Physiological and Pathological Regulation of Thyroid Cell Proliferation and Differentiation by Thyrotropin and Other Factors

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I. CELL PROLIFERATION IN NORMAL THYROID TISSUE IN VIVO
   A. Physiological Situation

   The thyroid tissue is mainly composed of thyroid follicular cells, the thyrocytes (70%), arranged in follicles and of their supporting mesenchymal tissue and cells, the endothelial cells of the capillaries (20%) and the fibroblasts (10%) (78). Scarce calcitonin-secreting parafollicular cells are located at the periphery of the follicles. After its differentiation in the fetus, the tissue grows roughly in parallel with body weight and remains at the same size throughout adult life. As the fetal thy-
which should be fully reversible (364). The desensitization of the thyroid cell secretates thyroid hormones that inhibit the secretion by pituitary thyrotrophs of thyrotropin (TSH), the thyroid-stimulating hormone. Whenever thyroid hormone secretion decreases, as in iodine metabolism defects, iodine deficiency, or after goitrogen or antithyroid drug administration, TSH secretion increases, causing an activation of thyroid function and growth (77, 82, 88, 177).

B. Growth Under Stimulatory Conditions

In vivo growth, as induced by goitrogen administration in rats, is followed by a progressive increase in thyroid weight, which plateaus after 3 mo (at \(\times 12\) the original volume). In terms of relative components of the tissue, it first involves a fall in follicular lumen space and nonvascular stroma and a rise in epithelial cells and blood vessel space, with no further changes after 7–10 days (366). The proliferation of epithelial cells precedes that of the thyrocytes (201), and capillary vascularization varies more than follicular cell mass with the level of thyroid stimulation (140, 299, 357). Global increase in epithelial cell space is due to cell hypertrophy and cell multiplication; it is accompanied by folliculogenesis (69, 70). The thyroid capsule also grows (356).

The fact that growth stops after 3 mo despite persistent high levels of TSH suggests an inherent limitation in the cells or, more simply, a desensitization of the cells to the effect of TSH (246, 258, 364, 365). Mitogenic effect of wounding in such a tissue shows that the desensitization is TSH specific (362, 367). As the mitotic rate at the plateau level remains elevated, the cell turnover itself remains higher than in control tissue. However, if goitrogen is withdrawn from the diet for 3 wk, bringing back TSH levels to normal, the mitotic rate returns to normal, and follicular cell number slightly decreases. Restimulation by goitrogens brings back all parameters to their values before withdrawal. This does not fit in with a simple TSH desensitization mechanism, which should be fully reversible (364). The desensitization is specific to TSH, since the in vitro effect of insulin at high concentrations but not of TSH persists (302). Other effects of TSH are not desensitized, which shows that the desensitization affects only the growth-promoting action of TSH (308, 365). Indeed, the TSH receptor itself desensitizes little, and the expression of the gene is not downregulated by chronic stimulation (Maenhaut and Dumont, unpublished observations). Growth desensitization is observed in cultured cells from treated animals, which excludes the hypothesis of a chalone (308). Similarly, under constant stimulation after partial thyroidectomy, rat thyroids do not recover their normal size (77). This shows an inherent, albeit relative, limitation of the growth potential of the thyrocyte population as in other cells (114). It might reflect a limited life span (number of possible divisions) of the thyrocytes: in primary cell cultures no more than five to six divisions can be obtained (P. P. Roger, M. Baptist, and J. E. Dumont, unpublished observations). Also in thyroidectomized rats, \(2 \times 10^3\) incubated thyrocytes cannot restore normal function, whereas \(6 \times 10^4\) can (76). However, there are several arguments against this “very limited life span” hypothesis. In the constantly stimulated rat thyroid, although the weight and presumably the total number of cells do not increase further after 3 mo, the labeling index, although lower than at peak growth, is still elevated, which rather suggests a dynamic equilibrium, i.e., continuous stimulated cell proliferation compensated by increased cell death rate (366). Similarly, in dogs chronically stimulated by goitrogens the labeling index is still 8% after 2 mo, corresponding to a doubling time of 4–5 days, whereas the weight of the gland is barely increased (Maenhaut and Dumont, unpublished observations). In transgenic mice expressing a physiologically constitutive adenosine receptor activating adenylate cyclase, a labeling index of 6–8% (control <0.5%) is found after 3–7 mo (corresponding doubling time ~4 days), and thyroid weights up to 400 mg (normal weight 1 mg) are observed after 9 mo (C. Ledent and J. E. Dumont, unpublished observations). It is interesting that this labeling index is normal and is barely increased in very young transgenic mice. The inherent limitation of thyroid cell number is therefore tighter than the limitation of the cell’s life span. This suggests increased cell death in chronically stimulated gland. Cell death or cell loss has been demonstrated in normal and chronically stimulated rat thyroids (44). The role and mechanism of such a process is unexplained, although it is interesting to note that in leukemia cells, adenosine 3',5'-cyclic monophosphate (cAMP), which in the thyroid mediates TSH effects, induces programmed cell death (apoptosis) (176). In rat thyroids in culture, TSH decreases the persistence of [3H]DNA, which suggests it induces cell death (305). Thus, although the life span of the thyrocyte is certainly limited, it is probably higher than the limit reached under in vivo physiological situations or in vitro.

C. Coordination Between Various Thyroid Cell Types

Parenchymal, stromal, and endothelial cells (in thyroid capillaries, in the proximal part of veins and lym-
FIG. 1. Schematic representation of possible cell interrelations in thyroid tissue. Input, known extracellular signals acting on thyrocyte. Output, release of extracellular signals by thyrocyte. These can act on thyrocyte itself (autocrine loops) or on fibroblasts, endothelial, or calcitonin-secreting (C) cells (paracrine loops). Products of those cells may also influence thyrocytes. cGRP, calcitonin growth factor; FGF, fibroblast growth factor; GII, growth hormone; I-, iodide; IGF, insulin-like growth factor (somatomedin); ILs, interleukin-6, IT, iodotyrosine; PGE, prostaglandin E; T3, triiodothyronine; T4, thyroxin; Tg, thyroglobulin; TGF-β, transforming growth factor-β; TH, thyroxin and iodotyrosine of thyroglobulin; TSH, thyrotropin; X, putative unknown signal; ?, hypothetical.

phatics, and in the distal segment of thyroid arteries) all proliferate during growth (53, 201, 299, 357, 365). Even the capsule greatly increases in thickness in stimulated rat thyroid (356). After iodide treatment the vascularization of the hyperstimulated thyroids regresses earlier than thyrocyte function in rats (274). These facts imply a cross signaling between these cells. Presumably, it is the thyrocyte that must coordinate the response of the other cell populations of the thyroid (70,115; Fig. 1). It is the obvious site of control of the information about TSH concentration and action, iodine supply and metabolism, and thyroid hormone formation. The role of the thyrocyte is supported by the induction by grafted donor thyrocytes of angiogenesis from cells of the recipient nude mice (215).

The intercellular paracrine signals generated by the follicular cells are not known, although several potential candidate molecules are secreted by thyrocytes in culture: insulin-like growth factor I (IGF-I) (14,196), plasminogen activator (199), fibroblast growth factor (FGF) (92,117), transforming growth factor-β (TGF-β) (120,217), interleukin-6 (345), and probably atrial natriuretic factor (6,292). Other candidates should be considered: adenosine, the oxygen-sensitive signal (3,289), nitric oxide (216), and angiogenic factors (101). The possible paracrine relations between calcitonin-secreting (C) cells and thyrocytes have not been studied, although effects of calcemia on thyrocytes may well be mediated through C-cells (113, 157), and the parallelism between mitotic index of C-cells and follicular cells during the circadian rhythm suggests a relationship (156).

D. Stem Cells

Although, as in the liver (291), most of the thyrocytes in the thyroid appear to be able to respond by a few divisions to a proliferation stimulus in vivo and in vitro, the existence of a population of stem cells able to replenish the pool of fully differentiated and proliferation-limited thyrocytes has been postulated. This hypothesis rests on indirect but suggestive evidence: 1) the growth of thyroid transplants in recipient animals requires the injection of a minimal number of cells (219), which might measure the relative frequency of stem cells; and 2) in cloning assays in radiobiological experiments, foci formation occurs but with very low efficiency. From both types of experiments the frequency of stem cells would be estimated at most to be 1/1,000. Such stem cells could be primary candidates for transformation and tumorigenesis, but the arguments in favor of their existence are indirect, and until now, we and presumably others have failed to demonstrate it directly. Certainly in dog thyroid cells in primary culture, in the absence of stimulation, double labeling with \[^{3}H\]thymidine and bromodeoxyuridine at several days interval is exceedingly rare (P. P. Roger, M. Baptist, and J. E. Dumont, unpublished observations).

II. METHODOLOGY AND MODEL SYSTEMS FOR STUDY OF THYROID GROWTH

A. Model Systems

In the study of organ function and growth the investigator may resort to many different systems, from humans in vivo, who are our ultimate subject, to the purified enzyme. Often when we choose one particular experimental model we tend to be skeptical about the others. This should not necessarily be so if we respect two caveats. Going down the ladder from the complex physiological in vivo system to the clean in vitro purified systems, at each step the data become more precise, the relations simpler, and therefore the conclusions...
clearer. However, at each step we lose some of the characteristics of the in vivo situation (in terms of controls, environment, and structure). Data collected in vivo may lead to erroneous conclusions because of the complexity of the systems involved; data obtained on simplified preparations may have no relevance to the in vivo situation.

Clinical experience with patients treated with antithyroid drugs or thyroid hormones, now backed by sophisticated and precise instrumentation, demonstrates every day the basics of thyroid growth control in humans in vivo. In vivo work on animals in which \(^{3}H\)thymidine has been injected has provided us our concepts on the in vivo regulation of growth as well as the parameters of the cell cycle of thyroid cells. Exogenous transplants of human tissue in nude mice are in an in vivo environment, subject to endocrine but not neural control, with a reconstituted normal follicular architecture. They have allowed researchers to distinguish hyperfunction and growth due to the environmental milieu (Graves' disease) and to an intrinsic cell property (autonomous adenoma) (152).

In vitro perfusion and the slices system lose the nervous control, and probably the paracrine controls, as extracellular signal molecules are diluted in the medium. However, such systems retain the normal tissue architecture and some physiological properties (secretion, thyroid hormone synthesis, TSH control). Studies of such systems have allowed the demonstration of the rich diversity of the patterns of neurotransmitter action from one species to another but also of the intracellular signals and cascades involved in the regulation of function. Although some first steps in the promotion of growth can be studied (such as ornithine decarboxylase induction), the short lifetime (4-7 h) of the system never allows researchers to verify if the end result, true growth, DNA synthesis, or mitosis, would have occurred. However, it must be said that in many studies on growth control in cell cultures this is not verified either. In the literature, the relationship of so-called early mitogenic events to proliferation is often only the fact that they are induced by growth factors.

Primary cell cultures have allowed for the demonstration of the direct mitogenic effect of TSH through cAMP in dog, rat, and human thyroid cells. The time limitations of these systems (~2 wk) have not allowed more than a few rounds of mitoses to be demonstrated in these cells. If cultured in the right conditions, these cells can regain their polarity and the asymmetry of their normal tissue architecture. They become then the best model for studies of transport.

