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The Effect of Thyrotropin (TSH) Levels on Follicular Cluster Formation from Grafted Monodispersed Rat Thyroid Cells[1,2] (41603)

HIROMITSU WATANABE,[3] MICHAEL N. GOULD, DAVID P. ROSE, AND KELLY H. CLIFTON

Departments of Human Oncology and Radiology, Wisconsin Clinical Cancer Center, University of Wisconsin, Madison, Wisconsin 53792

Abstract. The effect of experimental conditions which alter TSH levels on the number and size of the multifollicular thyroid clusters which develop from inoculated monodispersed thyroid cells was explored. Five week-old male Fischer rats were used as thyroid cell donors and recipients. The recipients were totally thyroidectomized (Tx) 1 week before thyroid cell transplantation (group A), hemithyroidectomized (hemi Tx) 1 week before transplantation (group B), or Tx 1 day before grafting (group C). Additional groups of recipients included intact animals maintained on low-iodine diet (LID) (group D), and on normal diet (ND) (group E). The FD_{50}, the number of grafted cells required to produce at least one follicular cluster in 50% of the transplant sites, was the lowest in group A and the highest in group E. The TSH levels in the serum on the day of cell inoculation were highest in group A and lowest in group E. Large follicular clusters tended to be more frequent in groups A and C than in group B, but the data were variable; no large clusters were found in groups D or E. The TSH levels in groups A and C 28 days after cell implantation were higher than in group B. The FD_{50} is thus dependent on the concentration of circulating TSH for initiation of follicle formation; large follicular clusters result from continued elevation of TSH levels.

Techniques for the preparation and quantitative transplantation of monodispersed suspensions of rat mammary (1) and thyroid epithelial cells (2–6) have been described previously. Normal thyroid follicular units (FU) develop when sufficient numbers of viable monodispersed rat thyroid cells are inoculated into the subcutaneous fat pads of histocompatible recipients. The number of cells required for FU formation in 50% of the graft sites (FD_{50}) is less in hypothyroid than in euthyroid animals (4, 5); in intact recipients the FD_{50} depends on donor and recipient age and sex (6). FU in thyroidectomized (Tx) animals are larger than those in intact animals (4, 5). In this study, we investigated the effects of experimental manipulations that alter TSH levels on both the number and size of thyroid follicle clusters.

Materials and Methods. *Animals and experimental groups.* Male Fischer rats, 5 weeks old on the day of grafting, were used throughout. Some intact recipients were fed commercial rodent chow (ND) and acidified deionized water *ad libitum.* Thyroidectomized and hemithyroidectomized animals and some intact animals were maintained on a Remington low-iodine diet (LID) (ICN Nutritional Biochemicals, Ohio) and deionized drinking water supplemented with calcium–glucose. All were housed in a temperature- and humidity-controlled room with a 12-hr light:12-hr dark cycle.

The recipients were divided among five groups. In one, the rats were totally Tx under light ether anesthesia 1 week before thyroid cell transplantation (group A); in another, the right thyroid lobe only (hemi Tx) was removed 1 week before grafting (group B); a third group was Tx 1 day before grafting (group C). In an additional group, the recipients were intact animals maintained on LID from the day of grafting (group D); a final group, also intact, was fed ND (group E). Extra rats were included in groups B and E; these were killed

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[3] Present address: Research Institute for Nuclear Medicine and Biology, Hiroshima University, Kasumi 1-2-3, Hiroshima 734, Japan.

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on Day 0 (day of grafting) for determination of thyroid lobe weights. Some experimental groups were replicated several times.

