Autoimmunity and thyroid growth: 
Methods, concepts and misconceptions

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Graves' disease is characterized by hyperfunctioning and hyperplasia of the thyroid which lead respectively to thyrotoxicosis and goitre. Functional hyperactivation of the gland has been clearly related to the presence in plasma of immunoglobulins stimulating the TSH receptor and its adenylate cyclase. With regard to growth, several facts have suggested that additional mechanisms may be involved. There is no simple relationship between level of function in Graves' disease and goitre size, indeed there are cases of hyperthyroidism with little increase in thyroid size and others with slight thyrotoxicosis and large goitres. Moreover the presence of circulating growth stimulation factors in euthyroid patients with goitre has been reported. On the other hand, depending on the species, several biochemical pathways may be involved in the regulation of thyroid cell proliferation: 1) the TSH activated adenylate cyclase-cyclic AMP cascade, 2) the cyclic AMP independent epidermal growth factor mechanism probably involving protein tyrosine phosphorylation, and 3) as in other types of cells, the phosphatidylinositol-Ca\(^{2+}\) cascade. It is therefore quite conceivable that some factors may activate growth of thyroid cells without influencing the rate of hormone synthesis and secretion.

Thyroid biologists have employed experimental systems using different species and end points to demonstrate the existence of circulating thyroid growth stimulators in various thyroid pathologies. Using these tests, the results obtained so far by clinical investigators are contradictory and confusing. In this commentary we wish to show that much of the confusion stems from overinterpretation of data leading to erroneous semantics and reasoning and unwarranted extrapolation of results from one system to another. When biological mechanisms are still unknown or controversial a fundamental principle to be followed is to avoid general terminologies and to restrict oneself to operational definitions.

The first pitfall is the term growth itself as it may cover thyroid cell hypertrophy as well as thyroid tissue hyperplasia. Mere increase in macromolecules and organelles synthesis may cause the former whilst DNA replication and mitosis are also necessary for the latter. Obviously investigators are interested in tissue growth, as measured by the increased number of cells, in stimulated thyroids which itself results from increased mitogenesis and proliferation. To avoid confusion the latter two terms should be preferred. Although we wish to detect hormonal factors which stimulate growth in vivo, for practical reasons the assays used are all in vitro thyroid preparations or cell cultures.

A first source of confusion in the study of proliferation factor stems from the measurement used to quantify this variable. The induction or stimulating of proliferation resulting in an increase in cell number is, by definition, best evaluated by cell counting or DNA content. However, these reliable indices of cell proliferation have not
been frequently applied to studies of thyroid cell regulation and a fortiori even less in clinical investigation.

The frequency of nuclei labelled with tritiated thymidine, as demonstrated by autoradiography, measures the number of cells in which DNA has been synthesized de novo i.e. 'stricto sensu', in a quiescent population, the proportion of cells which have entered into the S-phase of the cell cycle. As it is known that, with a few notable exceptions, once engaged in this phase the cell will achieve mitosis, this could be a reliable index of the proportion of the cells which are undergoing one round of cell multiplication.

The frequency of mitoses, especially after treatment with a microtubule inhibitor which blocks the cells at this step, is also a direct measurement of the number of cells entering into mitosis during the blockade i.e. of the mitogenic action of a drug/factor. The length of the blockade should be short enough (a few hours) not to interfere in the pre-replicative phase but not too short or the number of mitoses counted may be low and the assay consequently insensitive. The 4 aforementioned types of measurement are therefore reliable indices of the proliferative action of a factor in a given system. Nevertheless, in the study of the biology of thyroid cell proliferation it has been judged necessary to combine several of them to demonstrate a true proliferative action.

Tritiated thymidine uptake into DNA has also been proposed as an easy and reliable index of growth. However, as has been known for many years to cell biologists, labelled nucleoside incorporation into RNA or DNA of cells reflects in addition to the rate of nucleic acid synthesis, 1) its transport, 2) its dilution in intracellular pools 3) the relative rate of catabolic versus anabolic pathway. Most trophic factors or hormones increase [3H]uridine uptake into RNA by an effect on trapping long before any effect on general RNA synthesis. Thus an increased total [3H]thymidine uptake into DNA might reflect increased transport, decreased de novo synthesis and decreased thymidine phosphate catabolism as well as increased DNA synthesis. The converse is possible, especially when cold nucleotides are used or if exogenous thymidine is rapidly degraded. Thus, while such an assay may elicit false positives, false negatives may also ensure. Such assays are used in cell biology for routine screening, but their results should be checked by unambiguous demonstration of a mitogenic response for positive effectors. The same rule could be applied in thyroid clinical investigations.

Feulgen histochemical staining of guinea pig thyroid cells after 5 h exposure to serum samples has also been used as an index of growth. The procedure stains DNA and can be used to quantify cellular DNA provided that only intact nuclei are measured. However, when used in thyroid fragments after 5 h exposure to a stimulating agent, there can be no true increase in DNA content as thyroid cells require at least 15 h to initiate new DNA synthesis. What may be being measured under such conditions is DNA accessibility i.e. an index of nuclear activation. This parameter could reflect one of the biochemical phenomena of the pre-replicative phase. However, the relationship between such measurements and mitogenesis is at best conjectural.

