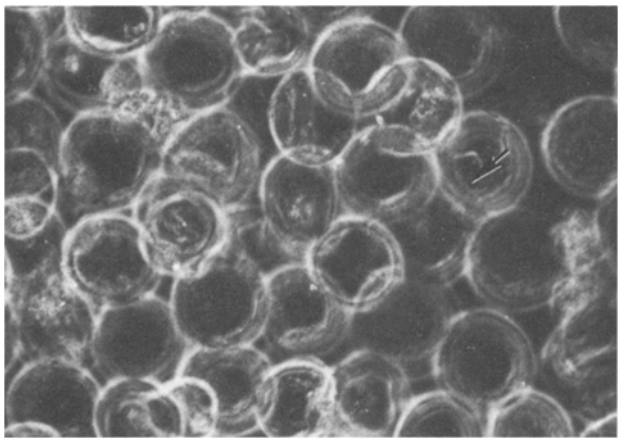

Dark field picture of part of a sample of isolated zonae pellucidae suspended in a liquid medium. Concentric circles in several units correspond to the various planes of phocus (arrow).

order to collect the components set free by disruption of the ovaries; intact follicles, naked oocytes, empty ZP and follicle cells. After this step the sample was centrifuged, washed in PBSS and centrifuged again at 200 rpm, (2 min each), in order to separate the population of follicle cells.
The sample was then resuspended in 2 ml of PBSS and gently deposited on the top of a discontinuous gradient composed of 2 layers. The one at the bottom (A, 3 cm high) is a 20% sucrose in PBSS and the intermediate layer (B, 6 cm high) is a phenol red colored PBSS + 8% sucrose.
Differential sedimentation of the various components according to their mass occurs in about 20–30 min. After this period the upper half of the intermediate layer (B) was pipetted out and centrifuged briefly. The supernatant was discarded and the pellet placed in a culture dish to check the quality of the sample at the light microscope level.
Some of the samples were fixed in 2.5% glutaraldehyde in phosphate buffer, (pH: 7.2) for 1 h at 4 °C and postfixed in 1% buffered OsO_4 followed by embedding in Durcupan ACM (Fluka). Sections were made in a Sorvall MT2 ultramicrotome, stained in uranyl acetate and examined in a Siemens Elmiskop 1.

Results and discussion. Using this method, which is a modification of the one already published for the isolation of ovarian follicles[6] and ovarian oocytes of mice[7], it is possible to obtain 15–25 entirely free ZP per ovary. The total number of ZP depends only on the number of ovaries used in each squashing experiment. The time required after the ovaries are placed on the mesh until the ZP are free and completely isolated from other ovarian components is no longer than 1 h.
In these experiments most of the free ZP were found in the upper half of the intermediate layer (B), well preserved oocytes were found in the lower half of the same layer and intact follicles were found in layer (A). Only a part of the free follicle cells were found in the top layer (original sample).
Examination of the samples at the light and electron microscope level showed that cytoplasmic components were absent from isolated ZP. The figure included in this paper illustrate at the light microscope level, the appearance of ZP in a fresh sample.
Collection of ZP from small mammals by the method proposed here offers the following advantages; ZP can be easily obtained in desirable quantities; the procedure is not time consuming and does not require special equipment and reagents; careful handling of the samples yields ZP almost entirely free of contaminants from ooplasm or from follicle cells, and other ovarian cell types can be simultaneously obtained for other studies.

