. We have cultured the follicular cells of
thyroids of several species including rat, beef, pig,
sheep (Eggo et al. 1984) and humans (Errick et al.
1986). Of these, we have found that the system
best able to maintain all aspects of thyroid differen-
tiated function is the sheep. Cells maintain the
ability to trap iodide, organify iodide on thyroglobu-
lin and to release the thyroid hormones T₃ and
T₄. The amounts of thyroid hormones produced
per day per cell are comparable to those produced
in vivo. Synthesis of the hormones is subject to the
same controls as in vivo e.g. excess iodide results
in inhibition of thyroglobulin iodination and thy-
roid hormone release (Becks et al. 1986).

The ability to synthesize and secrete thyroid
hormones sets this culture system apart from rat
cell culture systems including the FRTL-5 cell
strain (Grollman et al. 1986). Similarly the pri-
mary dog thyroid cell culture system traps iodide
but rapidly loses the ability to organify (Magnus-
son & Rapoport 1985). More success in maintain-
ing full differentiated thyroid function has been
obtained with primary cultures of pig thyroid
follicles, and results obtained with this system,
where studied, are in a large part comparable to
those obtained with sheep (Gärner et al. 1985).
Human thyroid cells, while maintaining the important differentiated function of thyroglobulin synthesis, do not synthesize thyroid hormones de novo (Errick et al. 1986).

Although we have shown that TSH mediates many aspects of the differentiated function of sheep thyroid cells including follicle formation (Mak et al. 1986a), protein glycosylation (Eggo & Burrow 1982), thyroglobulin synthesis (Errick et al. 1985), iodide uptake and organization (Eggo et al. 1984) and thyroid hormone synthesis (Becks et al. 1986), we have not found TSH to be a growth factor. We have assayed for TSH effects on growth by cell counting, DNA content and thymidine uptake. Although TSH in some experiments produced a slight increase in cell numbers, in other experiments a decrease was found. Short term assays measuring labelled thymidine incorporation into nucleic acid usually showed inhibition of incorporation. This absence of trophic effect of TSH is also seen in human (Errick et al. 1986) and pig thyroid (Gärtnert et al. 1985) cell culture systems.

We have examined the effects of other known growth factors on labelled thymidine incorporation into DNA in thyroid cells. This parameter was chosen as an assay for growth in our system for the following reason. Growth curves, while providing much data in cell lines, are not as suitable for studies with primary cultures. Primary cultures contain mixed populations of cells and selecting under pressure for growth may isolate a cell type atypical of the differentiated cell. We prefer to use cells entering the plateau phase when the culture has become confluent. In this condition the synthesis of specialized rather than structural proteins is thought to be favoured. Although cell growth is reduced under these conditions, proliferation at a reduced rate will continue well beyond confluence resulting in multilayers of cells (Freshney 1983). Therefore, our contention is that we have highly differentiated cells that are in a uniform environment with respect to cell:cell contact and that incorporation of labelled thymidine into DNA is consistent with a commitment to growth. Our reservations regarding this and any other culture system to measure thyroid cell growth, lie in the extrapolation from the cell culture dish with its artificial constraints to the situation occurring in vivo.

We have examined the effects of insulin, an obligatory hormone for most serum-free culture media, putative stimulators of protein kinase C, and the role of the endogenously produced insulin-like growth factors (IGF's) and their binding proteins. Insulin promotes the uptake of glucose and amino acids and may in part owe its mitogenic effects of this property (Freshney 1983). The insulin-like growth factors, as their name implies, can influence these parameters although at lower potencies. However, IGF's have also been shown to be important in enhancement of cell differentiation and are thought to be the mediators of the growth stimulating effects of growth hormone. Although present in $2000-3000 \times$ the levels of insulin in serum, IGF's are found complexed to carrier proteins which may inhibit access to membrane-bound receptors (see Zapf et al. 1984 for review).

Epidermal growth factor (EGF) has been shown to stimulate growth in many cell types including the thyroid (Eggo et al. 1984). Although the structural domains of the receptor have been elegantly mapped, the mechanisms of action of EGF after receptor binding are not clear. Whether EGF activates protein kinase C in the thyroid remains unknown. One stimulator of this kinase is the tumour promoting phorbol ester, tetradecanoyl phorbol acetate (TPA) (Nishizuka 1984). We (Bachrach et al. 1985) and others (Roger et al. 1985) have shown that TPA stimulates thyroid cell growth. However, its interaction with other growth factors is not known.

Materials and Methods

Submaxillary gland EGF was a generous gift of Dr J. E. Kudlow, Department of Medicine, University of Toronto. Human IGF-I and II, purified by high performance liquid chromatography, were generous gifts of Drs Bala and Bhaumick, Department of Medicine, University of Saskatchewan, Saskatoon, Canada. Synthetic human growth hormone (hGH) was from Genentech. Other hormones and enzymes were obtained from the Sigma Chemical Company.

