Naturally Occurring Clones of Cells with High Intrinsic Proliferation Potential within the Follicular Epithelium of Mouse Thyroids

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ABSTRACT

The proliferation pattern of some scattered clones of naturally occurring follicular cells with an exceedingly high intrinsic growth potential was investigated in the mouse thyroid gland. In particular, evidence was sought to demonstrate that the high propensity to replicate is a stable trait transmitted from the progenitor cells to their offspring. We hypothesize that these cell clones are at the origin of the multiple adenomas that invariably arise in chronically stimulated thyroid.

Growth stimulation was induced either by hemithyroidectomy or by methimazole feeding. In a first series of experiments, involving hemithyroidectomized animals, \( ^3\text{H} \) thymidine was administered continuously for 3 weeks by means of osmotic minipumps, so that all cells entering the mitotic cycle during that time were labeled. Hemithyroidectomy led to a 3-fold increase of the fraction of labeled cells in the remaining lobe. The increase was prevented by thyroxine treatment in thyroid-stimulating hormone-suppressing doses. Autoradiographs of contiguous serial sections across whole follicles showed that roughly 75% of the labeled cells were clustered in groups of 3 or more, rather than being randomly distributed.

In a second set of experiments, glands stimulated by methimazole-induced thyroid-stimulating hormone hypersecretion were pulse-labeled by a single i.p. injection of \( ^3\text{H} \) thymidine. Animals were sacrificed either 2 h or 3 weeks after the administration of the label. The thyroids were excised and the fate of labeled thyroid cells was analyzed autaradiographically. In the 2-h exposure, about 95% of all labeled follicular cells were single and the remaining 5% were in pairs. In contrast, about 50% of all labeled cells were clustered in groups of 3 to 12 cells 3 weeks after the pulse labeling. The number of silver grains per nucleus was compared to that of the identically exposed controls. The intensity of label per cell appeared to be decreased in proportion to the size of the labeled clusters, indicating that clusters had generated several subsequent generations of cells.

The results support previously produced evidence that highly growth-prone cells naturally occur within the normal thyroid and demonstrate, in addition, that their high intrinsic growth rate is a stable, inheritable trait. Cells which replicate at a rate faster than that of the average epithelial cell have a tendency to overgrow during goitrogenesis. They may be at the very origin of the nodules and adenomas commonly found in experimentally produced and naturally occurring goiters.

INTRODUCTION

In the last few years, a number of new and important concepts have been gaining increasing interest in research on tumorigenesis. Among them are inter- and intracloinal heterogeneity of tumor cells and even of postdifferential heterogeneity in normal cells, homeostasis of tumor growth in three-dimensional tissue structure, pathogenesis of autonomy, and regression of autonomous and malignant growth (1-4). The bulk of information on these topics has been obtained from tissue culture and from sophisticated in vivo experiments with benign and malignant tumors, but little is known about the growth of normal tissue in its natural three-dimensional structure (2).

In the course of systematic studies on the mechanism which creates the notorious interfollicular heterogeneity whenever a normal thyroid gland enlarges to become a goiter, we increasingly realized some unique possibilities to investigate, in a naturally growing organ, processes such as the generation of diversity in the progenies of single epithelial cells. We have shown previously that normal human and mouse thyroid epithelial cells are heterogeneous, that the glands contain a few clones of cells with a growth potential much higher than average, and that some of these cells may replicate autonomously, i.e., in the absence of TSH (5). In this paper, we further analyze the intrinsic growth pattern of the rapidly dividing cell clones in normal mouse thyroid glands, their response to gentle TSH stimulation, and the generation of progenies of equally growth-prone cells. We suggest that the rapidly dividing cells may be at the origin of the multiple adenomas that invariably develop in thyroids exposed to long-term TSH stimulation. Many of these adenomas evolve into autonomous tumors and eventually become malignant (6).

MATERIALS AND METHODS

BALB/c mice, approximately 4 months old (25 ± 3 g body weight), were kept at 20°C in a 12-h-light, 12-h-dark cycle and fed standard pellets (EWOS, Södertälje, Sweden) and tap water ad libitum.

Mice were surgically hemithyroidectomized or were sham-operated during pentobarbital anesthesia and allowed to recover for 3 days. Nine hemithyroidectomized and 3 sham-operated mice were selected for the experiment. The hemithyroidectomized animals were divided into 2 groups: group A, hemithyroidectomy only (n = 6); and group B (n = 3), hemithyroidectomy plus thyroxine administration (0.4μg L-thyroxine/ml of drinking water). With a water intake of about 5 ml/24 h this provided approximately 7.4μg of thyroxine/100 g body weight/24 h.