Thyroid cell preparation and inoculation. Details of the experimental methods have been reported (2–5). Briefly, donor thyroid glands were dissected free from the trachea, pooled, and minced in cold Medium 199. The minced tissue was then incubated for 2 hr in 300 U collagenase/ml solution (Type II, Worthington Biochemical Co., N.J.) at 37°C followed by 1.25% Pronase (Calbiochem-Behring Co., Calif.) for 90 min at 4°C and then passed through a 53-μm cloth filter to remove cell aggregates. A small amount of Deoxyribonuclease I (Sigma Chemical Co., Mo.) was added to reduce cell clumping. Appropriate dilutions of known numbers of cells were prepared in fresh Medium 199 with the aid of a hemocytometer and 400× phase microscope. Only nucleated cells with intact plasma membranes were counted as morphologically "viable." Appropriate serial dilutions were then prepared in Medium 199 and mixed with an equal volume of brain homogenate so that the desired number of cells could be injected in a volume of 0.06 ml. Each recipient received one injection at each of five sites on Day 0, one into the interscapular (i.s.) white fat pad and two into each of the medioventral and laterodorsal inguinal fat pads; all five sites in a single recipient were injected with inocula from the same cell dilution. Generally, six different cell dilutions were each injected into 15 to 24 transplant sites in each experiment. Four weeks after cell transplantation, the rats were autopsied and their fat pads were removed for analysis of the graft sites.

Analysis of thyroid growth. As previously described (2–6), the fat pads were fixed in Bouin's solution, stained with hematoxylin, cleared, and examined under a dissecting microscope for the presence of thyroid follicles. Positive transplantation was scored by the presence of at least one follicular unit (FU), defined as being comprised of two or more follicles. The fraction of positive transplant sites within each cell dilution group was determined and the data were fitted with the aid of a computer program designed after the terminal dilution transplantation assay model of Porter *et al.* (3–5). This model states that:

$$P = 1 - e^{-M}$$

and

$$\log M = \log K + S \log Z$$

where P is the probability of one or more FU, M is the average number of clonogens inoculated, K is the clonogenic fraction, Z is the number of morphologically viable thyroid cells in the inoculum, and S is the slope of the linear portion of the cell-dose–FU-response relationship. Log K and S were generated from the data with a computerized iterative method using a maximum likelihood procedure and were then used to calculated FD_{50} values, i.e., the average number of morphologically viable cells required to produce one or more FU in 50% of the transplant sites.

In addition, the number of follicular clusters in each site was recorded and cluster size was measured. Follicular clusters greater than 300 μm in diameter were classed as large; those less than 300 μm were classed as small. Statistical evaluations of the data were made by Student's t test and analysis of variance.

Thyrotropin (TSH) determination. TSH levels were determined in the sera of animals from each experimental group. Two milliliters of blood was collected by retroorbital puncture at the time of transplantation and from the abdominal aorta before autopsy 4 weeks after cell transplantation. Animals were lightly anesthetized with Ketamine HCl (Ketalar, Parke-Davis, Morris Plains, N.J.), during blood collection. The blood collections were made at 9:00–10:00 AM because of reports of a circadian rhythm in serum TSH levels (7–9). The samples were centrifuged and the serum was removed and stored at −60°C until analyzed. TSH concentrations were determined by double antibody radioimmunoassay, with the materials and procedure provided by the Pituitary Hormone Distribution Program, National Institute of Arthritis, Kidney and Digestive Diseases. The reference hormone preparation used was NIAKDD-rat TSH RP-1. Goat anti-rabbit antiserum was purchased from Calbiochem-Behring Corporation, La Jolla, California.

Results. The TSH assay results and thyroid lobe weight data indicate the following. In group A, TSH titers were elevated sevenfold at the time of grafting, and had doubled again by the end of the experiment (Table I). The FD_{50} was the least (Fig. 1) and the number of