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0014-4754/83/040420-02$1.50 + 0.20/0

Control of growth in the rat thyroid – an example of specific desensitization to trophic hormone stimulation

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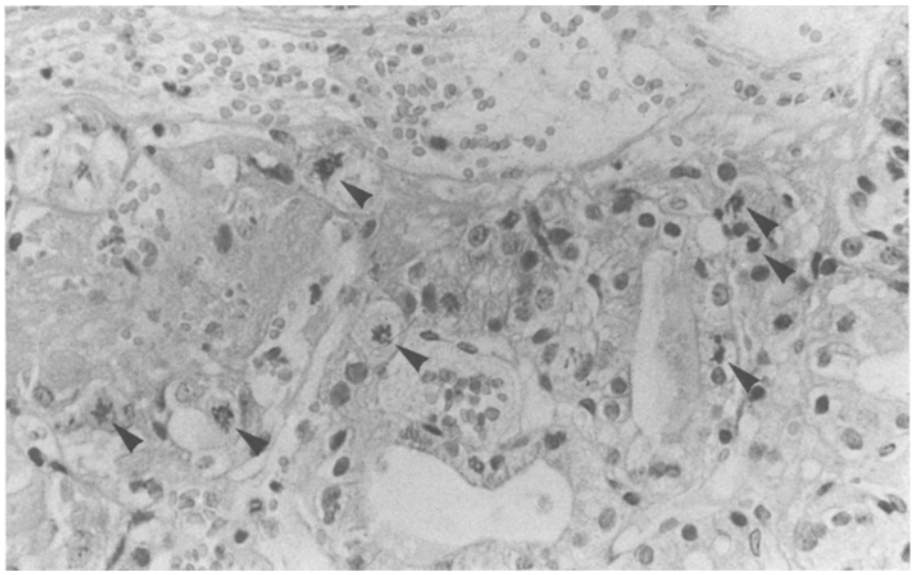
August 13, 1982

Summary. Rat thyroid follicular cells desensitized to the mitogenic action of TSH by prolonged stimulation retain the capacity to respond to the mitogenic stimulus of wounding. The implications for thyroid growth control and neoplasia are discussed.

Prolonged growth stimulation leads in many tissues to tumor formation[1-3]. The dramatic initial response to trophic hormone however is usually short-lived[4-6] and a period of quiescence follows before tumors develop. The thyroid, with its easily-manipulated trophic stimulus – thyrotrophin (TSH) – provides an excellent model for studying this phenomenon. Sustained elevation of serum TSH leads to a limited period of thyroid growth[7,8] followed by a 'plateau' before tumors appear[9]. We have shown[8] that the lack of a continued growth response of the normal follicular cell to TSH is due to a decline in mitotic activity rather than to an increase in cell death rate. We have also shown[8] that this occurs despite a sustained increase in follicular cell function, thus demonstrating that there is an uncoupling of the growth and functional responses to TSH during prolonged stimulation.
To test the specificity of this loss of mitotic responsiveness, we have now investigated the effect of an alternative stimulus – wounding – on these chronically TSH-stimulated thyroids.
Materials and methods. Seven male Wistar rats aged 12 weeks were treated with the goitrogen aminotriazole



Photomicrograph of wound edge 2 days after incision of thyroid isthmus. Many follicular cell mitoses (arrowed) can be seen in follicles adjacent to the wound (which forms the top half of the field). (Hematoxylin and eosin, ×372.)

(0.1% in the drinking water) for 4 months, a regimen which blocks all detectable thyroid hormone synthesis[10], and gives a sustained 5-fold elevation of serum TSH[8].

Anesthesia was induced with ether and maintained with halothane. The neck was opened by a midline incision and the strap muscles separated. The thyroid isthmus was incised with sharp scissors and the neck was closed with skin clips. Two days later the animals were injected i.p. with the metaphase-arrest agent vincristine (1 mg/kg) and sacrificed after a further 4 h, using urethane - 16.8 mmoles/kg i.p. The thyroids were resected on the trachea, fixed in buffered formalin, and then carefully dissected off and embedded in paraffin wax. Serial sections were cut through the isthmus at right angles to the wound edge. Beginning with the first complete isthmus profile, 3 sections were taken at 100 µm intervals and stained with haematoxylin and eosin.