In vitro primary culture of dog thyroid cells, TSH triggers, in the absence of serum, or enhances, in the presence of serum, the proliferation of thyrocytes. Under maximal stimulation [i.e., with TSH, serum, and epidermal growth factor (EGF)], almost all the thyrocytes synthesize DNA, which shows that, as in vivo, most thyrocytes are able to respond (270). However, even under such stimulation, they do not divide more than four to six times. This limitation could be due to an artifact (e.g., a necessary factor is missing) of the system. However, it is tempting to relate this limited life span to the known in vivo limits.

Thyroid cell lines are obviously very remote from physiology. The Fisher rat thyroid cell line, FRTL-5, that is mostly used (8) loses, for instance, the growth control by EGF, the Ca\(^{2+}\)-phosphatidylinositol (PI) response to TSH, and iodide binding to proteins (89, 91). Cell lines may also have acquired new non-physiologically relevant controls, for example, \(\beta\)-receptors (93). Nevertheless, such cells are very useful not only for their simplicity and reproducibility but because they allow permanent transfections and genetic experiments. Moreover, they are perfectly suitable for the study of the metabolisms and the controls that they have retained, such as the control of growth by the cAMP pathway.

In cell cultures, except perhaps for whole follicles in suspension or polarized monolayers, the control of growth by the extracellular matrix is probably disturbed or nonexistent. It should also be realized that culture per se has a profound effect on cell proliferation: while in vivo the labeling index of dog thyrocytes is \(4 \times 10^{-4}\) and in quiescent cultures it is \(5 \times 10^{-8}\) (49).
or absence of colchicine), the fraction of nuclei labeled with \[^{3}H\]thymidine or bromodeoxyuridine, or the fraction of the cells between the diploid and tetraploid stage (as observed with cell sorter) measure the number of cells reaching a defined stage of the cell cycle (mitosis, DNA synthesis) after the no-return decision to go through one round of divisions. Applying these techniques on in vivo-obtained specimens immediately after removal gives information on the fraction of the cells that are at a particular stage of the mitotic cycle in vivo. However, such data as well as histological examination are snapshots; they only give information at one point in time. Similar examination 1 h or 1 mo after could reveal that the same cell or follicle that looked active at one time may be quiescent later (139).

In general all the techniques used to measure cell proliferation have drawbacks; therefore none should be used alone in the assessment of proliferation rate (87, 121). These methodological considerations are now accepted by a growing fraction of workers in the thyroid field, if not in others (it is striking to see how many so-called reputable journals accept articles on proliferation based only on \[^{3}H\]thymidine uptakes in DNA).

Apparently negative results or discrepancies between results obtained by valid methodology with different models should be examined critically. 1) In dog and rat thyroid cells and in other cell cultures the mitogenic effects of TSH and EGF require the presence in the medium of IGF-I and/or insulin. This shows that an absence of effect in a system may always be due to the absence of a required factor. 2) The concentration-effect relationship of TSH is often biphasic. 3) Due to polarity of cells in some systems, receptors may not be accessible to the culture medium. 4) For the control of function as well as of growth, species differences may exist. In dog thyroid, the cAMP system is negatively controlled by norepinephrine through N_i at the level of the cyclase and the Ca\(^{2+}\)-PI cascade is activated by ace-tylcholine through muscarinic receptors. The converse situation is found in mouse thyroids. Thryotropin activates Ca\(^{2+}\)-PI cascade in human but not in dog thyroid cells or in the FRTL-5 cell line. 5) The history of the investigated cells may modify their responses to a given agent. Depending on the pretreatment of pig thyroid cells in primary culture, receptors or GTP-binding transducing proteins appear or disappear (206, 284). One should therefore, as always, remain very cautious about negative results. It is easy not to observe a phenomenon.

C. Working Definition: Growth Control Ratio

When mitochondria are incubated with ADP their respiration is increased. The ratio of respiration in the presence versus in the absence of ADP is called the respiratory control. In other systems this ratio of activity in the presence of maximal concentrations of stimulant versus in its absence is called the stimulation ratio. The respiratory control and the stimulation ratio define the tightness of a control. Damaged mitochondria have a higher basal respiration and a lower respiratory control than intact mitochondria (loose coupling). The damage is not characterized by a lower activity but by loss of control or uncoupling. In systems like proliferating cells or cells expressing genes, the coupling or tightness of control better characterizes the performance of the system than the basal activity of the measured process. In thyroid cell culture systems, the ratio of growth with or without TSH (growth control ratio) may vary between 1 and 100. In some cases (dog thyroid cells in the absence of serum, FRTL-5 cells) the control is almost all or none.

III. DIFFERENTIATION, DETERMINATION, DIFFERENTIATION EXPRESSION

A. Thyroid System

In many systems proliferation and differentiation are antagonistic processes. As used in cell culture work these concepts may introduce confusion. In embryology when a cell, by an epigenetic mechanism, begins a new more differentiated lineage the process is called determination. On the other hand, a determined cell may express more or less the characteristics of its type; we call this modulation, differentiation expression. In thyroid cell culture, incubation with 12-O-tetradecanoylphor-bol-13-acetate (TPA) or EGF shuts off the specialized thyroid genes (thyroglobulin, thyroperoxidase). However, these cells remain and remember that they are thyroid cells. Treated with TSH or with any agent enhancing cAMP accumulation, they will again fully express their specialized genes. When treated with EGF and TPA the cells remain determined but lose their differentiation expression and recover the latter when their cAMP increases. These concepts find their counterpart at the level of the proteins involved in specific thyroid cell control and of the chromatin. To remain determined and inserted in their physiological regulatory network the follicular cells must recognize the signals addressed to them and respond properly. Indeed, the TSH receptor mRNA levels are remarkably stable in thyroid cells in vivo and in vitro. Differdifferentiation with EGF and phorbol esters in vitro never abolishes TSH receptor mRNA expression, whereas it suppresses thyroperoxidase and thyroglobulin gene expression (Maenhaut and Dumont, unpublished observations). Similarly, in thyroid tumors, TSH receptor mRNA remains present when the other two have disappeared (234, G. Brabant, C. Maenhaut, and J. E. Dumont, unpublished observations). One would expect the same stability for the key proteins and genes involved more distally in the specific response of the thyroid cell to its physiological signals [e.g., thyroglobulin transacting factor 1 (TTF-1)] (46). At the level of chromatin, active differentiation-expressing dog thyroid cells exhibit two DNase-sensitive sites in the promoter of thyroglobulin. When treated with EGF they lose the downstream site; the upstream site remains open in the cells that are still
determined; it is not present in the chromatin of non-thyroid cells (122).

The stage of determination without differentiation expression is different from the undifferentiated state preceding differentiation in the embryo. It is probably during the transition between these states that the upstream DNase-sensitive site of the thyroglobulin promoter, specific for thyroid tissue, appears (122).

In vivo the integrity or even the presence of the hypothalamic hypophysis system is not necessary for the full differentiation and early growth of the thyroid in the embryo. Complete normal morphological development of the thyroid (e.g., follicular structure, with colloid) is observed in the absence of TSH in vitro (363) in anencephalic human fetuses missing the hypotalamus and hypophysis, in decapitated rabbit fetuses (158), and in chick embryos (134). In the latter case, thyroid growth and accumulation of thyroglobulin were present but diminished in the absence of the pituitary. This suggests that pituitary hormones stimulate but do not induce development of the thyroid and do not affect its differentiation in vivo. However, it should be pointed out that in none of these experiments were blood pituitary hormones (TSH, growth hormone) actually measured, and therefore the remote possibility of such an action by extrapituitary factors has not been excluded.

B. Model for Thyroid

Our present knowledge of the various stages of thyroid cell differentiation in vivo and in vitro can be summarized in the scheme outlined in Figure 2. This scheme should be considered only as a working model, but it has the merit to provide a conceptual framework for further investigations. It certainly grossly oversimplifies the very complex and multistage pathway of differentiation. It distinguishes the stages of the totipotent embryonic cell (I), the determined cell that has acquired its thyroid differentiation (V), the cell expressing this differentiation after the start of thyroid function in the fetus (VI), the hyperfunctioning cell under intense physiological or pathological (e.g., hyperthyroidism) conditions (Vlc), or the resting cell relieved of TSH positive control (e.g., in pituitary myxoeclema) (VIa).

The totipotent embryonic cell (I) has by definition no characteristics of the thyroid cell. The determined cell is a postulated stage (V) in which the genes of the proteins that normally insert the thyroid in its physiological network are expressed, e.g., the TSH receptor and the transacting factors conferring specific thyroid gene expression (such as TTF-1) (46, 296). This cell knows or “remembers” its differentiation (344). However, none of the specific functional genes (e.g., thyroglobulin) or functions are expressed. Dog thyroid cells in primary culture incubated without TSH correspond to the “resting” stage, and those incubated with TSH correspond to the hyperfunctioning stage. Slices from the thyroids of experimental animals, reflecting their in vivo situation, correspond to the physiological conditions but can (333) in the presence of TSH reproduce the stimulation in vivo or in culture by this hormone for a short time period (hours).

The phosphorylations of many proteins in cells or slices take place within minutes. They represent the rapid posttranslational effects of TSH involved in the acute regulation of function. Phosphorylations of histone H1 (169a), H3, or high-mobility group (HMG) 14/17 proteins (55, 56) also observed under these conditions may reflect a first step of the more delayed transcriptional effects of TSH that lead to growth and chronic hyperfunction. Phosphorylation of HMG 14 inhibits its interaction with nucleosomes and therefore modifies the structure of chromatin (304).

In cell culture the passage from the determined cell to the cell expressing differentiation requires several days (86, 261, 262, 266, 273, 334) and in the case of the thyroglobulin gene is enhanced by insulin (108). In slices
in vitro or in rats in vivo the activation of thyroglobulin expression only requires 1 h (108, 109). Thus the delays involved in the transition from quiescent to stimulated states are much faster than the transition from determined cell to the cell expressing differentiation. Until now, in dog thyroid cells at least, all the effects of TSH on this differentiation pathway were reproduced by the activation of the cAMP cascade. They are assumed to represent similar cAMP-mediated steps of differentiation in the embryo (247). Both EGF and phorbol esters when applied to cells pretreated with TSH or forskolin dedifferentiate them to the "determined cell" stage (V).

IV. EXTRACELLULAR SIGNALS INVOLVED IN CONTROL OF PROLIFERATION OF THYROID CELLS

A. Thyrotropin

In general the major element controlling thyroid growth in vivo is the level of TSH (82). In addition to the classic endocrinological evidence, much new experimental physiology supports this concept. Proliferation, as evaluated by mitotic activity in young rats, follows TSH levels: 1) it increases by a factor of five in goitrogen-treated rats (TSH × 54) (366), 2) it follows the circadian rhythm of TSHII (363), and 3) its circadian rhythm disappears with the TSH rhythm in goitrogen-treated rats (363). The growth of thyroid grafts in recipient animals greatly increases in thyroidecomized animals (greatly decreasing the influence of donors' age). It increases and then decreases with the age of the recipient in parallel with serum active TSH levels. It increases in male versus female recipients but not in gonadectomized animals in parallel with TSH levels (341).