TABLE I. SINGLE THYROID LOBE WEIGHTS AND TSH TITERS IN THE TREATMENT GROUPS

		Day 0			Day 28		
		Thyroid lobe weight		TSH	Thyroid lobe weight		TSH
Group	Treatment (Day)[a]	mg (n)[b]	mg/100 g	μg/ml (n)[b,c]	mg (n)[b]	mg/100 g	μg/ml (n)[b,d]
A	Tx, LID (−7)	—	—	4.8 ± 0.9 (8)	—	—	11.0 ± 1.2 (6)
B	hemi Tx, LID (−7)	6.0 ± 0.8 (21)	10.0 ± 1.5	1.3 ± 0.1 (5)	24.4 ± 7.5 (20)	17.8 ± 5.4	0.6 ± 0.1 (6)
C	Tx, LID (−1)	—	—	1.6 ± 0.7 (5)	—	—	9.7 ± 1.6 (6)
D	LID (0)	—	—	—	12.9 ± 3.8 (19)	8.6 ± 2.1	0.6 ± 0.1 (6)
E	ND	4.4 ± 0.7 (19)	4.8 ± 0.6	0.7 ± 0.1 (6)	6.0 ± 0.6 (6)	3.6 ± 0.3	—

[a] Tx indicates complete thyroidectomy; hemi Tx indicates unilateral thyroidectomy; LID and ND are low-iodine and normal diets, respectively; numbers in parenthesis indicate 7 days before, 1 day before, and day of grafting, respectively.

[b] Mean ± SD. Numbers in parenthesis indicate number of observations. Note: Differences between thyroid lobe weights of groups B and E (Day 0) and among thyroid lobe weights of groups B, D, and E (Day 28) all significant at $P < 0.001$.

[c] A vs B, C, or E, $P < 0.001$. B vs E, $P < 0.001$. C vs E, $P < 0.02$.

[d] A or C vs B or D, $P < 0.001$.

follicular clusters the greatest in this group (Fig. 2).

In groups B and C, TSH levels were significantly higher than in intact rats of group E at the time of grafting (Table I). The FD_{50} values and total number of follicular clusters per 1000 cells were similar in these two groups; the FD_{50} values were significantly greater and the numbers of follicular clusters less than in group A (Figs. 1 and 2). In group B, however, compensatory hypertrophy of the remaining thyroid lobe was evident by the day of grafting. With continued compensatory hypertrophy of the remaining host thyroid tissue, a near-euthyroid condition was reestablished, as indicated by normal TSH levels at the termination of the experiment. In group C, hypothyroidism became progressively more severe during the experiment, and final TSH

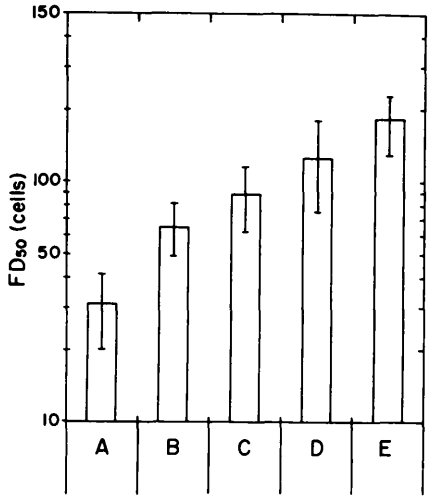

FIG. 1. The FD_{50} values following various Tx and diet conditions. Vertical lines indicate 95% confidence limits. See Table I for treatment regimens. Significant differences ($P < 0.05$): A vs B, C, D, or E; E vs B or C.

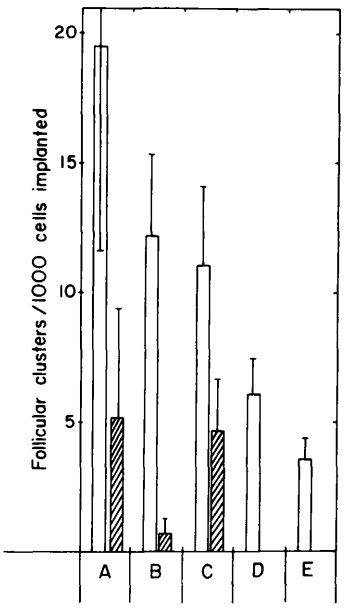

FIG. 2. Effect of Tx and diet on the number of follicular clusters per 1000 cells implanted per injection site. Open bars: total follicular clusters; hatched bars: large follicular clusters; lines: SD. See Table I for treatment regimens. Significant differences, total follicular clusters ($P < 0.002$): A vs B, C, D, or E; B or C vs D or E; D vs E.