The number of follicular cell metaphases and follicular cell nuclei was counted manually in square fields of side 300 µm using a ×40 objective and their ratio, the metaphase index, used as an index of mitotic activity[11]. For each section, in both lobes, 3 alternate fields were quantified adjacent to the wound edge, and 3 random fields from the body of the lobe, giving a total per animal of 18 'wound edge' fields and 18 'background lobe' fields.

Results. At sacrifice the mean body weight (± SEM) of the rats was 480±31 g, with thyroid weights of 291±34 mg. The mean follicular cell metaphase index (± SEM) in the background thyroid was 0.1±0.02%, which was close to that found previously in unoperated animals treated for 4 months with aminotriazole. In follicles immediately adjacent to the wound edge there was a dramatic increase in mitotic activity to 2.3±0.3% ($p < 0.001$), a level of activity which was higher than the peak response (1.5±0.23%) seen throughout the thyroid shortly after introduction of goitrogen treatment[6] (fig. 1). (The mean metaphase index in normal controls of similar age was found previously to be 0.012±0.002%[8].)

Discussion. This result clearly demonstrates that follicular cells of these goitrous thyroids, which have become desensitized to the mitogenic action of TSH following prolonged stimulation, retain the ability to respond to the alternative stimulus of wounding. It is not known whether the effect of wounding in the thyroid is mediated by the local release of a mitogenic 'wound hormone', such as platelet-derived growth factor[12], or through a local decline in the concentration of a mitotic inhibitor, as has been shown in the skin[13]. We can conclude, however, that the mechanism which limits thyroid growth during long-term TSH stimulation is not a nonspecific limitation of the number of divisions that a follicular cell may undergo (analogous to the 'Hayflick limit'[14]), but is a specific desensitization to the mitogenic action of its trophic hormone, which could be mediated at prereceptor, receptor, or post-receptor level[15].

It is, of course, possible that this growth limiting control mechanism does not operate equally on all follicular cells so that a small minority retain a persistently high mitotic rate, and that it is from this sub-population that tumors subsequently arise. We have found no evidence, however, for a focal distribution of the residual mitotic activity in the 'plateau' phase of pre-neoplastic thyroid growth. We will therefore assume here that all cells are equally desensitized. This leads us to postulate that the initial step in thyroid tumor formation is simply a sporadic breakdown of this stimulus-specific control, giving rise to a clone(s) of cells which will continue to divide in the presence of TSH. This hypothesis explains the known hormone-dependency of early experimentally-induced thyroid follicular cell tumors[16].

Similar patterns of growth limitation have been shown in a variety of other hormonally-regulated tissues, including prostate, seminal vesicle, and endometrium[4-6], and early hormone dependency is also a frequent feature of experimental tumors in such tissues[17-19]. We therefore suggest that specific desensitization of the mitotic response to a trophic hormone is a general mechanism of growth control, and that its breakdown may represent the initial step in the development of neoplasia in hormonally-controlled tissues. This hypothesis offers the encouraging implication that the early stages of neoplasia are in many cases due to a potentially readily identifiable defect in a single pre-existing control pathway.

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0014-4754/83/040421-03$1.50+0.20/0

Studies on the mechanism of the enhanced cold-induced TSH secretion in spontaneously hypertensive rats[1]

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Summary. Various noradrenergic and tryptaminergic antagonists as well as pinealectomy significantly inhibited cold-induced TSH secretion in SHR as in control rats.

Dysfunction of the central noradrenergic and tryptaminergic systems has been proposed in spontaneously hypertensive rats (SHR)[3-6]. Several abnormalities in the endocrine function of these animals have also been suggested[7]. Thyrotropin (TSH) levels in serum have been found to be higher in SHR than in normotensive control rats[8-10]. Our recent studies[11] indicated that the cold-induced TSH secretion is enhanced in SHR accustomed to +30 °C. The last finding suggests that the disturbance in the regulation of TSH secretion in SHR is located in the hypothalamus, since the TSH cold-response is known to be mediated through the activation of hypothalamic TRH neurons[12].