Thyrotropin-increasing treatments markedly enhance the growth of human thyroid tissue transplanted in nude mice (244). In several species, including humans, TSH promotes the proliferation of thyrocytes in culture (272). The lack of stimulatory or even the inhibitory effects observed on porcine and beef thyroid cells remain exceptions (106, 110, 130, 342) perhaps due to artifacts of the culture system or the absence of comitogenic factors.

There is some in vivo evidence that the stimulation of thyroid growth by TSH is partly dependent on other hormones. Growth effects of TSH are reduced in hypophysectomized (145) or adrenalectomized rats (150). In vitro, optimal growth effects of TSH require IGF or insulin as comitogenic factors in several systems (269, 270, 272, 303, 325-328, 353). Moreover the stimulus of iodine deficiency causes goiter in normal populations but not in pygmees, who are congenitally deficient in IGF-I (B.
Contemptre and J. E. Dumont, unpublished observations).

B. Iodine

Iodine deficiency in hypophysectomized animals also induces some thyroid growth. Iodine, as such, thus exerts a negative endogenous control on the thyrocyte growth. Moreover iodine deficiency increases the sensitivity of the thyroid to the goitrogenic effects of TSH (30, 204). Thus iodine deficiency may render a normal TSH concentration goitrogenic. That this occurs in the physiological range is shown by the inhibitory effects of moderate iodine supplementation in humans (41, 151). The relief of direct effects of iodine thus partly accounts for the goitrogenic action of antithyroid drugs in vivo (309). Conversely the stimulated thyroid is more sensitive to iodide (47). The inhibitory effect of iodide has been partially reproduced in FRTL-5 cells (279). An iodolactone has been proposed as a putative intermediate in iodide action, as it inhibits the proliferation of rat and porcine thyroid cells in vivo and in vitro, respectively (81, 249). However, because these cells require exogenous arachidonate to synthesize the iodinated derivative, the role of the iodolactone in this process remains debatable. The role of the iodinated lipid normally present in thyroid (2iodohexadecanal) has not yet been investigated (213).

C. Other Hormones

It would be interesting to investigate whether thyroid hormones by themselves exert a direct control on the growth of the thyroid gland. Thyroxine certainly increases the growth of transplanted autonomous thyroid tumors in rats in vivo (297), and thyroid cells contain many T₃ receptors (64).

Growth hormone, perhaps through IGF-I as an intermediate (14), induces thyroid growth but does not markedly enhance function as demonstrated in acromegaly (107, 168, 212), although some degree of autonomy in the goitrous acromegalic has been reported (359). In Snell dwarf mice, growth hormone induces cell proliferation but, contrary to TSH, no cell hypertrophy (68, 190).

Human chorionic gonadotropin and thus luteinizing hormone at high concentrations activate the cAMP cascade and consequently proliferation in FRTL-5 cells (62, 373, 374) and human thyroid cells (241). Because these effects are inhibited by TSH receptor-blocking antibodies, they are mediated by this receptor (372). The concentrations reached in patients with trophoblastic tumors or even in pregnancy (241, 374) are sufficient to activate the human thyroid (132, 159, 371).

D. Pathological Signals

Other plasma signals appear only in disease, such as the autoimmune immunoglobulins directed against thyroid cell membrane receptors. Thyroid-stimulating antibodies (TSAbs) and thyroid-blocking antibodies (TBAbs) bind to the adenylate cyclase-coupled TSH receptor. The TSAbs activate (4) and TBAbs block the stimulation by this receptor of function and growth. The TSAbs are responsible for Graves' disease hyperthyroidism; TBAbs are responsible for some idiopathic myxoedemas (192, 379).

The concept of specific thyroid growth immunoglobulins (TGI) arose in the 1980 (79, 351) from the acknowledgement that some TSH effects were not mediated by cAMP, which might be explained by the existence of different TSH receptors or effectors (83). Since then TGI activities have been reported by some groups, but the methodologies used raise questions (87). Although the concept may remain valid, its demonstration would require an unquestionable double-blind study using accepted methodologies (379). If accepted, the concept should provide an explanation for the thyroid specificity of TGI. Indeed all known growth factors and their receptors are remarkably ubiquitous, specificity being insured by local delivery through autocrine or paracrine mechanisms.

E. Local Signals

As shown, compensatory hypertrophy after thyroidectomy and goitrous hyperplasia in iodine deficiency or after goitrogens administration are caused by the operation of the classic thyroid hormone-pituitary-TSH feedback and thus are prevented by thyroid hormone treatment. However, other local controls of thyroid cell proliferation exist (77).

In the embryo the thyroid develops before TSH secretion, even in anencephalic, i.e., hypopituitary, fetuses (134, 153, 306). Similarly a lesion of the thyroid (wound or cell death and necrosis) provokes an important local wave of cell divisions (307) even in thyroids desensitized to TSH action (362). Growth occurs in some cases (iodine treatment after iodine deficiency) when TSH levels are actually decreasing or in other cases in the absence of TSH (200, 275). Compensatory hyperplasia in hemithyroidectomized mice also takes place, although at a reduced level, in dwarf mice that have a hereditary lack of growth hormone, prolactin, and TSH and in hypophysectomized animals (68, 188, 189). These growth processes are obviously independent from pituitary control.

Thyrocytes, as other cells, also respond in vitro to a number of paracrine factors, i.e., factors secreted by neighboring cells. Some of these factors are also synthesized and secreted by the thyrocytes themselves (autocrine secretion). Several growth factors have been shown to be mitogenic or comitogenic (permissive) for thyrocytes (29, 92, 96, 98, 110, 160, 196, 235, 261, 263, 265, 269, 270, 272, 273, 303, 325-329, 347, 349, 353). EGF, FGF, IGF-I, the secondary factor secreted in response to growth hormone, IGF-II, and insulin, even at physiological concentrations. Insulin-like growth factor-I is produced by sheep thyroid (14), IGF II by FRTL-5 cells.
(196), and FGF by porcine thyrocytes (92, 117). A possible role of insulin and/or IGF-I in modulating thyroid growth response in vivo has been known for a long time (149). The action of growth hormone on thyroid cell proliferation in Snell dwarf mice congenitally deficient in pituitary hormones is probably mediated by IGF-I (189). The removal of submaxillary glands in mouse, which presumably greatly decreases serum EGF, has been reported to lead to thyroid regression (311), whereas perfusion of fetal sheep with EGF results in a considerable enlargement of the thyroid gland (324). Thyroxine increases plasma EGF levels and increases them in the thyroid, which rather suggests a functional negative feedback in mice in vivo (237).

At least one local hormone has been shown to inhibit growth, TGF-β (120, 217, 330, 360), as in other epithelial cells (195). It also induces extracellular matrix deposition, cell spreading, and the organization of the thyrocyte actin microfilaments network in bundles (104).

The panel of factors active on thyrocyte growth may vary from one species to another. Of course, such local hormones (IGF-I, FGF) also act on the endothelial cells and fibroblasts of the thyroid and may thus represent the mediators of the cross signaling that must exist to allow balanced growth of the gland. As the number of known growth factors increases, one may expect the progressive unraveling of a complex network of cell cross signaling.

In pathological situations, cytokines such as interleukin-1, interferon-γ, and tumor necrosis factor might be secreted in the gland by cells of the immune system (345). These cytokines strongly influence FRTL-5 and human thyroid cell proliferation and metabolism (94, 162, 166, 167, 211, 256, 369, 375, 377).

As in other cell types the extracellular matrix also probably exerts a local control (presumably negative). This is suggested by the growth response after wounding and by the inverse relation of proliferative response versus cell density in primary thyrocyte cultures. Such local controls would have great importance in pathology, generating diverse patterns from one area or one cell to another, i.e., tissue heterogeneity (111, 142-144). It is known that in thyrocytes (118), as in other cell types (381), a modulation of cytoskeleton organization modifies the activity of the cAMP cascade.

V. PATHWAYS REGULATING THYROID CELL PROLIFERATION

A. General Considerations

It is probable that the key machinery in the immediate control of the decision of a cell to divide will turn out to be very general. Indeed the first studies of complementation of proliferation-deficient yeasts with mammalian genes have already allowed the identification in mammalian cells of genes able to perform the same function. One of these genes, cdc 21 kinase has been shown to be a part of the well-known maturation promoting factor involved in the triggering of meiosis in oocytes (233). On the other hand, the regulatory circuits allowing the control of mammalian cells by extracellular signals vary in importance and significance from one cell type to another and within one cell type from one species to another. It is therefore highly improbable that the role of these circuits in the control of the decision to divide, i.e., in the control of the key machinery, will be conserved in all mammalian cells. Indeed, examples of opposite effects of the same cascade on proliferation and differentiation in different cell types abound: the phorbol ester protein kinase C pathway, which in the thyroid induces proliferation and dedifferentiation, exerts the opposite effects in keratinocytes and in colon cancer cells (40). The CAMP cascade that negatively regulates proliferation in fibroblasts and lymphocytes stimulates it in keratinocytes and thyrocytes. Thus the role of a given cascade in the control of cell proliferation and differentiation has to be considered for each cell type of each species. Generalizations are unwarranted in this field.

Reviews on the control of vertebrate cell proliferation generally consider two signal cascades as the main pathways between an extracellular mitogenic signal and mitogenesis itself: the growth factor receptor-protein tyrosine kinase pathway and the PI-Ca^2+-diacylglycerol-protein kinase C cascade (38, 293). In such reviews, the first demonstrated signal cascade, the cAMP system, is either not considered or merely ascribed a role of inhibitory pathway (16). This general picture derives from the fact that growth and proliferation have mostly been studied on "easy experimental objects," fibroblasts and cell lines of mesenchymal origin. As often in science, the choice of experimental subject has biased the outlook on the whole subject. In fact, in several epithelial cells and in yeast cells the cAMP cascade is a positive regulatory pathway of cell growth and proliferation (84, 86). In the thyroid we consider at least three well-defined distinct pathways (84, 170, 197): the hormone receptor-adenylate cyclase-cAMP protein kinase system, the hormone receptor-tyrosine protein kinase pathway, and the hormone receptor-phospholipase C-diacylglycerol protein kinase C-calcium calmodulin protein kinase cascade (Fig. 3). In dog thyrocytes, the receptor-tyrosine kinase pathway may be subdivided in at least two main branches: some growth factors, such as EGF, induce proliferation and repress differentiation expression, whereas others, such as FGF or IGF-I, are mitogenic or are necessary for the proliferation effect of other factors without being mitogenic by themselves, but they do not inhibit differentiation expression (250).