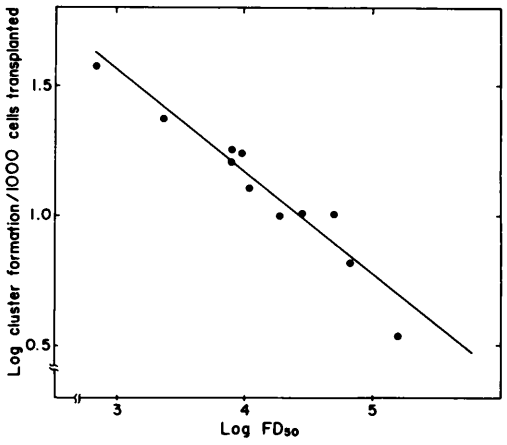

FIG. 3. The correlation of follicular clusters per 1000 grafted cells with the FD_{50} values. ($r = -0.95$, $y = 2.73 - 0.389x$, $P < 0.01$)

titers were very high (Table I). In groups A, B, and C, in contrast to groups D and E, large follicular clusters were observed (Fig. 2), but there were no statistically demonstrable differences in their numbers among these three groups.

In group D, compensatory thyroid hypertrophy appears to have compensated for the reduced iodine of the low iodine diet, and final TSH titers were normal (Table I). The FD_{50} value and the number of clusters observed in the group were not significantly different from those of the euthyroid group E (Figs. 1 and 2). No large follicular clusters were observed in either of these two groups.

Discussion. Mulcahy *et al.* (4) have presented evidence that the proportion of potential thyroid clonogens "triggered" to give rise to FU after transplantation is dependent on the levels of TSH; i.e., the FD_{50} values in rats Tx 1 day before grafting were approximately one-eighth those of grafted euthyroid animals. The higher FD_{50} values in euthyroid hosts were not due to loss of thyroid clonogens from the graft site; FD_{50} values of recipients Tx 1 day before grafting, fed a normal iodine diet, and autopsied 4 weeks after grafting were insignificantly different from FD_{50} values of recipients grafted, TX 4 weeks later, fed a normal diet throughout, and autopsied 8 weeks after grafting, 4 weeks after Tx (4). Mulcahy *et al.* (4) also presented evidence that the inclusion of some dietary iodide is necessary for optimal initiation of FU formation.

This early marked effect of Tx, presumably mediated through elevated TSH titers, is on the *proportion* of clonogens triggered to FU formation. Once FU development is initiated it proceeds similarly in euthyroid and Tx animals; *i.e.*, the morphology of early individual FU development is indistinguishable in the presence of normal or elevated TSH levels until normal follicles are distinguishable 7 days after grafting (3). Thereafter FU morphology is again dependent on TSH titers.

Recently, Watanabe *et al.* (6) have related age- and sex-dependent differences in FD_{50} values to published reports of TSH titers. The current experiments were designed to further test this hypothesis, and to determine the relationship among the numbers and sizes of follicular clusters and FD_{50} values 4 weeks after grafting, and the TSH levels. We conclude (a) that, in accord with Mulcahy *et al.* (3, 4), "triggering" of potential thyroid clonogens to form FU is directly related to the levels of TSH acting over a relatively short time span, (b) that the subsequent growth of these FU to form large follicular clusters is dependent on sustained elevated TSH titers, and (c) that the number of follicular clusters per 1000 inoculated cells may be a useful index of clonogen survival.