Hemithyroidectomized (groups A and B) and sham-operated mice (group C, n = 3) were given tritiated thymidine ([methyl-\( ^3\text{H} \)] thymidine; specific activity, 6.7 Ci/mmol; New England Nuclear, Boston, MA; concentrated to 5 mCi/ml) continuously for 22 days by means of miniosmotic pumps (Alzet Model 2001; Alzo Corp., Palo Alto, CA) implanted into the peritoneal cavity. Each pump delivered 0.925 μl/h, corresponding to a release of 37 μCi of \([\text{H}]\) thymidine/8-h period (7).

In a second experiment involving 12 mice, endogenous TSH secretion was gently stimulated by adding 0.075% of MMI (Fluka AG, Buchs, Switzerland) to the drinking water. Two weeks after initiation of increased TSH secretion by means of antithyroid treatment, each animal was given an i.p. injection of a single dose of 50 μCi of \([\text{H}]\) thymidine. Groups of 3 animals were sacrificed 2 h, 1 week, 2 weeks, and 3 weeks, respectively, after administration of the thymidine label. MMI administration was continued throughout this time.

Thyroid tissue was fixed by perfusion via the left ventricle of the heart with a solution of 2% glutaraldehyde and 2% formaldehyde in 0.04 M sodium cacodylate buffer, pH 7.2, containing 0.1% sodium azide (8, 9) and dextran T-70 (Pharmacia, Uppsala, Sweden). The thyroid lobes were excised and immersed in the same fixative for 48 h.
at 4°C. After dehydration in a graded series of ethanol and propylene oxide, the tissue was embedded in methacrylate (JB-4 embedding mixture; Polysciences Inc., Warrington, PA). Up to 20 contiguous 3-μm sections were cut at different levels of the thyroid lobes, mounted on glass slides, and dipped in Kodak NTB-2 nuclear emulsion (Eastman Kodak Co., Rochester, NY) for autoradiographic demonstration of [3H]thymidine incorporation into the nuclei of the proliferating cells. After appropriate exposure times the slides were developed with Kodak D-19 developer and counterstained with nuclear fast red. All sections of a single experiment were processed together, thus ensuring semi-quantitative analysis of the data as described previously (5).

The fraction of [3H]thymidine-labeled cells was determined by evaluating 1000 follicular cells/gland and the mean value was calculated for each group. Cells containing 5 or more silver grains were considered to be labeled. The distribution pattern of the proliferating cells within the follicular epithelium was analyzed statistically. The numbers of labeled and unlabeled cells were assessed in 20 different follicles using a modified binomial test procedure which takes into account the variable follicle size. The statistical analysis is described in detail elsewhere (5, 10). In addition, the spatial distribution of the proliferating cells within individual follicles was assessed by reconstruction of entire follicles from contiguous serial sections by means of a projection microscope (Visopan; Reichert Jung, Vienna, Austria) (5).

RESULTS

Fraction of Labeled Follicular Cells and Distribution of Labeled and Unlabeled Cells in Gland Continuously Exposed to [3H]Thymidine for 3 Weeks. In group A (hemithyroidectomy only) 30.4 ± 4.5% (SD) of all follicular cells were labeled. This figure is significantly higher than the 10.2 ± 2.5% of labeled cells present in the follicular epithelium of the sham-operated animals (group C) \((P < 0.001)\) and the 4.2 ± 1.8% observed in hemithyroidectomized animals treated with thyroxine (group B) \((P < 0.001)\).

In none of the thyroids, except those of the 2-h single-shot [3H]thymidine experiment, were the labeled follicular cells randomly scattered among the follicles (deviation from random distribution, \(P < 0.05\)). Rather, a few newly generated follicles contained a much higher than average fraction whereas others contained only a few, if any, labeled cells. Moreover, assessment of the spatial distribution of the labeled and unlabeled cells within individual follicles based on autoradiographed serial sections confirmed the nonrandom distribution of labeling and revealed a strong tendency of the labeled cells to remain in cohorts within the follicular shell (Figs. 1, 2, and 4). In the hemithyroidectomized group (group A), the fraction of labeled cells in individual follicles ranged from nearly 0% in a few follicles to almost 100% in others (Fig. 1). Labeled cells are easily mistaken as being single or paired if only one autoradiographed section is assessed; they show up to be part of labeled clusters only when followed on subsequent contiguous serial sections (Fig. 2). Roughly 75% of all labeled cells were clustered in groups of 3 or more cells. In some follicles, part of the proliferating cells formed protrusions from the follicular shell (Fig. 3). In serial sections these cells were found to form solid buds at the outside of the follicular shell. In group C (sham-operated), labeled cells were present in only about 1 of 10 follicles. Within these follicles, the cells with high replicating activity again remained clustered after multiple divisions (Fig. 4), so that cohorts of cells with a similar growth potential were formed.