As we might have guessed when we look at the present complexity of the regulation of a simple organism like the phage, this simple scheme is now daily expanding and becoming more sophisticated. First, new regulatory pathways are being defined, e.g., the atrial natriuretic factor-guanylate cyclase-guanosine 3',5'-cyclic monophosphate, the hormone-phospholipase A_2-arachi-
donate, and the hormone-phospholipase C-phosphatidylcholine cascades (24). These pathways have not yet been considered in the regulation of thyroid cell proliferation. Second, at each level, any of the regulatory pathways may be directly controlled by any step of this or the other pathways. Thus, in the dog thyroid, calcium through calmodulin activates a cyclic nucleotide phosphodiesterase and diacylglycerol inhibits phospholipase C and the prostaglandin E, stimulation of adenylate cyclase and potentiates TSH activation of this enzyme. Third, the level of the proteins involved in each pathway may be controlled at gene transcription or distal to it by the same or other pathways. Fourth, the stimulation of a regulatory pathway in one cell may stimulate the production and release of factors that control these same cells (autocrine mechanisms) or neighboring cells (paracrine mechanisms). A possible example of such interactions is the secretion by pig thyroid cells of an FGF that can act on thyrocytes, on fibroblasts, and on endothelial cells (105). Therefore different mechanisms concurring to the same end will probably coexist in each system, and the demonstration of one such mechanism does not exclude the possibility of another. Moreover, at each level the circuits may be different from one species to another (257). Therefore if data should be coherent within one system, they may vary from one system to another.

B. Mitogenic and Differentiating Action of Thyrotropin

Although there is no doubt that TSH in vivo stimulates the proliferation of thyroid cells, there was in the 1970s little evidence that this was a direct effect (82). Indeed, the adrenocorticotropic hormone (ACTH) trophic effect on the adrenal appears to be indirect (186). The study of early steps of growth in slices (213) showed that TSH enhances ornithine decarboxylase activity in dog thyroid cells as it does in vivo and in vitro in rat thyroid (205, 286), which is generally considered as a preliminary to growth. The effect was mimicked by cAMP analogues and inhibited by agents inhibiting cAMP accumulation. As these results were against the current dogma, they were generally ignored.

To get at the problem of proliferation, it was necessary to use primary cultures. A technique derived from References 99, 164, and 255 was used, with a serum-free medium supplemented as proposed by Ambesi-Impimbiato et al. (8). With the use of several methods, it was demonstrated that TSH stimulates proliferation of the dog thyroid cells (262, 265, 269). More recently this result was confirmed in normal human thyroid cells (272). Despite earlier negative studies using inadequate culture conditions or pathological tissues (96, 331, 332, 346), the mitogenic effect of TSH on human thyroid cells is now well established in vitro (137, 352, 353). Other results obtained in various culture systems were sometimes contradictory. Although in dog thyroid cells in primary culture, in rat thyroid follicles in suspension (232, 303), in ovine cell lines (OVNI) (98), and in rat cell lines FRTL-5 (8, 73) and WRT (29) TSH has been demonstrated to enhance or induce cell proliferation, to our knowledge no such effect has been obtained in porcine (97, 106, 130, 342), calf (110), or ovine (90) thyroid cells in primary culture. Whether this is due to inaccessibility of the TSH receptor(s), lack of an essential element in the culture medium, alteration of cell program in culture, or true unresponsiveness to direct TSH action is...
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not known. The latter hypothesis is plausible, as in the very similar system of the adrenal cells the stimulating effects of ACTH on the proliferation in vivo (240) have not been convincingly reproduced in vitro (112). In this case, there are even arguments that the stimulating effect may be indirect: ACTH would induce the synthesis and secretion of growth factors by the adrenal cells, which would then, acting as extracellular signals, trigger the cell proliferation.

In porcine thyroid cells, TSH through cAMP induces EGF receptors, making these cells more responsive to EGF (12, 348, 350). In rat thyroid, propylthiouracil-induced goitrogenesis is accompanied by an increased concentration of IGF II receptors (251). In FRTL-5 cells TSH greatly increases the effects of IGF-I, serum, and TPA on 3H-thymidine incorporation into DNA (314). In the control of thyroid cell proliferation, complementary mechanisms as well as differences of strategy from one species to another are possible (23).

In dog (269, 270) and human (236, 272, 353) thyroid cells, IGF-I or insulin is generally required for the mitogenic action of TSH or EGF but does not by itself stimulate proliferation. It has been known for a long time that TSH by itself does not stimulate thyroid growth in hypophysectomized rats (145). In rats, full growth response to TSH requires adequate levels of another hormone(s) that can be replaced by insulin (149, 150). In FRTL-5 cells IGF-I potentiates the activation of adenylate cyclase by TSH (31). In FRTL-5 and rat cells, IGF-I is weakly stimulatory per se (303, 326), although this has not been found by another group (283). In pig thyroid cells, IGF-I produces a stronger mitogenic signal (281).

In the promotion of growth by TSH, increased protein content results from increased protein synthesis (82) and decreased protein catabolism (75).

It should be noted that in dog thyroid cells TSH directly stimulates proliferation while maintaining the expression of differentiation. Differentiation expression, as evaluated by iodide transport or thyroperoxidase and thyroglobulin mRNA content or nuclear trans-}

C. Effect of Factors Other Than Thyrotropin on Proliferation and Differentiation

iodide inhibits many metabolic steps in thyroid cells. Most of these effects, such as the inhibition of adenylyl cyclase, are relieved by treatments that block the penetration of iodide in thyroid cells (e.g., perchlorate) or its oxidation (e.g., methimazole). They are therefore ascribed to a postulated iodinated inhibitor (836). It inhibits both the activation of the cAMP cascade and the phosphatidylinositol bisphosphate (PIP2) cascade (178, 336) but also, independently, other steps, such as protein iodination and iodothyronine synthesis. This negative role also extends to proliferation in some thyroid cells (such as the FRTL-5 cells) and to gene expression. An indication for the latter effects is the stimulation by methimazole of thyroglobulin and thyroperoxidase mRNA accumulation in FRTL-5 (183).

Epidermal growth factor also induces proliferation of dog thyroid cells (261, 263, 270, 273) as well as other cells (36). It also stimulates the growth of thyroid cells from other species in culture (e.g., porcine, ovine, bovine, and human but not of the FRTL cell line that lacks EGF receptors) (96, 110, 319, 347, 349). This effect is often weaker than the effect of TSH. However, the action of EGF is accompanied by a general and reversible loss of differentiation expression (50, 90, 173, 252, 261, 263, 272, 273, 347) assessed as described. Similar results have been obtained in sheep in vivo (57, 324) and in newborn rat thyroids transplanted in nude mice (238). The effects of EGF on differentiation can be dissociated from their proliferative action. Indeed, they are obtained in cells that do not proliferate in the absence of insulin (172, 250), in human cells in which the proliferative effect is weaker (173), and in pig cells at concentrations lower than the mitogenic concentrations (343). Finally other growth factors,FGFs, and serum induce dog thyroid cell proliferation without exerting the dedifferentiating effects of EGF (264). A similar effect of IGF-I in TSH-primed FRTL-5 cells has been reported (313). However, FGF and serum in calf and porcine thyroid cells act as EGF in dog cells, inducing proliferation but repressing differentiation (92, 110).
Finally, the tumor promoting phorbol esters, the pharmacological probes of the protein kinase C system, and analogues of diacylglycerol also enhance the proliferation and inhibit the differentiation of dog as well as other thyroid cells (15, 125, 191, 266, 272, 319). These effects are transient because of desensitization of the system by protein kinase C inactivation (260). The activation of the PIP$_2$ cascade by physiological agents, such as carbamylcholine and Bradykinin, in dog thyroid cells does not reproduce all the effects of phorbol esters. In particular, prolonged stimulation of the cascade inhibits rather than stimulates proliferation (E. Raspé and J. E. Dumont, unpublished observations) as well as induction of ornithine decarboxylase (213). Thus we cannot necessarily equate effects of phorbol esters and prolonged stimulation of the PIP$_2$ cascade. Similarly, prolonged enhancement of the intracellular Ca$^{2+}$ level might explain the mitogenic effects of IGF-I on FRTL-5 cells (313) but does not stimulate growth in dog thyroid cells (Raspé and Dumont, unpublished observations).

The dedifferentiating effects of phorbol esters do not require their mitogenic action either. Thus the effects of TSH, EGF, and phorbol esters on differentiation expression are largely independent of their mitogenic action.

In several thyroid cell models, very high insulin concentrations are necessary for growth even in the presence of EGF (8, 269, 270, 272, 303, 326). We now know that this mainly reflects a requirement for IGF-I (58, 272). It is interesting that in the FRTL-5 cell line (196), as in cells from thyroid nodules (354), this requirement may disappear as the cells secrete their own somatomedins and thus become autonomous with regard to these hormones. However, low physiological concentrations of insulin can replace IGF-I to allow growth stimulation by TSH in dog (270) but not in human thyrocytes (272).

Serum and FGF also induce growth in dog and calf thyroid cells (25, 110, 263). In addition, IGF-I per se also stimulates the proliferation of FRTL-5 cells or WRT rat cells (277, 326). Although serum fully inhibits differentiation expression in calf thyrocytes and FRTL-5 cells (110, 378) and partially inhibits it in dog thyroid cells, IGF-I and insulin have no such effect. In fact IGF-I and insulin acting through IGF-I receptors have some positive effects on specialized gene expression in FRTL-5 cells (283), and insulin even at low concentrations is a moderate inducer of thyroglobulin gene expression in dog cells (108, 250). In FRTL-5 cells IGF-I also potentiates the stimulation of adenylate cyclase by TSH (31). This therefore represents another type of receptor-tyrosine protein kinase pathway that leads to mitogenesis and to some extent to differentiation expression.

### D. Role of Adenosine 3’,5’-Cyclic Monophosphate, Phosphatidylinositol Bisphosphate, and Tyrosine Protein Kinase Cascades in Thyrocyte Proliferation

The TSH effects on the proliferation and differentiation of thyroid cells are mediated by cAMP. Thyrotrpin induces within minutes a striking morphological change in dog thyroid cells in culture, a rounding up following the disruption of the actin network (231, 255, 263, 268). All the cells are affected. In addition, TSH also enhances the accumulation of cAMP in these cells within <5 min; it remains elevated for 48 h in the continuous presence of the hormone. In the dog thyroid cells, analogues of cAMP as well as general cyclase activators (forskolin, cholera toxin) reproduce all the effects of TSH: acute morphological changes, proliferation, expression of differentiation (263, 265, 267, 269, 271, 273). Microinjection of the catalytic subunit of cAMP-dependent protein kinase also reproduces the disruption of the actin network (267). All the protein phosphorylations induced by TSH are also caused by forskolin (54, 181, 337). The pattern of protein synthesis induced by TSH is also fully reproduced by cholera toxin (171). Moreover, combinations of cAMP analogues that are synergistic on the two cAMP-dependent kinases iso-enzymes are also synergistic on these effects (338). Therefore cAMP is a general intracellular positive signal for function, proliferation, and differentiation in the dog thyroid cells.

For proliferation, similar results have been obtained with human (165, 272) and rat thyroid cells in culture (361) and, despite a first contradictory report (331, 332), in FRTL-5 cells (73, 89, 148, 260). In the latter cells, thyroid blocking antibodies inhibit in parallel TSH binding and TSH-induced cAMP accumulation and $[^{3}H]$thymidine uptake (34). It is interesting that in cloned dedifferentiated tumorigenic FRTL-5-derived cells, cAMP, as in fibroblasts, inhibits proliferation (93). Thus changing the phenotype of these cells may reverse the role of cAMP. In other cloned mutated FRTL-5 cells, growth becomes independent of TSH and cAMP (329).