Sheep thyroid follicles were isolated as described previously (Eggo & Burrow 1983; Eggo et al. 1984). FRTL-5 cells were a generous gift from Dr L. Kohn (NIH, Bethesda, MD) in 1983 and were used as previously described. Cell counting was performed using a Coulter counter. Iodide and $99m$ pertechnetate uptake and iodide organization studies were performed as previously described (Eggo et al. 1986) in the presence of $10^{-7}$ M carrier Na.
Results

Effects of components of the culture medium
Table 1 shows the effects of some of the components of the serum-free culture medium on sheep thyroid cell DNA synthesis. TSH alone or in combination with 5S did not stimulate \(^{3}\)HdT incorporation into DNA. In fact the incorporation by cells cultured in 5S + TSH was considerably lower than that in 5S alone. As can be seen from Table 1, insulin (10⁻⁶ M) was responsible in some part for the greatly increased uptake of 5S treated cells. However, 5S cells showed much higher incorporation than cells incubated with insulin alone implying that other components of 5S e.g. transferrin, thought to be essential for iron uptake, are important. These data emphasize the difficulty in ascertaining whether a factor stimulates growth per se or maintains health.

In FRTL-5 cells, for whom this medium was optimized, previous data from our laboratory have shown similar cooperative effects (Eggo et al. 1985) of these additives. However, for these cells TSH or other stimulators of cyclic AMP are potent stimulators of DNA synthesis (Eggo et al. 1986). These effects of TSH and stimulators of cyclic AMP are greatly augmented by insulin and more so by 5S.

| Table 1. Effect of medium components on \(^{3}\)H]thymidine incorporation in sheep cells. |
|---------------------------------|-----------------|-----------------|
|                                | Methyl \(^{3}\)H]thymidine incorporation into TCA precipitable material dpm x 10⁻³ |
|                                | 0-24 h           | 24-48 h         |
| Control                        | 13.14 ± 1.84     | 11.57 ± 1.67    |
| TSH 1 µU/ml                    | 11.75 ± 1.51     | 9.4 ± 1.80      |
| Insulin 10⁻⁶ M                 | 28.33 ± 2.98     | 25.44 ± 6.25    |
| 5S                             | 61.82 ± 6.75     | 46.62 ± 1.48    |
| 5S + 1 µU/ml TSH               | 34.21 ± 9.09     | 41.68 ± 10.47   |

The serum-free culture medium contains insulin 10 µg/ml, transferrin 5 µg/ml hydrocortisone 10⁻⁸ M, somastatin 10 ng/ml and glycyxl histidyl lysine 10 ng/ml and is referred to as 5S in the Table.

After 3 days incubation in 5S + 0.5% serum cells were washed with HBSS and the additions as noted, added. \(^{3}\)H]dT-labelling and incorporation into DNA were as noted in methods.

Effect of the tumour promoting phorbol ester, TPA

The effects of TPA, on FRTL-5 thyroid cell growth are shown in Fig. 1. TPA 10⁻⁷ M, stimu-

EFFECT OF 10⁻⁷ M TPA AND 10⁻⁹ M TSH ON CELL NUMBER IN FRTL5 CELLS AFTER 7 DAY INCUBATION

![Graph showing cell numbers](image)

Fig. 1.

FRTL-5 cells were incubated in control medium which is 5S + 5% bovine calf serum, in control medium + 10⁻⁷ M TPA or in control medium + 1 µU/ml TSH or a combination of TPA and TSH. Cells were counted in a Coulter counter 7 days later.
Effect of TPA on \(^{3}\text{HdT}\)-incorporation into DNA in FRTL-5 cells cultured in basal, 5S + serum and 5S + TSH + serum medium.

<table>
<thead>
<tr>
<th></th>
<th>dpm × 10(^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) TPA</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>(+) TPA</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Basal</td>
<td></td>
</tr>
<tr>
<td>5S + serum</td>
<td>2.4 ± 1.2</td>
</tr>
<tr>
<td>5S + serum + TSH</td>
<td>41.3 ± 1.4</td>
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</tbody>
</table>

Basal and 5S + serum cells were incubated in 5S + serum for 13 days prior to the start of the experiments. 6S cells were incubated in 6S + serum for 72 h prior to the start of the experiment. The cell layer was washed and medium changed to the conditions as noted. Incubation in \(^{3}\text{HdT}\) continued for 54 h.