Our current findings are consistent with those of Mulcahy *et al.* (3, 4) on triggering of potential clonogens, and with results of others. Previous reports show that complete thyroidectomy causes an increase in TSH levels within 24 hr, and that this increase is sustained (10–13). Hemithyroidectomy has been found to be followed rapidly by compensatory hypertrophy of the remaining lobe presumably under the influence of elevated TSH titers; this procedure has been employed to measure the capacity of the pituitary gland to respond to thyroid hormone deficiency (14).

Plasma TSH (15–17) and thyroid weight (18–20) have both been reported to increase in animals on restricted iodine, and TSH stimulates proliferation of thyroid epithelium *in vivo* and *in vitro* (21–24). Indeed, the degree of increase in both TSH and gland weight has been shown to be directly related to the iodine content of the specific diet employed (25, 26).

In the current studies, a modest elevation in TSH titers in the hemi-Tx group B as compared to intact group E was apparent on the day of grafting, and was reflected in a signif-

icantly lower FD_{50} value, greater number of follicular clusters, and the occurrence of large follicular clusters. However, by the time of autopsy 4 weeks after grafting and 5 weeks after hemi-Tx, TSH titers in group B were normal. These findings suggest that given an adequate bulk of thyroid tissue resulting from compensatory hypertrophy under transient elevation of TSH, there was adequate iodide in the low-iodine diet employed to reestablish a balanced pituitary–thyroid axis.

Mulcahy *et al.* (4) found that thyroxine levels did not fall below the normal range in such rats until 3–4 weeks after initiation of a low-iodine diet. In the current studies, compensatory thyroid hypertrophy had occurred in group D by autopsy 4 weeks after initiation of the low-iodine diet. This was presumably in response to a transient elevation in TSH levels which had returned to normal by the time of autopsy, in agreement with the results in group B. The elevation in TSH was not adequate, however, to significantly decrease the FD_{50} in group D as compared to euthyroid group E, but it did significantly increase the number of follicular clusters which developed from triggered clonogens.

The inverse relationship between the number of follicular clusters per 1000 inoculated cells and the FD_{50} values was found to be highly significant (Fig. 3). This suggests that these two are parameters of the same phenomenon, the triggering of potential clonogens to FU formation. Under controlled conditions, the number of follicular clusters per 1000 grafted cells might be a useful clonogen assay endpoint.