The stimulation of proliferation by cAMP is mediated by the activation of cAMP-dependent protein kinases. Indeed analogues of cAMP that are selective for the two sites of each kinase and for each kinase must be used in the combination that activates these kinases to elicit the mitogenic effects in dog thyroid cells (338). The data even suggest a predominant role for type I cAMP kinase in this process. This is also supported by the fact that desensitization to the cAMP growth effect coincides with type I kinase downregulation (32) and other in vivo results in other systems supporting a positive role of this kinase in growth (52). The inhibitory effect of iodide on thyroid cell proliferation in vivo can also be explained by its well-documented inhibition of thyroid adenylate cyclase (336).

One argument that cAMP may be the mediator of rat thyroid cell proliferation in vivo is the fact that methylxanthines, inhibitors of cAMP phosphodiesterases, even at doses that do not further enhance serum TSH levels or decrease serum thyroid hormones, greatly potentiate the goitrogenic action of propylthiouracil (355). The action of methylxanthines is abolished by a high-iodine diet or hypophysectomy (355). The effect of methylxanthine could be due to inhibition of adenosine receptors. As thyroid mostly contains $A_1$-receptors negatively coupled to adenylate cyclase, the goitrogenic ef-
The phenomenology of EGF, TPA, and TSH proliferation into nuclear DNA of dog thyroid cells are very similar for TSH, forskolin, EGF, and phorbol esters (TPA) (270). Whatever the stimulant, there is a similar minimal delay of 16–20 h before the beginning of the labeling, i.e., of DNA synthesis. This is the minimal time required to prepare the necessary machinery. Only for the combination of TSH, EGF, and serum is the delay shortened to 14 h. For the cAMP pathway, the stimulatory agent has to be present during this whole prereplicative period: any interruption of the stimulation (e.g., by forskolin washing) greatly delays the start of DNA synthesis (271).

The time sequence of biochemical phenomena between the stimulation and the beginning of DNA synthesis in these dog thyroid cells in primary culture suggests a causal sequence (Fig. 3). However, many such postulated causal relationships remain to be proved (239).

The phenotypology of EGF, TPA, and TSH proliferative action on dog quiescent cells has been studied in detail with the aim to identify steps in this action. Three
biochemical aspects of the proliferative response occurring at different times of the prereplicative phase have been considered. The pattern of protein phosphorylation induced within minutes by TSH is reproduced by forskolin and cAMP analogues (54). The phosphorylation of at least 11 proteins is increased or induced. Treatment of the gels with NaOH does not reveal any remaining phosphorylation on these proteins suggestive of tyrosine phosphorylation. The phosphorylation of seven proteins is decreased. In EGF-stimulated cells, the phosphorylation of five proteins is stimulated, two of them are phosphorylated on tyrosines (42,000 mol wt). These two proteins are similar (isoelectric points, approximate molecular weight, composition in phosphorylated amino acids) (54) to the two 42,000-mol wt proteins described in other systems, which have been implicated in the mitogenic response to diverse agents and recently identified as the mitogen-activated protein (MAP)-kinase (276). This kinase phosphorylates the S6 kinase II that is involved in the control of protein synthesis at the ribosome level. Phorbol esters induce the phosphorylation of 19 proteins, including the tyrosine-phosphorylated proteins mentioned. There is no overlap in the patterns of protein phosphorylation induced by TSH and cAMP enhancers, on the one hand, and by EGF and phorbol esters, on the other hand (54). Similarly the stimulation by EGF of Na+·H+ exchange in porcine thyroid cells has not been found in TSH-stimulated dog thyroid cells (318; E. Raspé, unpublished observations).

The expression of c-myc and c-fos has been studied by Northern analysis of RNA extracts (259). As in other types of cells (155), growth factors, EGF, and TPA enhance first c-fos and then c-myc mRNA expression (130, 259). On the other hand, TSH or forskolin enhances strongly but for a short time c-myc mRNA concentration and, with the same kinetics as for EGF/TPA, c-fos mRNA concentration. In fact, cAMP first enhances and then decreases c-myc mRNA accumulation. This second phenomenon is akin to what has been observed in fibroblasts in which cAMP negatively regulates growth (129). The enhancement has been observed for TSH and agents increasing intracellular cAMP in FRTL-5 cells, but the second phase has not been investigated or observed (71, 325). In pig thyroid cells, TSH and cAMP are not mitogenic and do not enhance c-myc and c-fos gene expression (130). The effect of TSH and cAMP on c-fos gene expression in FRTL-5 cells is transcriptional (60). It is easily explained by the presence of two cAMP-dependent response elements in the promoter of c-fos gene. The dyad symmetry element accounts for the effects of EGF, serum, and phorbol esters. In dog thyroid cells the effects of TSH on c-myc and c-fos expression do not require the presence of insulin but are potentiated by insulin. The absence of growth in the absence of insulin thus shows that the TSH enhancement of c-myc and c-fos expression may be necessary but is not sufficient for the mitogenic response. The fact that expression of antisense c-fos inhibits FRTL-5 cells proliferation suggests that indeed expression of c-fos is necessary (102). The expression of c-jun is enhanced by EGF and phorbol esters but inhibited by TSH and forskolin. This is similar to results obtained with NIH 3T3 cells in which phorbol esters stimulate proliferation while cAMP inhibits it (S. Reuse and J. E. Dumont, unpublished observations). Enhancement of c-jun expression is therefore not required for the induction of proliferation by TSH and cAMP. Thyrotropin, through cAMP, also enhances the mRNA expression of another protooncogene, c-ras, in rat cells (72).

In general, although initially distinct, the mitogenic pathways activated by the growth factor receptor tyrosine kinases and by the PI cascade overlap and converge on several early events: stimulation of the PI cascade in some systems, but not in dog thyrocytes, in response to receptor tyrosine kinase activation; phosphorylation of 42,000-mol wt proteins on tyrosine following protein kinase C activation; common stimulation of Na+·H+ antiport; and similar control of c-fos and c-myc protooncogene expression. In contrast, as delineated in the dog thyrocyte system, the activation of the cAMP cascade is sufficient to induce DNA synthesis but is essentially distinct from the cAMP-independent pathways. Differences include divergent patterns of protein phosphorylations and thus utilization of different protein kinases and second messengers and different kinetcs of protooncogene expression.

The pattern of proteins synthesized in response to the various proliferation stimuli has been studied (172, 182). Again two patterns emerge. Both TSH and forskolin induce the synthesis of at least eight proteins and decrease the synthesis of five proteins. Epidermal growth factor, phorbol ester, and serum induce the synthesis of at least two proteins and decrease the synthesis of two proteins. The only overlap between the two patterns concerns the decrease in the synthesis of a protein (18,000 mol wt) that is also reduced by EGF after proliferation has stopped. Only one protein has been shown to be synthesized in response to the three pathways, proliferating cell nuclear antigen (PCNA), the auxiliary protein of DNA polymerase δ (17). However, the kinetics of this synthesis are very different, with an early synthesis in the cAMP cascade (consistent with a role of signal) and a late (S phase) synthesis in the other cascades (172). Finally the synthesized proteins satisfying several criteria for signal proteins involved in the induction of DNA synthesis (biphasic kinetics, no induction in cells at confluence, synergy of induction by effectors of the other pathways), i.e., PCNA and protein 1, are specifically induced in G1 by TSH and cAMP and by EGF and phorbol esters, respectively (171, 172). Thus obviously two different phenomenologies are involved in the proliferation response to TSH through cAMP, on the one hand, and EGF and phorbol ester, presumably through protein tyrosine phosphorylation, on the other hand. Although this conclusion needs to be further substantiated, it certainly suggests that the proliferation of dog thyroid cells is controlled by at least two largely independent pathways. The effects of TSH, EGF, and phorbol esters on protein synthesis can be obtained in the absence of insulin in the medium, except for the
induction of PCNA synthesis; some are enhanced by insulin. Thus these effects also are not sufficient to induce mitogenesis (172).

F. Role of Autocrine Loop

The studies of protein phosphorylation, protooncogene expression, and protein synthesis in dog thyrocytes allow for discrimination between two models of cAMP action on proliferation in this system, a direct effect on the thyrocyte or an indirect effect through the secretion and autocrine action of another growth factor. If the effect of TSH through cAMP involved such an autocrine loop, one would expect a faster kinetics of action of the growth factor and at least some common parts in the patterns of protein phosphorylation and protein synthesis induced by cAMP and the growth factor. The results do not support such an hypothesis, at least for the growth factors we have tested (Fig. 4).

It has been suggested, for example, that part or all of the TSH growth effects on FRTL-5 cells are secondary to the autocrine secretion of IGF-II and other factors (314). This is not general as IGF-II, which is secreted by FRTL-5 cells and is mitogenic for them (196), is not a growth factor for dog thyroid cells by itself. Moreover, dog thyrocyte cultures are in general carried out in the presence of high concentrations of insulin that would saturate the IGF-I receptor (270). In the FRTL-5 cells themselves, anti-IGF antibody (Sm 1.2) only inhibits the effects of low concentrations of TSH (196) and does not inhibit the synergism between TSH and high concentrations of insulin. Moreover, there is no evidence that TSH does stimulate the production of IGF-II or other autocrine factors in these cells. An effect of TSH and cAMP through protein kinase C activators or EGF is also very unlikely in dog thyroid cells. The kinetics of action of TSH or forskolin are similar for the end point of DNA synthesis for the three types of agents. Moreover, the kinetics of protooncogene c-myc and c-fos expression are not delayed for TSH and cAMP. Finally the patterns of protein phosphorylation and protein synthesis induced by EGF and phorbol esters show partially common responses, whereas there was no overlap with the pattern of TSH or cAMP action. There is no evidence either that, as in Swiss 3T3 cells, the effect of TSH might be secondary to a TSH-induced release of prostaglandins. Thyrotropin does not induce such a release, and indomethacin does not inhibit the mitogenic effect of TSH (264). Thus there is no evidence in favor of the involvement of an autocrine loop with a growth factor or prostaglandin in the action of TSH and cAMP on dog thyroid cell proliferation. In FRTL-5 cells a cAMP-independent TSH stimulation of arachidonate release has been reported. Arachidonate derivatives were therefore suggested to play a role in the induction of proliferation by TSH (312). However, in this system the action of TSH is fully reproduced by activation of the cAMP cascade. This does not exclude that such a mechanism may operate in thyroids of other species, as suggested by the induction by TSH of EGF receptors in porcine thyroid cells (12, 348, 350).