Distribution of the Labeled Follicular Cells after Single-Shot Labeling during Continuous MMI Administration. Two hours after the administration of a single injection of 50 μCi [3H]thymidine, 0.1% of all follicular cells were labeled, and 190 of 200 labeled follicular cells evaluated on 45 serial sections were randomly distributed as single cells with no clustering, while 10 cells were in pairs of 2 adjacent cells. Clusters composed of 3 or more cells were not observed in this group (Fig. 5A).

In glands examined 3 weeks after administration of the single [3H]thymidine injection, 104 of 200 labeled follicular cells assessed on 45 serial sections occurred as pairs of labeled cells, whereas the remaining 96 cells formed clusters of up to 12 labeled cells (Fig. 5B). This indicates that some of the cells had passed through only one cell division since the time of the pulse labeling, whereas others must have divided several times. Accordingly, the density of silver grains in paired cells was very high, whereas it appeared to be diluted in the larger cohorts of labeled cells (Fig. 5).

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Fig. 1. Autoradiographed section from the remaining lobe of hemithyroidectomized animal labeled continuously with [3H]thymidine for 3 weeks. Nearly all cell nuclei of follicle 1 are labeled with [3H]thymidine. In contrast, follicles 2 and 3 contain no labeled cells. Follicle 4, shown in part only, contains a small cluster of labeled cells. All findings were confirmed in contiguous sections across the entire follicle.
Fig. 2. Three autoradiographs selected from 21 serial 3-μm sections from a thyroid of a hemithyroidectomized animal. In A, follicle 1 (1) is tangentially cut. Nearly all cells of this follicular region are labeled. B and C, cut at lower levels, show the lumen of follicle 1. If only B had been assessed, it would appear that the follicular shell contains two independent groups of proliferating cells. However, screening of all sections interposed between A and C reveals that the labeled cells form one single coherent cluster. In contrast, follicle 2 (2) is virtually unlabeled on all sections. Follicle 3 (3), if considered only in B, would appear to contain two apparently isolated cells. However, as in follicle 1, serial sections reveal a large family of labeled cells, the center of which appears in A with tentacle-like protrusions running down the shell of the follicle.

DISCUSSION

Proliferation of thyroid follicular cells, as well as that of other cellular elements of the thyroid gland, is promoted by TSH (9, 11–15). Hemithyroidectomy induces an elevation of TSH (15) along with a compensatory hyperplasia of the remaining gland, whereas thyroxine administration results in an inhibition of the postthyroidectomy rise in plasma TSH (16). The thyroid is therefore well suited for in vivo studies of cellular proliferation under controlled conditions.

The concept of the polyclonal composition of thyroid epithelial cells, initially suggested by Feder (17), has provided a possible basis for the understanding of functional heterogeneity of the thyroid follicular epithelium (18, 19). Epithelial cells with varying functional properties can be observed within a single follicle, as demonstrated by intercellular variations of iodine organification, peroxidase content, and endocytosis of colloid (5, 9, 20, 21). Therefore, the generation of new follicles from genetically different progenitor cells (5, 20) may be one
of the mechanisms which create the heterogeneous cohorts of hot and cold follicles commonly observed in human multinodular goiter (19, 22).

In a number of previous studies we have observed a nonrandom distribution of a fraction of preferentially replicating cells (5, 20). However, a replicating activity close to 100% was obtained in growing thyroid tissue in one study (18). We argued that the discrepancy between these studies could be due to differences in the level of TSH stimulation. While gentle stimulation could possibly induce proliferation of only the most sensitive cells (5, 20), intense stimulation would, presumably, force all but the most resistant cells into the replication cycle (11). The present study confirms the concept that not all epithelial cells have the same potential to replicate in response to a growth stimulus but that there are individual cells or cell groups located within some follicles which more readily enter the mitotic cycle than the average cell (5, 20). Clusters of highly labeled cells remaining in close association with each other were observed with continuous labeling for 22 days. The same phenomenon was observed after a single shot [3H]thymidine administration, provided that enough time was given to the proliferating fraction to replicate several times.