Previous studies indicate that central noradrenergic and tryptaminergic systems exert a stimulatory action on the TRH-TSH secretion in rats[13-16]. Also an intact pineal gland is necessary for the normal TSH cold-response[17]. In the present study we have tried to find out whether the potentiation of the TSH cold-response in SHR could be caused by an increased activity or sensitivity of noradrenergic or tryptaminergic neurons, or of the pineal gland. This was pursued by comparing the effect of various noradrenergic and tryptaminergic antagonists or pinealectomy on the TSH cold-response in SHR and in Wistar-Kyoto (WKY) control rats.

Materials and methods. SHR (blood pressure constantly between 150 and 210 mmHg) and WKY control rats (blood pressure less than 135 mmHg) were originally obtained from NIH and then inbred in our department. They were fed with ordinary laboratory pellets (iodine content 0.5–1 mg/kg) and tap water ad libitum. The animals were kept individually in plastic cages in a dark silent room with artificial illumination from 07.00 h to 19.00 h. The rats were adapted to +30 °C for 7 days before experiments were performed between 13.00 h and 15.00 h. All rats were exposed to cold (+4 °C for 30 min) to elevate serum TSH levels through the activation of hypothalamic TRH neurons[12]. The rats were decapitated immediately after the cold-exposure and the trunk blood was collected. Serum TSH concentration was measured by radioimmunoassay as described by Ranta[18]. Pinealectomy was performed 3–4 days before experiments as described earlier[17]. The following drugs were used: Ca-fusarate (Orion, Helsinki); p-chlorophenylalanine (pCPA, Sigma, St. Louis); metergoline (Farmitalia Carlo Erba, Milan), α-methyl-p-tyrosine methylester hydrochloride (α-MPT, Kistner, Goethenburg) and phenoxybenzamine hydrochloride (SKF, Welwyn Garden City). α-MPT and phenoxybenzamine were dissolved in 0.9% saline while pCPA and Ca-fusarate were suspended in 0.5% carboxymethyl-cellulose. Metergoline was dissolved in 1 drop of concentrated acetic acid, then diluted with 0.9% saline. All injections were given i.p. in a vol. of 1 ml/100 g of b.wt 1.5 h before sacrifice. An equal volume of diluent was given to the controls. The doses of the drugs given as salts refer to the respective acids or bases. Arithmetical means, SEMs and SDs were calculated. Student's t-test was used for comparison of 2 means. The p-values < 0.05 were considered statistically significant.

Results. Noradrenergic antagonists. α-MPT, an inhibitor of tyrosine hydroxylase[19], Ca-fusarate, an inhibitor of dopamine-β-hydroxylase and phenoxybenzamine, an α-receptor blocking drug[21] inhibited the TSH cold-response in both SHR and WKY control rats (fig. 1). The inhibition caused by Ca-fusarate and phenoxybenzamine was similar in both groups of rats whereas the effective dose of α-MPT was lower in SHR than WKY rats.

Tryptaminergic antagonists. pCPA, an inhibitor of tryptophan hydroxylase[22] and metergoline, a 5-HT receptor blocking drug[23] caused a significant inhibition of the cold-stimulated TSH secretion in SHR and WKY controls (fig. 2). Metergoline was as effective in both groups while the lowest dose of pCPA causing a significant inhibition was smaller in SHR than WKY control rats.

The effect of pinealectomy, performed 3–4 days before sacrifice, on the cold-induced TSH secretion in SHR and WKY control rats

Treatment	Serum TSH (ng/ml) SHR	WKY control rats
Intact	9546±833 (6)	3000±492 (6)
Pinealectomy	5748±972** (5)	1715±285* (6)

Mean±SEM. Number of animals in brackets. Statistics: *$p<0.05$; **$p<0.01$ vs corresponding intact control.