VI. APPARENTLY OPPOSITE PROGRAMS: PROLIFERATION AND DIFFERENTIATION EXPRESSION ARE INDUCED BY THYROTROPIN AND ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE CASCADE

The incompatibility at the cell level of a proliferation and differentiation program is commonly accepted

![Figure 4: Models of mitogenic activation by cAMP cascade. Model 1: direct activation of mitogenic pathway in cell. Model 2: induction or secretion of growth factor or growth factor receptor that by autocrine process will activate mitogenic pathway of this growth factor. Predicted consequences of model 2 are outlined. They are not realized in case of stimulation by TSH of dog thyrocyte. A, cAMP; eAPK, cAMP dependent protein kinase; GF, growth factor; GFR, growth factor receptor; H, hormone; PDGF, platelet-derived growth factor; Prot, protein; Protooncogene; R, rat; broken arrow with plus, stimulation; broken arrow with bar, inhibition.](image-url)
in biology. In general, cells with a high proliferative capacity are poorly differentiated, and during development such cells lose this capacity as they progressively differentiate. Some cells even lose all potential to divide when reaching their full differentiation; this is called terminal differentiation. Conversely, in tumor cells there is an inverse relationship between proliferation and differentiation expression. It is therefore not surprising that in thyroid cells the general mitogenic agents and pathways, phorbol esters and the protein kinase C pathway, and EGF (and in calf and porcine cells FGF) and the protein tyrosine kinase pathway induce both proliferation and the loss of differentiation expression (92, 110, 263, 273, 266). The effects of the cAMP cascade are in striking contrast to this general concept. Indeed TSH and cAMP induce proliferation of the dog thyrocytes while maintaining differentiation expression: both proliferation and differentiation programs can be triggered by TSH in the same cells at the same time (250). This is by no means unique: neuroblasts in the cell cycle may simultaneously differentiate (14). It is tempting to relate this apparent paradox to the role and expression of protooncogenes in these cells. The expression of c-fos is enhanced in a great variety of cell stimulations, leading to either proliferation or differentiation expression (220). On the other hand, if there is one generalization that could be made on protooncogenes, it is the dedifferentiating role of c-myc. A rapid and dramatic decrease in c-myc mRNA by antisense c-myc sequences induces differentiation of a variety of cell types (119, 126, 254). In rat thyroid cell lines, expression of myc oncogene abolishes differentiation expression (22). The dedifferentiation following establishment of thyroid cells in primary culture is accompanied by an increased c-myc expression (135). It is therefore striking that in the case of the thyrocyte in which the activation of the cAMP cascade leads to both proliferation and differentiation, the kinetics of the c-myc gene appear tightly controlled. After a first phase of 1 h of higher level of c-myc mRNA, c-myc expression is decreased below control levels. In this second phase, cAMP decreases c-myc mRNA levels, as it does in proliferation-inhibited fibroblasts. It even depresses EGF-induced expression (259). The first phase could be necessary for proliferation, whereas the second phase could reflect the stimulation of differentiation by TSH. This downregulation is suppressed by cycloheximide, which suggests the involvement of a neosynthesized or labile inhibitory protein at the transcriptional level or at the level of mRNA stabilization. Surprisingly the second downregulation of c-myc (51) has not been observed in FRTL-5 cells.

Preliminary results indicate that TSH downregulates at a posttranscriptional level the c-myc mRNA expression: as soon as TSH is in the medium, a destabilization of c-myc mRNA is observed in cells in which transcription is blocked by actinomycin D. It can therefore be hypothesized that the first rise of c-myc mRNA expression reflects a very high induction of transcription combined to a destabilization mechanism. Later, the positive transcription effect is repressed and the destabilization mechanism persists, leading to a downregulation of the c-myc mRNA level. The transcription could be repressed either at the initiation (124, 242, 322) or at the elongation level (209). It would be interesting to test whether cloned tumorigenic FRTL-5 cells, in which cAMP inhibits proliferation (98), have lost the first positive control of c-myc expression. In the feedback mechanism, the inhibitory neosynthetic protein could even be the c-myc protein itself, specifically modified at the posttranslational level by the cAMP pathway. Such an autoregulatory mechanism of blockade of transcriptional initiation requires additional transacting factors and could act as a homeostatic regulator of c-myc expression in vivo (242). It is interesting that transformed FRTL-5 cells have lost the positive effect of TSH and cAMP on proliferation and express only a negative control (93). This suggests that in normal cells a dual control exists with a dominant positive regulation.

In many cells stimulation of proliferation is accompanied by an increased expression of c-jun protooncogene. In cells in which proliferation is inhibited by the cAMP cascade, c-jun expression is also inhibited. The expression of c-jun has therefore been considered as a general marker of cell proliferation. In fact, in dog thyroid cells, TSH and cAMP enhancers both induce proliferation and inhibit c-jun expression (Reuse and Dumont, unpublished observations). There is therefore no parallel between c-jun expression and proliferation in the dog thyroid cells, but it is possible that c-jun could be another factor involved in the negative control of differentiation expression. The effects of cAMP on c-jun expression are difficult to fit with the current concept that expression of c-jun is inhibited by CREB and that this inhibition is relieved by the cAMP-dependent phosphorylation of CREB.

VII. GROWTH CONTROL AND GOITROGENESIS

A. General Concepts

Goiter and therefore abnormalities in thyroid growth control are common to most thyroid diseases. Whatever its cause, goiter is the long-term end result of a disease. The thyroid has become extremely heterogeneous, and the primary lesion, even if still present, is swamped by a whole set of secondary and late consequences. This is well exemplified by the heterogeneous nature of many tumors, presumably of monoclonal origin, or of the thyroid after long-standing Graves' disease, iodine deficiency, or congenital defects, all of which originate from a single cause. The normal thyroid already presents, at a given time, heterogeneity from follicle to follicle and cell to cell in terms of function (as demonstrated by iodine radioautography), of functional response to stimulatory agents (as demonstrated by induced macropinocytosis or by the presence or absence of colloid droplets), and of proliferation (as shown by the
fact that, at a given time, only a few cells synthesize DNA) (82, 85). However, this heterogeneity, known for a long time but reinvented periodically, should be interpreted with caution. The picture observed at one point in time is not necessarily permanent; in fact histological sections and autoradiographies may be compared with snapshots of a rapidly evolving scene. The demonstration that apparently "cold" follicles in aged mice contain the same amount of iodine as hot follicles is evidence that these cold follicles must have at one time taken up iodine (208). Similarly, in the case of proliferation, in stimulated dog thyroid cells in primary culture it can be shown by double-label autoradiography that cells that divide at day 4 may not divide at day 8 and vice versa (Ruger and Dumont, unpublished observation). As is well known in cancerology (220), given the dynamic heterogeneity of the thyroid, even a simple primary defect may lead to permanent heterogeneity due to many factors. 1) One is the different kinetics of general controlling factors (TSH, iodine) and of cell sensitivity (e.g., the cells that are more sensitive to TSH at a given time will react more to a TSH surge). Such differences in sensitivity to growth factors are even observed in cultures of homogeneous cell lines such as the Swiss 3T3 cells. These differences are not heritable in the long term but represent an unstable property lost after a few generations in high-serum medium (83). 2) In addition there are local factors, such as cell-to-cell contact (370), blood flow availability, inflammation, hemorrhage, necrosis, and scarring. Cell necrosis in thyroids in involution is associated with cell multiplication and folliculogenesis (200). In thyroids, toxic or autoimmune reactions induced by iodine or other factors would also contribute to heterogeneity (70). 3) There is also the cell's immediate environment: cytokines and growth factors induce cells to alter their surrounding matrix, but conversely adherence to matrix induces cells to make cytokines and controls how cells respond to cytokines. In such a complex interplay the fate of two neighboring cells may be completely different (229). 4) There are mutations, the probability of which increases with the number of mitoses (9, 123, 253). A good example of such an evolution is shown by the heterogeneous mammary or pancreatic neoplasia induced by the single targeted overexpression of TGF-α in transgenic mice (202, 282). Also heterogeneity soon appears in the stimulated thyroids of mice submitted to hemithyroidectomy or methimazole treatment (298) or in the thyroids of transgenic mice constitutively stimulated by an adenosine A2-receptor (Ledent and Dumont, unpublished observations). This heterogeneity is limited within one follicle by the intercellular communication of small molecules allowed by the gap junctions (223).

To understand the pathogenesis of goiter, it is thus advisable to study, as early as possible, simple lesions originated from a single cause. The lessons learned on simple models should help to understand complex and multifunctional diseases, just as the study of a congenital defect enlightens our understanding of all its phenotypic counterparts. We therefore mainly review goiter formation in simple diseases such as Graves' disease, hyperfunctioning adenomas, and adenomas with congenital defects.

The role of the signals and cascades controlling thyroid cell proliferation and differentiation in pathology has been until now little studied. In a few cases thyroid pathology can be explained in a straightforward manner within the framework just presented.

### B. Graves' Disease

The disease in which goiter is easiest to explain is Graves' disease. In this disease autoantibodies directed against the TSH receptor (TSAb) activate this receptor and consequently the whole cAMP cascade (148, 193, 376). At the highest concentrations reached in pathology, these TSAbs do not activate, as TSH does, the Ca2+-P1 cascade (180). Thus hyperthyroidism in Graves' disease appears to result from a chronic hyperstimulation of the cAMP cascade. As shown, the effects of this cascade on cultured thyroid cells are to enhance function and proliferation while maintaining differentiation, i.e., they represent the in vitro counterparts of what is observed in Graves' disease thyroids in vivo. Indeed TSAbs induce human thyroid cell growth and c-fos expression in vitro (188). It is interesting to note that, as in vivo or in vitro chronically stimulated thyroids, the growth of thyroid in Graves' disease is generally limited, although hyperfunction and thus hyperstimulation persist. The permanency of the stimulation is explained by the low desensitization of TSH receptors and the absence of downregulation of TSH receptor mRNA (61, 65; Maenhaut and Dumont, unpublished observations). This apparently simple and unicausal disease may lead in time to heterogeneous goiter. Also, in these chronically stimulated thyroids in which proliferation and the increasing number of mitoses are bound to allow the fixation of more mutations, cancer incidence has been shown to increase (207), although this is controversial (7). Thus even though the cAMP cascade itself maintains differentiation while promoting proliferation, the greater number of mitoses may give a higher probability of occurrence to the rare mutagenic events that lead to carcinogenesis. Moreover, in the V79 cell line, activation of the cAMP cascade increases the mutation frequency induced by mutagenic methylating agents (131).

### C. Congenital Defects

The goiter resulting from congenital defects in iodine metabolism by the gland is also simply explained by classic concepts of thyroid regulation. Deficiency in thyroid hormone formation resulting from the defect relieves the thyroid hormone feedback on the hypophysis and leads to increased TSH secretion and stimulation of the thyroid. In addition, a deficiency in iodine metabolism, at the level of trapping or iodination, will relieve the intrathyroid negative feedback of iodide and
increase the sensitivity of the gland to the TSH growth-promoting effect. Impaired iodination due to a congenital defect or to inhibition by antithyroid drugs has been shown to relieve the inhibitory effect of iodide on cAMP accumulation (66, 67). Defects of iodotyrosine coupling and iodotyrosine deiodination that also lead to iodine depletion will in time have the same effect. It is interesting to note that defects in iodination, which most severely affect the iodide inhibitory pathway, lead to the severest goiters, sometimes to follicular carcinoma (2).

It is also important to note that even in this case where a single identified cause of the disease and its goitrogenic consequences exists, prolonged stimulation of the thyroid will in time generate heterogeneity and its ultimate result, the multinodular goiter (163, 278, 301). Similar considerations apply to endemic goiter in which iodine deficiency and sometimes goitrogen intake phenotypically reproduce the congenital defects. However, in this case variations of the stimulation in time, e.g., by refeeding iodine to the animals, will lead earlier to macroscopic heterogeneity (116). After iodine administration in iodide-depleted glands, the death of many cells, the burst of cell proliferation, and the consequent remodeling of the thyroid may explain these phenomena (70, 275).