The FRTL-5 growth measured by cell number and labelled thymidine incorporation into DNA was decreased by TPA (Table 2). Subsequent experiments using a new preparation of TPA showed 10\(^{-7}\) M to be toxic particularly in the absence of serum. We are currently using 3 × 10\(^{-8}\) M TPA to examine effects on growth in FRTL-5 cells. The effects of TPA were seen in the presence of high concentrations of insulin implying that a separate pathway had been activated by TPA. Stimulation of growth by TPA did not approach the increases mediated by TSH alone or 5S + TSH. TPA is known to activate protein kinase C, a phospholipid dependent, calcium activated enzyme phosphorylating different substrates than the cyclic AMP-dependent kinases (Nishizuka 1984). In contrast to the results obtained with insulin we did not find the effects of TSH and TPA to be additive (Table 2).

Table 2.

<table>
<thead>
<tr>
<th></th>
<th>dpm × 10(^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) TPA</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>(+) TPA</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Basal</td>
<td></td>
</tr>
<tr>
<td>5S + serum</td>
<td>2.4 ± 1.2</td>
</tr>
<tr>
<td>5S + serum + TSH</td>
<td>41.3 ± 1.4</td>
</tr>
</tbody>
</table>

Table 3.

Effect of TPA on \(^{3}\text{HdT}\)-incorporation into sheep thyroid cells cultured in 4S, 4S + insulin and 4S + insulin + TSH medium for 24 h.

<table>
<thead>
<tr>
<th></th>
<th>dpm</th>
<th>dpm × 10(^{-3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>4S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5S</td>
<td>207</td>
<td>8.5 ± 2.5</td>
</tr>
<tr>
<td>6S</td>
<td>787</td>
<td>19.6 ± 1.9</td>
</tr>
<tr>
<td>3 × 10(^{-8}) M TPA</td>
<td>5.7 ± 2.6</td>
<td></td>
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</tbody>
</table>

Cells were incubated in medium containing 5S or 5S(-) insulin or 5S(+ TSH for 3 days. Cells were challenged for 24 h with 3 × 10\(^{-8}\) M TPA and \(^{3}\text{HdT}\).

Table 4.

<table>
<thead>
<tr>
<th>Specific counts bound × 10(^{-3})</th>
<th>(-) TSH</th>
<th>(+) TSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simultaneous addition</td>
<td>10.3</td>
<td>9.7</td>
</tr>
<tr>
<td>3 h pre-incubation</td>
<td>29.4</td>
<td>30.4</td>
</tr>
<tr>
<td>24 h pre-incubation</td>
<td>32.3</td>
<td>31.0</td>
</tr>
<tr>
<td>Isolated membranes from 48 h pre-incubated cells</td>
<td>7.5</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Table 4.

Effect of TSH \(^{[125]}\text{I}\)EGF binding to sheep thyroid cells and membranes.

TSH, 20 mU/ml was added to cells simultaneously with \(^{[125]}\text{I}\)EGF or 3 h or 24 h (0.5 mU/ml) prior to its addition. Membranes were isolated from cells incubated in 0.5 mU/ml TSH for 48 h. Binding studies were performed as described previously (Bachrach et al. 1985). Subtraction of nonspecific binding has been performed.
Table 5. Effect of TSH on IGF production by sheep thyroid cells.

<table>
<thead>
<tr>
<th></th>
<th>ng per ml per day</th>
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<tbody>
<tr>
<td>IGF-I</td>
<td>0.6</td>
</tr>
<tr>
<td>IGF-II</td>
<td>49.0</td>
</tr>
<tr>
<td>Control</td>
<td>1.4</td>
</tr>
<tr>
<td>100 µU per ml TSH</td>
<td>67.0</td>
</tr>
</tbody>
</table>

Control cells were incubated in 5S medium. 100 µU/ml TSH was added 3 days prior to medium collection. IGF-I was measured by radioimmunoassay and IGF-II by radioreceptor assay (Mak et al. 1986b).

receptor (Fern & King 1985) and we have found that TPA and to a lesser extent synthetic diacylglycerols significantly inhibit [125I]EGF binding in thyroid cells (Eggo & Bachrach 1986) we conclude that TSH does not act through this pathway in sheep cells. Similarly that the differentiating effects of TPA antagonize the differentiating effects of TSH is hard to reconcile with the hypothesis that TSH activates this kinase in sheep cells.

Insulin-like growth factor effects

We have recently shown that both IGF-I and IGF-II are produced by the primary cultures of sheep thyroid cells (Mak et al. 1986b). The regulation of the production of both by TSH is shown in Table 5. Treatment with 100 µU/ml TSH increased the production of IGF-I and IGF-II implying that the principal producers of these factors are the TSH-responsive thyroid follicular cells. The demonstration of TSH-dependence is important since fibroblasts, which are a potential contaminant of any primary cell culture, also produce IGF's. FRTL-5 cells did not produce measurable quantities of IGF's.