The strong tendency of the thymidine-labeled cells to remain clustered so as to form large families indicates that a given sensitivity of a progenitor cell toward the events triggering the mitotic cycle is transferred to the cells of subsequent generations. This is further confirmed by the gradual dilution of the tracer among the cells of a single family derived from an individual cell (Fig. 5). Thus, the actual growth potential appears to be an inheritable quality. The newly generated cells in the thyroid gland may not only be used for apoptosis, i.e., replacement of dead cells, but may also serve to increase the size of the epithelial monolayer forming the follicular shell (Fig. 3) (5) or else to generate cell buds from which new follicles arise (5, 20).

The true nature of the epithelial cells with the higher growth potential is not known. They may represent stem-like cells in a favorable position for growth reaction on a physiological stimulus or a clone or clones of more growth-prone cells which in low concentration are scattered in the epithelial bed. Hampering but not excluding the first assumption is the observation that upon strong stimulation almost all epithelial cells are within or have passed the mitotic cycle after 20 days (11) and that mild growth stimulation results in wide cell clusters in which some of the replicating cells must be located at a considerable distance from the original favored position of the mother cell (5) (Fig. 5B). Hampering the second interpretation is the fact that cells with an inheritable high growth potential would be selected and constitute the major fraction of the epithelial cell population. However, the difference in growth responsiveness may be so small that a considerable part of the life span of an organism will be passed before the heterogeneity becomes apparent. A mild growth stimulation would preferentially stimulate the more growth-prone clone and unmask the varying growth responsiveness within a relatively shorter period of time.

In a few cells the intrinsic propensity to multiply appears to be strong enough to allow cell division even when TSH is virtually suppressed (5, 23). These cells are considered to grow autonomously. They occur in large numbers in human nodular goiter (5). In the present experiments 4.2% of all follicular cells were found to be labeled after 22 days of [3H]thymidine administration to thyroxine-treated hemithyroidecotomized mice (group B). The latter fraction of cells with a particularly high growth potential may not only be the progenitors of new thyroid follicles generated in the course of goitrogenesis (5, 18, 20) but may also be at the very origin of the true monoclonal or oligoclonal thyroid adenomas (24) which grow autonomously and invariably arise in chronically stimulated thyroids (6), including human nodular goiters (5). Indeed, the high incidence of adenomas in experimental goiters (6) is perhaps the strongest single element supporting the concept that the particular growth advantage of some individual epithelial cells is not merely a transient characteristic but rather a heritable trait (20, 21, 24). Further evidence is provided by the disproportionate proliferation of rapidly dividing subpopulations of epithelial cells in human goiter transplants growing in chronically TSH-stimulated recipient nude mice (5).

Each cell may conceivably have its own characteristic growth sensitivity. Our results do not indicate whether the threshold of a particular cell to enter the mitotic cycle is the same in respect to all or any of the known thyroid growth factors (12, 25), such as epidermal growth factor (26, 27), fibroblast growth...
factor (28), prostaglandin E₂ (25), and insulin-like growth factor (29), or whether each cell has its own individual pattern of response toward each growth factor.

If new follicles are late offsprings of a single cell, they should theoretically behave like a clonal cell line. However, intercellular heterogeneity may again arise (5, 21, 24, 30). The mechanisms by which stable intraclonal diversity is generated (2, 3) are under intense investigation in our laboratories. It appears that there is no organ like the thyroid gland which allows a more convenient access to investigations on the regulation of clonal growth in intact three-dimensional organs.

The polyclonality of the normal follicular epithelium readily explains many aspects of the functional heterogeneity of human goiters (22). For instance, the individual inheritable growth capacity of each cell may be the biological basis for the dramatic differences in local growth rates clinically apparent whenever the thyroid is forced to grow for long periods of time such as in human (5, 19) and animal (6, 24) goiters.

An obvious question is whether other organs may also contain cell clones with high intrinsic replication power. Certainly, this holds true for tissue with a high natural cell turnover, such as bone marrow or intestine. Foci of cells with high replicative potential have also been observed in the terminal glandular units of the mouse mammary gland (31). If other tissues with slow natural cell replication should, like the thyroid, contain some cells with a high intrinsic growth potential, this might well bear on the pathogenesis of benign tumors other than thyroid adenomas.

Further studies on the exact relationship between the rapidly dividing subpopulations of thyroid epithelial cells and the ultimate growth of autonomous and dependent adenomas and eventually of malignant tumors are under way in our laboratories.

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