D. Thyroid Adenoma

Thyroid adenomas, which by definition are monoclonal, i.e., are constituted by the progeny of one mutated cell, also result from a single initial biochemical lesion. A lesion can be called monoclonal if all cells of the lesion are derived from one cell that initiated the lesion. These cells have some common hereditary properties different from those of normal cells. The primary cell that acquired these properties first is mutated. Although this concept is clear, the terms oligo- and polyclonal are fuzzy; a lesion is monoclonal or is not monoclonal.

Adenomas are well-encapsulated homogenous neoplasia, whereas nodules are benign neoplasia that do not fit this definition (323). In mice Thomas et al. (323) have demonstrated that adenomas constitute the progeny of one mutated cell, i.e., are monoclonal; nodules are non-clonal. This has been recently confirmed for human thyroid lesions (133, 226).

There is ample indirect and direct evidence that monoclonal adenomas of various types exist in humans. 1) The demonstration of a common biochemical defect in the whole lesion can best be explained by a genetic defect by somatic mutation in the cell at the origin of a clone. Defects entirely analogous to congenital defects have been demonstrated in some well-studied adenomas: iodide trapping defects with normal iodination and iodination defects with normal trapping (66, 67, 100), constitutive activation of the GTP-binding protein that positively controls adenylate cyclase (175), enhanced iodide trapping and lack of protein substrate of cAMP-dependent protein kinase in autonomous ade-
In autonomous adenomas the function without TSH must be higher than in normal tissue without TSH (otherwise the "tumor" would behave normally) but can be set at different levels. If it is assumed that a given cell has a maximal level of activity [in terms of enzyme kinetics, its maximum uptake rate ($V_{max}$)], autonomy can be defined as the ratio between basal activity without TSH ($V_0$) and $V_{max}$. It varies from a minimal value of $V_0$, corresponding to the basal activity of the normal tissue, to 1, where $V_0 = V_{max}$ and $V_0/V_{max} = 1$. Defined in this way autonomy is the inverse of the stimulatory ratio. A tumor may lose activity, but if it loosens its control and becomes autonomous, it can only do so by increasing $V_0/V_{max}$ and thus its basal activity $V_0$. Fetal tissue is relatively autonomous for growth with regard to TSH (245).

2) Autonomy is also relative in a qualitative sense. The whole biology (function and growth) can be autonomous: one would expect this from a constitutive activation (mutation) upstream in the common steps in the hormone receptor cascade (e.g., receptors, $G$ proteins, cyclase). On the other hand, some elements of the activating cascade may become autonomous (e.g., function or one step of the metabolism, such as iodide trapping, iodination, or growth). These latter mechanisms would not lead to adenomas as an increase in function only and would not confer any growth advantage.

The simplest example of a somatic mutation leading to autonomous hyperfunctioning adenomas has been demonstrated by Bourne’s group in the hypophysis of acromegalic rats. In the rat somatotrophs, as in dog and human thyroid cells, the activating hormone, growth hormone-releasing hormone, acts by activating adenylate cyclase and the cAMP cascade, which leads to functional activation and growth. In hyperfunctioning, autonomous adenomas of the somatotrophs (175) they demonstrated a mutation in $G_i$, that causes constitutive activation of this transducing protein and consequently of the whole cAMP cascade. A systematic search for similar lesions in other tumors has allowed the demonstration of several identical mutations in the $G_i$ of thyroid adenomas (194). Of course, any somatic mutation leading to the constitutive activation of the first elements of the cAMP cascade (TSH or other stimulating receptor, cyclase, protein kinase) or to the inactivation of a negative controlling element (inhibiting receptor, $G_i$), and a postulated iodine inhibitor XI) could result in a similar phenotype of constitutive activation of the cAMP cascade and consequently of the function and growth of the affected cells, i.e., in an autonomous thyroid adenoma. Similarly the expression in the thyroid of transgenic mice of a constitutively activated adenosine $A_2$-receptor, which stimulates cyclase, induces hyperfunction, growth, and autonomy of the whole thyroid (Ledent and Dumont, unpublished observations). Thus although available data on the cAMP system in the human autonomous thyroid adenoma suggest that the constitutive $G_i$ or adenosine $A_2$-receptors will only explain a minority of cases, they provide a useful paradigm for the study of other mechanisms of autonomy.

Autonomy in these adenomas corresponds to an uncoupling of thyroid function from the level of activation by TSH but not to a total loss of control: the adenomas still contain TSH receptor mRNA (G. Brabant and J. E. Dumont, unpublished observations) and respond to TSH, but the basal level of iodide trapping and thyroid secretion is high and the stimulation ratio much decreased (337). The synthesis of DNA is also high but can be stimulated by TSH (221, 222). Activating mutations of $G_s$ have also been found in differentiated thyroid tumors, suggesting that they contributed to the tumorigenic phenotype (310). As pointed out earlier, not all autonomous nodules observed in vivo are monoclonal adenomas (227). The autonomy of some nodules has been shown to be reversible, which would be difficult to explain in a lesion caused by a mutation (295). Moreover, areas of autonomy may be spread in the whole tissue of a multinodular goiter (210).

Similar mutations inducing constitutive activation in the elements (genes or proteins) of the Ca$^{2+}$-PIP$_2$ cascade of cells secreting by an exocytic process could explain autonomous functional adenomas of such exocrine or endocrine organs. It will be of interest to look for such defects in calcitonin secreting cell tumors.

Well-defined mutations of two of the ras genes have been demonstrated in some adenomas (228). These mutations induce a constitutive activation of the Ras proteins, i.e., of GTP-binding transducing proteins. In fact the hydrolysis of GTP by the protein that abolishes its activity is no longer possible. Although the effector system(s) of the Ras proteins has not been identified yet, its permanent activation leads to a proliferation program and to transformation. Again the autonomy of growth corresponds to a loss of control. The type of ras mutation encountered depends on the cause of the tumor, lesions of Ha-ras in carcinogen-induced tumors and mutation of Ki ras in radiation induced tumors (184). A similar pattern is found in human tumors (358).

The isolated defects in iodide trapping and of iodination in a few well-studied “cold” adenomas could perhaps also explain the growth of these adenomas (66, 67, 294). In the absence of iodine trapping or oxidation, the negative control of iodide is relieved, which might confer a selective advantage to the affected cells, favoring the appearance of new mutations and of tumorigenesis.

Finally, response to heterotypic hormones (e.g., ACTH) would confer autonomy to the affected cells. Such heterotypic responses have been observed in adrenal tumors (203) and with platelet-derived growth factor receptors in an anaplastic thyroid carcinoma (127). However, in thyroid autonomous adenomas, only an increased response to β-adrenergic agents has been found in some cases (337). Undoubtedly, these mechanisms do not account for all thyroid adenomas, and other causes will be found. It should be noted that even in such well-defined clonal lesions as these adenomas the histology may be or become very heterogeneous: local factors such as blood supply and new mutations generate heterogeneity.
E. Sporadic Goiter

It is striking that the cause and mechanisms of the most common form of goiter in developed countries, sporadic goiter, are still completely unknown. The great heterogeneity of such goiters, especially late in their evolution (multinodular goiter), does not exclude a single initial homogeneous cause for each. Indeed, investigation of a series of patients with sporadic goiter suggests a continuous growth and increasing nodularity in the natural history of the disease (20). Moreover, we have seen that the evolution of goiters due to simple causes, such as a defect in iodine metabolism or iodine deficiency, ends up in a similar heterogeneity. Relative iodine deficiency and heterozygocity of congenital defects have long been suspected to play a role in sporadic goiters (5, 103). The introduction of a simple gene for TGF-α with a mammary tissue-specific promoter is sufficient to induce very complex and heterogeneous tumors in the affected transgenic mice (202, 282). In the mechanisms generating this heterogeneity the first is certainly that fixation of mutations requires cell division and thus that the mutation load depends on the number of past mitoses. Any mutation causing a loss of control, i.e., a degree of autonomy, will give a selective advantage to the affected cell. This explains why the permanent stimulation by TSH of the cAMP cascade in human thyroids, i.e., of proliferation and differentiation, eventually leads to an increase in the frequency of thyroid cancers. In this regard secretion of autocrine or paracrine growth factors or modulation of the number of receptors to those factors per cell will, as in many tumors, play a large role. It is also probable that local factors, such as irrigation, or its decrease by sclerosis or autoimmune reactions will greatly modify the tissue biology from one locus to another. It has been well shown in endothelial cells that local factors, such as cell tension and shape, that are controlled by the extracellular matrix greatly influence the proliferation of the cells and their responses to growth factors (141–144). In human thyroid cells a set of changes in protein synthesis induced by TSH and cAMP is reproduced by just culturing the cells in dense aggregates (60). Conversely, treatment of dog cells with TSH or phorbol esters but not with EGF induces a disorganization of the actin network (268) and a switch in the expression of tropomyosin isoforms.

VIII. HYPOTHETICAL AND EXISTING EXAMPLES OF CONSTITUTIVE ACTIVATION OF ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE CASCADE

As shown, among the mitogenic cascades the cAMP pathway is the only pathway that in some cell types induces both proliferation and differentiation and also activates function (84). In the thyroid and hypophysis its permanent stimulation leads to hyperfunctioning hypertrophied lesions. These involve the whole organ in the case of Graves’ disease, where the whole thyroid is constantly stimulated by thyroid-stimulating immunoglobins, or an adenoma in cases where a somatic mutation causes constitutive activation of one element of the cascade in the cell of origin. Many possible somatic mutations could conceivably cause constitutive activation of the cAMP cascade and hyperfunctioning adenomas. Any permanent enhancement of a positive control or suppression of a negative control could give a selective advantage to the affected cells. This advantage would be expressed by a higher mitogenic rate and therefore a higher probability of mutations and of further progression of the cells to autonomy and tumorigenesis. However, only constitutive enhancement of a positive control could lead to autonomy from the normal regulatory feedback control (such as the thyroid pituitary feedback). Similar somatic mutations in cells in which cAMP is a negative signal for growth would of course have the opposite effect; they would select against the affected cells. In the thyrocytes, the following models can be proposed (Fig. 5).

A. Adenylate Cyclase-Coupled Receptors

There are several ways by which receptors could activate adenylate cyclase chronically. Overexpression of the receptor could lead to permanent stimulation if the unoccupied receptor had some basal activity or at least could render the thyrocyte more sensitive to TSH and thus give it a selective advantage. Mutation of the receptor could induce or increase its constitutive activity as has been shown for the EGF receptor in v-erbB oncogene transformation (339). Heterotypic expression of a nonthyroid receptor (such as the β-adrenergic re-
receptor) would make the cells independent of the normal thyroid pituitary feedback. Heterotypic expression of receptors coupled to the PI cascade [serotonin 5-HT$_{1c}$ (154)], a putative angiotensin receptor, i.e., the mas oncogene (147), leads to fibroblast transformation in vitro. Heterotypic expression of platelet-derived growth factor receptors has been demonstrated in anaplastic thyroid carcinoma (127). Similarly, expression of a receptor to a metabolite (e.g., adenosine) or a signal (e.g., a prostaglandin) that is constantly produced by the cell would also cause apparently constitutive activation. Such physiologically constitutive activation has been conferred in culture on various types of cells by the expression of the high-affinity adenosine A$_3$-receptor (198). Expression of this gene in the thyroid of transgenic mice leads to hyperfunction and thus hyperthyroidism and increased proliferation and thus goiter (Ledent and Dumont, unpublished observations). These causes of chronic activation would of course have to override the normal desensitization mechanisms, but these are weak in the thyroid (65; Maenhaut and Dumont, unpublished observations). On the contrary, low levels of thyroid stimulation increase the sensitivity of thyroid cells to TSH (315).