The binding proteins of the IGF's which can modulate their biological function were also produced by the thyroid follicular cells (Mak et al. 1986c). Secretion of binding proteins was indirectly shown by data from the radioreceptor assay for IGF-II performed by Drs Bala and Bhaumick. This assay measures not only the hormone but also proteins capable to binding to IGF's. Since we found IGF-II activity in acid extracted proteins of molecular weight too high to be IGF-II or its precursors, we deduced that binding proteins were present and contributing up to 90% of the assayable IGF-II activity. Binding protein activity was found to copurify with urokinase-like plasminogen activator (uPA) on immunoaffinity chromatography using antibodies to uPA coupled to sepharose. Since we have previously shown PA activity to be regulated by TSH (Mak et al. 1984), we conclude that syntheses of both the IGF's and their binding proteins are regulated by TSH.

We have also found that both sheep and FRTL-5 thyroid cells have receptors for IGF's shown by their ability to bind radioiodinated derivatives of the hormone and also by their growth response (see Table 6). In this experiment sheep thyroid cells were incubated in basal medium alone without addition of 5S or serum for 3 days prior to challenge. Both IGF-I and IGF-II produced several fold increases over control. The effects of insulin and IGF's were not additive. Also shown in Table 6 are the effects of exogenously added IGF's on thyroid iodine uptake. Unlike the results with TPA or EGF, iodine metabolism was unaffected by IGF's in high concentration.

Effect of growth hormone

Since IGF production is partially regulated by GH, we examined the effects of synthetic hGH on labelled thymidine incorporation into DNA in sheep thyroid cells. Labelled thymidine incorporation increased in a concentration dependent manner with growth hormone, a 54% increase being seen at 10 µg. Although this is a supraphysiologic concentration (Zapf et al. 1984) this may reflect the instability of the stored peptide rather than the real biologic potency. Whether

Table 6. Autocrine roles of IGF-I and IGF-II. Effects on 3HdT-incorporation and [125I]uptake.

<table>
<thead>
<tr>
<th></th>
<th>3HdT %</th>
<th>[125I] cpm × 10^{-3}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100 ± 15.0</td>
<td>25.9 ± 3.5</td>
</tr>
<tr>
<td>IGF-I</td>
<td>278 ± 22.7</td>
<td>20.9 ± 3.1</td>
</tr>
<tr>
<td>IGF-II</td>
<td>233 ± 15.6</td>
<td>25.0 ± 1.5</td>
</tr>
<tr>
<td>EGF</td>
<td>N.D.</td>
<td>5.5 ± 1.8</td>
</tr>
</tbody>
</table>

For 3HdT-incorporation studies, cells were incubated for 2 days in basal medium. Cells were washed to remove endogenously produced IGF's, and incubated with 10 ng/ml IGF-I or 10 ng/ml IGF-II and 1 µCi/ml 3HdT for 24 h. For [125I]uptake studies cells were cultured in 5S medium for 3 days with IGF's.
this is a direct of hGH on sheep thyroid cells or an indirect effect on IGF production is not clear, but the effects were seen within 24 h of growth hormone addition which would implicate the former mechanism. hGH did not stimulate labelled thymidine uptake in FRTL-5 cells.

Conclusions

Regulation of thyroid cell growth appears to be a multifaceted phenomenon. Not only are there several effectors, as shown here, but also each species appears to have evolved unique interconnections between the pathways to effect growth. In sheep and human primary thyroid cell cultures TSH does not directly stimulate growth measured either by cell counting or labelled thymidine incorporation. However, the discovery of the production of IGF's by sheep thyroid cells and the regulation of their production by TSH raises the possibility of an indirect pathway of TSH growth stimulation. We have shown that receptors for these hormone do exist on thyroid cells and that these cells are able to respond with increased growth to IGF's. In the primary sheep culture system we have found IGF binding protein activity to be regulated by TSH and insulin which may explain why no growth stimulating effects of TSH are seen in these cultures. In vivo it may be possible to stimulate IGF production separate from binding protein activity, and hence an autocrine role for these hormones may exist.

The effects of TPA are different from insulin and the IGF's. TPA stimulates growth over and above that stimulated by insulin and 5S and antagonizes TSH-mediated differentiated functions. What the in vivo stimulators of thyroidal protein kinase C are, is not known. However, since thyroid cells have protein kinase C the enzyme is probably a second messenger and warrants further study. Similarly the effects of EGF which stimulates thyroid cell growth at physiologic concentrations and as TPA inhibits differentiated function, are also fruitful areas of research.

Acknowledgments

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References


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