1. Gould MN, Biel WF, Clifton KH. Morphological and quantitative studies of gland formation from inocula of monodispersed rat mammary cells. Exp Cell Res **107**:405–416, 1977.
2. Clifton KH, DeMott RK, Mulcahy RT, Gould MN. Thyroid gland formation from inocula of monodispersed cells: Early results on quantitation, function, neoplasia and radiation effects. Int J Radiat Oncol **4**:987–990, 1978.
3. Mulcahy RT, DeMott RK, Clifton KH. Transplantation of monodispersed rat thyroid cells: Hormonal effects on follicular unit development and morphology. Proc Soc Exp Biol Med **163**:100–110, 1980.
4. Mulcahy RT, Rose DP, Mitchen JM, Clifton KH. Hormonal effects on the quantitative transplantation of monodispersed rat thyroid cells. Endocrinology **106**:1769–1775, 1980.
5. Clifton KH. Quantitative studies of the radiobiology of hormone responsive normal cell populations. In: Meyn RE, Withers HR. eds. Radiation Biology in Cancer Research. New York, Raven Press, pp501–513, 1980.
6. Watanabe H, Gould MN, Mahler PA, Mulcahy RT, Clifton KH. The influence of donor and recipient age and sex on the quantitative transplantation of monodispersed rat thyroid cells. Endocrinology **112**:172–177, 1983.
7. Rookh HV, Azukizawa M, DiStefano JJ, Ogihara T, Hershman JM. Pituitary–thyroid hormone periodicities in serially sampled plasma of unanesthetized rats. Endocrinology **104**:851–856, 1979.
8. Jordan D, Pigeon P, McRae-Degueurce A, Pujol JF, Mornex R. Participation of serotonin in thyrotropin release. II. Evidence for the action of serotonin on the phasic release of thyrotropin. Endocrinology **105**:975–979, 1979.
9. Jordan D, Rousset B, Perrin F, Fournier M, Orgiazzi J. Evidence for circadian variations in serum thyrotropin, 3,5,3′-triiodothyronine, and thyroxine in the rat. Endocrinology **107**:1245–1248, 1980.
10. Davis SL, Borger ML. The effect of thyroidectomy on the secretion of prolactin and on plasma levels of thyrotropin luteinizing hormone and growth hormone in lambs. Endocrinology **92**:1736–1739, 1973.
11. Connors JM, Hedge GA. Effect of continuous thyroxine administration on thyrotropin secretion in thyroidectomized rats. Endocrinology **108**:2098–2102, 1981.
12. Salaman DF. Thyrotropic hormone in the plasma and anterior pituitary of thyroidectomized rat. J Endocrinol **29**:283–291, 1964.
13. Obregon MJ, Mallol J, Del Rey FE, de Escobar GM. Presence of L-thyroxine and 3,5,3′-triido-L-thyronine in tissues from thyroidectomized rats. Endocrinology **109**:908–913, 1981.
14. Clifton KH, Meyer RK. Effect of food intake on secretion of thyrotropin during diethylstilbestrol treatment. Endocrinology **58**:681–685, 1956.
15. Fukuda H, Yasuda N, Greer MA, Jutas M, Green SE. Changes in plasma thyroxine, triiodothyronine, and TSH during adaptation to iodine deficiency in the rat. Endocrinology **97**:307–314, 1975.
16. Riesco G, Taurog A, Larsen PR, Krulich L. Acute and chronic responses to iodine deficiency in rats. Endocrinology **100**:303–313, 1977.
17. Nataf BM, Fragu P, Othman SB. Relationship between peroxidase activity and serum TSH, T4 and T3 levels in rats in the course of iodine deficiency. Acta Endocrinol **88**:499–505, 1978.
18. Ekpechi OLV, van Middlesworth L. Iodinated compounds in thyroids of the offspring of rats maintained on low-iodine diet. Endocrinology **92**:1376–1381, 1973.
19. Fragu P, Nataf BM. Thyroid peroxidase activity in iodine deficient rats. Acta Endocrinol **82**:535–543, 1976.
20. Levine H, Remington RE, von Kolnitz H. Studies

on the relation of diet to goiter. II. The iodine requirement of the rat. J Nutr **6**:347–354, 1933.
21. Kippen AA, Loeb L. The relation between the quantity of thyroid stimulating hormone of the anterior pituitary gland administered and the proliferative activity and hypertrophy of the thyroid acini in guinea pigs. J Pharmacol Exp Therap **24**:246–257, 1935.
22. Cutting WC. Changes in growth and function of the thyroid after thyrotropic stimulation. Endocrinology **25**:286–287, 1939.
23. Isler H. Loss of mitotic response of the thyroid gland to TSH in hypophysectomized rats and its partial restoration by anterior and posterior pituitary hormone. Anat Rec **180**:369–376, 1974.
24. Pawlikowski M, Kunert-Radek J, Mroz-Wasilewska Z. Biphasic inhibitory and stimulatory effects of thyrotropin on the mitotic activity of thyroid explants cultured *in vitro*. Endokrinologie **73**:186–190, 1979.
25. Riesco G, Taurog A, Larsen PR. Variations in the response of the thyroid gland of the rat to different low-iodine diets: Correlation with iodine content of diet. Endocrinology **99**:270–280, 1976.
26. Okamura K, Taurog A, Krulich L. Elevations of serum 3,5,3′-triiodothyronine and thyroxine levels in rats fed Remington diets: Opposing effects of nutritional deficiency and iodine deficiency. Endocrinology **108**:1247–1256, 1981.

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