B. Cyclase-Activating GTPase-Binding Protein

Constitutive activation by mutational impairment of the GTPase activity of G~s~ or its subunit ~s~ would, as in the inhibition of this activity by cholera toxin, lead to permanent constitutive activation of adenylate cyclase. This type of somatic mutation, discovered by Landis et al. (175) in pituitary adenomas, has been described earlier. An inactivating mutation of the inhibitory subunit $\beta$ of G~s~ would have the same effect. Overexpression of G~s~ could possibly induce a permanent activation of adenylate cyclase.

C. Adenosine 3',5'-Cyclic Monophosphate-Dependent Protein Kinase

Inactivating mutations of the inhibitory regulatory subunit of cAMP protein kinase lead to constitutive activation of the enzyme. Such mutants have been produced in yeast (185).

D. Negative Controls of Adenosine 3',5'-Cyclic Monophosphate Cascade

Mutations in any negative control element could lead to activation of the affected cell and would confer it a selective advantage, for example, 1) negative feedbacks in cAMP action (phosphorylated protein inhibitor as postulated in autonomous nodules) (397), 2) the receptor-G~i~ pathway, and 3) the iodide inhibitory pathway (at the level of its action on adenylate cyclase iodide transport, oxidation, or at the level of the synthesis of the inhibitory iodinated derivative XI) (48). It is interesting in this regard that adenoma with defects in some steps in the latter pathway have been demonstrated (66, 67, 100).

IX. CONCLUSIONS

Apart from the few well-defined pathologies described, our expanding knowledge on the secretion in the thyroid of growth factors and local hormones and on the effects on thyrocytes of such factors, of cytokines, and of hormones has not been really translated in the study of disease until now. To investigate the role of such factors it will be necessary to ascertain, in primary cultures, which growth factors, local hormones, and neurotransmitters act on human thyroid cells. The recent separations and cloning of these factors and of their receptors now give the tools necessary to investigate, by immunohistochemistry and in situ hybridization on individual cells in thyroid sections, the local pathogenetic process involved in such common diseases as simple goiter and thyroiditis. This will allow researchers to correlate directly the various histological aspects with glimpses of the biological situations of the cells that these tools will provide. Also it will be necessary to investigate by double-labeling methods whether the pictures that histology and autoradiography provide correspond to a more or less permanent situation of a cell or to a snapshot at one point in time of the dynamic evolution of these cells. Concretely, will the follicle that takes up iodide now become quiescent later and vice versa; will the cell that synthezises DNA now become quiescent for long time or will it keep dividing? The next review on the subject will certainly involve a great deal of such information.

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REFERENCES


7. AHUJA, S., and H. ERNST. Hyperthyroidism and thyroid care-


9. AHUJA, S. N., and L. S. GOLD. Too many rodent carcinogene-

10. AOUANI, A., S. HOVÉSPIAN, and G. FAYET. Multithoroidal regula-

11. ASHIZAWA, K., S. YAMASHITA, Y. NAGAYAMA, H. KIMURA, H. HIRAYU, M. IZUMI, and S. NAGATAKI. Interferon-
γ inhibits thyrotropin-induced thyroidal peroxidase gene expres-

12. ATKINSON, S., and P. KENDALL-TAYLOR. Effect of thyrotro-
pin on epidermal growth factor receptors in monolayer cultures of

13. AVVEDIMENTO, V. E., A. M. MUSTI, M. UEFFING, S. OBICI, A. GALLOW, M. SANCHEZ, D. BEBRASI, AND M. E. GOTTES-
MAN. Reversible inhibition of a thyroid-specific trans-acting

14. BACHRACH, L. K., M. C. EGGO, R. L. HINTZ, AND G. N. BURROW. Insulin-like growth factors in sheep thyroid cells: ac-

15. BACHRACH, L. K., M. C. EGGO, W. W. MAK, AND G. N. BURROW. Phorbol esters stimulate growth and inhibit differen-

16. BASERGA, R. The Biology of Cell Reproduction. Cambridge, MA: 


18. BECKS, G. P., M. C. EGGO, AND G. N. BURROW. Organic iodide
inhibits deoxyribonucleic acid synthesis and growth in FRTL 5

19. BELLUR, S., K. TAHARA, M. SAIJ, E. F. GROLLMAN, AND L. D. KOHN. Repeatedly passed FRTL-5 rat thyroid cells can develop insulin and insulin-like growth factor-I-sensitive cyto-
exoxygenase and prostaglandin E2 isomerase-like activities to-

20. BERGHIOUT, A., W. M. WIERINGA, N. J. SMITS, AND J. L. TUVEK. Interrelationships between age, thyroid volume, thy-
roid nodularity, and thyroid function in patients with sporadic

21. BERTLINGERI, M. T., A. M. MUSTI, V. E. AVVEDIMENTO, R. DI LAURO, P. P. DI FIORE, AND A. FUSCO. The block of thyro-
globulin synthesis, which occurs upon transformation of rat thy-
roid epithelial cells, is at the transcriptional level and it is asso-

22. BERTLINGERI, M. T., C. PORTELLA, M. CRIEGO, M. SAN-
TORE, AND A. FUSCO. Cooperation between the polyomavirus
middle-T-antigen gene and the human c-myc oncogene in a rat
thyroid epithelial differentiated cell line: model of in vitro pro-

23. BIDEY, S. P. Control of thyroid cell and follicle growth. *Tendo-

24. BILAH, M. M., AND J. C. ANTHE. The regulation and cellular
functions of phosphatidylcholine hydrolysis. *Biochem.* 7: 203-
211, 1990.

25. BLACK, E. G., A. LOGAN, J. R. E. DAVIS, AND M. C. SHEP-
PARD. Basic fibroblast growth factor affects DNA synthesis and
cell function and activates multiple signalling pathways in rat
thyroid FRTL-5 and pituitary GM3 cells. *J. Endocrinol.* 127: 30
46, 1990.

26. BLAU, H. M., AND D. BALTIMORE. Differentiation requires con-

27. DONE, E., L. D. KOHN, AND P. CIOMCZYNSKI. Thyroglobulin
gene activation by thyrotropin and cAMP in hormonally depleted
1266, 1986.

28. ROSWALD, J. M., S. HARASIM, AND R. MAURER-SCHULZ. Tracer
dose and availability time of deoxyribonucleic acid and bromodeox-
yuridine: application of bromodeoxyuridine in cell kinetic stud-

29. BRANDI, M. L., C. M. ROTELLA, C. MAVILIA, F. FRANCES-
CHELLI, A. TANINI, AND R. TOCCAFONDI. Insulin stimulates cell
growth of a new strain of differentiated rat thyroid cells. *Mol.

30. BRAY, G. A. Increased sensitivity of the thyroid in iodine-de-

31. BRENNER, G. A. Increased sensitivity of the thyroid in iodine-de-

32. BRENNER, G. A. Increased sensitivity of the thyroid in iodine-de-

33. BRENNER, G. A. Increased sensitivity of the thyroid in iodine-de-

34. BRENNER, G. A. Increased sensitivity of the thyroid in iodine-de-


80. DUMONT, J. E., G. VASSART, AND C. REFETOFF. Thyroid disorders. In: The Metabolic Basis of Inherited Disease, edited by...
108. 107. 103. 690 98. 93. 92. VAN SANDE, J. E. DUMONT, donic acid in the regulation of cell proliferation of isolated por-

phenoidal surgery. other parameters of thyroid function in acromegaly after transs-

GERARD, C., A. LEFORT, D. CHRISTOPHE, F. LIBERT, and G. N. BURROW. Cultured thyroids. Is immortality the an-


98. 93. 92. EMOTO, O., I. ISOZAKI, M. ARAI, H. MURAKAMI, K. SHI-

ZUMI, A., M. TASHIMA, and M. DEMURA. Identification and characterization of basic fibroblast growth factor in


92. 91. ENDO, T., T. SHIMURA, T. SAITO, and T. ONAYA. Cloning of malignantly transformed rat thyroid (FRTL-5) cells with thyro-


91. 90. ENOMOTO, T., H. SUGAWA, S. KOSUGI, D. NOUFE, T. MORI, and T. SHIMURA. Prolonged effects of recombinant human inter-


89. 88. ERMANS, A. M., and M. CAMUS. Modifications of thyroid func-


88. 87. ERRICK, J. E., K. W. A. ING, M. C. EGOO, and G. N. BURROW. Growth and differentiation in cultured human thyroid cells: ef-


85. 84. FAYET, G., M. MICHEL-BECHET, and S. LISSITZKY. Thyro-

tropin-induced aggregation and reorganization into follicles of isolated porcine thyroid cells in culture. Eur. J. Biochem. 24: 100-

111, 1971.


82. 81. GARBI, C., G. COLLETTA, A. M. CIRAFICI, P. C. MARCHESSIO, and L. NITSCH. Transforming growth factor beta induces cyto-


80. 79. GÄRTNER, R. W. GREI, P. DRHMART, and K. HORN. Involvement of cyclic AMP, iodine and metabolites of arachi-


79. 78. GEELEHOED-DUIJVESTIJN, P. H. L. M., J. K. BUSSEMVERK, and F. ROELPSEMA. Changes in basal and stimulated TSH and other parameters of thyroid function in acromegaly after transs-


76. 75. GÉRARD, C. M., P. R. ROGER, and J. E. DUMONT. Thyroglobulin,


73. 72. GILLET, C., J. CORVILAIN, J. MATTE-HIRIART, D. WIL-

LEMS, and P. BERGMANN. Effect of acute hypercalcemia on thyrotopin (TSH) and triiodothyronine responses to TSH-


71. 70. GOLDSTEIN, S., J. W. HAMMACHER, J. MARK, C. H. HELDIN, and J. ERRICK. The labelling index is not always reliable. Discrepan-

cies between the labelling index and the mitotic rate in the rat


70. GRUBECK-LOEBENSTEIN, B., G. BUCHAN, R. SADEGHI, M.


68. HAAKJOLD, E., S. B. REFSUM, R. BJERKES, and T. O.

PAULSP. The labelling index is not always reliable. Discrepan-

cies between the labelling index and the mitotic rate in the rat


66. HAYE, B. J. L. AUBLIN, S. CHAMPION, B. LAMBERT, and C.


65. HEIKKILA, R., G. SCHWAB, S. WICKSTROM, S. LOONG

L. R. BACHRACH, G. FAYET, J. ERRICK, J. E.

HAMMACHER, J. MARK, C. H. HELDIN, and J. ERRICK. The labelling index is not always reliable. Discrepan-

cies between the labelling index and the mitotic rate in the rat


64. HELDIN, C. M., P. R. ROGER, and J. E. DUMONT. Thyro-


198. MATSUI, Y., S. A. HALTER, J. T. HOLT, B. L. M. HOGAN, AND


REGULATION OF THYROID CELL PROLIFERATION AND DIFFERENTIATION