It has been shown that TSH activates the hexose monophosphate pathway in the thyroid of most species. The two main functions of this pathway are provide NADPH₂ reducing equivalents for biosynthesis and pentose phosphate for nucleic acid synthesis; thus it was hypothesized that this action of TSH might be involved in the growth promoting action of the hormone. However, it was clear that the mechanism of activation mainly involved an increased NADP i.e. an increased NADPH₂ oxidation. Histochemical measurement of the activity of glucose-6-phosphate dehydrogenase i.e. the first enzyme in the pentose phosphate pathway, in thyroid fragments exposed for 5 h has also been proposed as an index of thyroid cell proliferation. However, the mechanism of the stimulation by TSH of the pathway did not involve an activation of the enzyme itself. Moreover, in some species (beef, man) the effects of TSH on the pathway in intact cells were observed at concentrations much higher than those required for other hormonal effects. Finally the relationship between the activity of the pentose phosphate pathway and a fortiori glucose-6-phosphate dehydrogenase activity and mitogenesis has never been demonstrated. Whether such an activation is a required step in the pre-replicative chain of events leading to mitosis is dubious.

A second major source of confusion in the investigation of thyroid growth stimulating factors is the variety of experimental systems used to assay them. Obviously the ideal subject for such studies is the human thyroid in vivo. The use of
cell cultures involves the loss of intercellular communication especially of the possible cooperation between thyocytes, fibroblasts and endothelial cells in tissue growth. Moreover, cells in primary culture may lose receptors; cell lines, by definition have lost some of the controls of normal cells, and their regulatory circuits may be very different from those of their parental cells. Similarly a factor which is required but not limiting in vivo e.g. insulin, may be under the right conditions required and thus stimulating in vitro. Finally differences in the mechanisms of cell proliferation control should be considered:

At least three mechanisms of proliferation control should be considered:

1) TSH induces the proliferation of dog and rat thyroid cells and with the exception of one report to the contrary, this effect is mediated by cyclic AMP, even in the FRTL cell line. No such effect is observed in pig and sheep thyroid cells.

2) EGF through a pathway which involves protein tyrosine phosphorylation, but not cAMP or the phosphatidylinositol Ca<sup>2+ </sup>cascade induces the proliferation of dog, pig, ovine and bovine thyroid cells in primary cultures. FRTL cells are not activated by this pathway.

3) In other cell types, various growth factors (e.g. PDGF) induce proliferation through the activation of the phosphatidylinositol-Ca<sup>2+</sup> cascade. As TSH activates the turnover of phosphatidylinositol in most thyroid cells, it is conceivable that it may stimulate the hydrolysis of phosphatidylinositol diphosphate and induce the proliferation of thyroid cells in some species.

As the mechanisms of proliferation control in human thyroid cells are not known, the assay system or species used may lead to erroneous conclusions. For instance, let us assume that the effect of TSH on human cell proliferation involves the phosphatidylinositol and not the cAMP cascade; then agent stimulating cAMP but not proliferation in human cells, would, in a system in which proliferation is induced by cAMP, e.g. dog, rat, FRTL be classed as a growth factor. Conversely if in the human, thyroid cell proliferation was induced by TSH solely through cAMP, current assays for TSI (thyroid stimulating immunoglobulin) which measure cyclic AMP produced by human thyroid cells would be valid assays of proliferative factors acting via the TSH receptor. It is therefore obvious that with regard to experimental systems and species it would be advisable when reporting results to restrict ourselves to operational definitions (Table 1).

Table 1.
Proposed nomenclature.

<table>
<thead>
<tr>
<th>Operational definition</th>
<th>Assay</th>
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<tbody>
<tr>
<td>FRTL</td>
<td>Cell number of DNA increase in cultured FRTL cells</td>
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<tr>
<td>Proliferation stimulating factor</td>
<td>Number of mitoses in colchicine treated FRTL cells after 24 h exposure</td>
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<tr>
<td>Mitogenic factor</td>
<td>Number of cells labelled with [³H]thymidine after 48 h exposure</td>
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<tr>
<td>DNA synthesis stimulatory factor</td>
<td>[³H]thymidine uptake in DNA of FRTL cells after 24 to 48 h exposure</td>
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<tr>
<td>Thymidine uptake stimulating factor</td>
<td>Feulgen staining in fragments of guinea pig thyroids after 5 h exposure</td>
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<tr>
<td>Guinea pig</td>
<td>Staining of glucose-6-phosphate dehydrogenase in guinea thyroid fragments after 5 h exposure</td>
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<tr>
<td>Thyroid Feulgen stimulating factor</td>
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<td>Thyroid glucose-6-phosphate dehydrogenase</td>
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