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Long-term culture and functional characterization of follicular cells from adult normal human thyroids

(thyrotropin/cAMP/hormonal growth control)

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ABSTRACT **We have obtained long-term cultures of differentiated proliferating follicular cells from normal adult human thyroid glands. *In vitro* growth of such human cells has been sustained by a modified F-12 medium, supplemented with bovine hypothalamus and pituitary extracts and no added thyrotropin. Cultures have been expanded, cloned, frozen, successfully retrieved, and characterized. Functional characterization of these cells shows constitutive thyroglobulin production and release and thyrotropin-dependent adenosine 3′,5′-cyclic monophosphate production, the latter apparently not associated with significant increases in DNA synthesis or cell proliferation. Genetic characterization of these cells by chromosome counting showed the normal diploid chromosome number. The ability to cultivate differentiated human thyroid follicular cells in long-term culture opens possibilities for investigating the transduction pathways of thyrotropin stimulation in normal and pathological human tissues, developing clinically relevant *in vitro* assays, and considering cellular and molecular therapies.**

The number of presently available long-term *in vitro* proliferating cultures of normal human cells of nonmesenchymal origin is low.

Several years ago we reported the establishment of the permanent differentiated thyroid follicular cell strains FRTL and FRTL5, originated from normal adult rat thyroid glands (1–3). These cell strains release thyroglobulin (Tg) and respond to thyrotropin (TSH) by adenosine 3′,5′-cyclic monophosphate (cAMP) production, iodide trapping, and cell proliferation. Their TSH-dependent growth suggested a key role of the hormone as a mitogenic factor for thyroid cells. An important role may also be played by autocrine (4, 5) or indirect paracrine influences (6).

The role of cAMP as a second messenger involved in TSH stimulatory effects has been evaluated by different approaches, using various cell systems with, in some instances, apparently contradictory conclusions and is the subject of a very large literature.

All these studies dealt with thyroid cells from different animal species or from human pathological samples, so discrepancies may be due to differences among species, to the various pathological conditions, or to adaptation of the cells to culture conditions.

The few studies on reportedly normal donor tissues were limited to primary cultures, with very little evidence of follicular cell proliferation (7–11).

To solve this and other problems of thyroid pathophysiology in humans many investigators, including ourselves, have repeatedly but unsuccessfully attempted to establish proliferating thyroid follicular cell cultures from normal human donors. We now report the development of such cultures. This achievement has been made possible by substantial modifications of the culture medium: our formulation is largely based on a further modification of the F-12 modified medium (mF-12) previously developed by us for the rat thyroid cells (12, 13).

Here we describe a cloned strain of human thyroid follicular cells derived from a normal gland: HNTB-2K. This clone has been propagated *in vitro* for at least 40 generations after cloning, while maintaining the diploid chromosome number and differentiated phenotype. At the time of submitting the manuscript, we found that the proliferation rate slowed down, after the cells reached about population doubling level (PDL) 45.

MATERIALS AND METHODS

Culture Medium. The basic culture medium composition is similar to that used for the rat thyroid cells as previously reported (1). It consists of mF-12 (14), further varied to contain 0.48 mM $MgCl_2$ and 3 mM KCl. Fetal calf serum (FCS) (GIBCO) was added to 5%. Other additives were as follows: sodium insulin (Elanco, Indianapolis), 1 μg/ml; bovine transferrin (GIBCO), 5 μg/ml; hydrocortisone, 10 nM; selenous acid, 2 ng/ml; triiodothyronine, 3 pg/ml (all from Sigma). The mixture of sodium insulin at 1 μg/ml, bovine transferrin at 5 μg/ml, 0.01 mM hydrocortisone, glycyl-L-histidyl-L-lysine acetate (Sigma) at 10 ng/ml, and somatostatin (Sigma) at 10 ng/ml has been designated as 5H mixture or, when 10^{-9} M bovine TSH (Sigma) was added, as 6H. Freshly frozen bovine hypothalamus and bovine pituitary (Pel Freez Biologicals) extracts were added to a final concentration of 75 and 5 μg of protein per ml, respectively, and prepared as previously described (12, 13). Briefly, the frozen tissues were mixed in a Waring blender with 200 mM Hepes buffer, pH 7.2, in a ratio of 1:3 wt/vol. The mixture was then left at 4°C for 30 min, mixed again, left 30 additional min at 4°C, centrifuged at 6000 × *g* for 1 h, and clarified by centrifugation at 125,000 × *g* for 1 h, always at 4°C. The supernatants were aliquoted, frozen in liquid nitrogen, and stored in the vapor phase above liquid nitrogen.

Primary Cultures. Cell dissociation procedures, all performed under sterile conditions, were similar to those used with rat cells (1). Typically, human normal thyroid tissue obtained from an organ donor was freed from adherent connective tissue, cut into small (less than 1-mm diameter) pieces, and washed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) by a 5 min centrifugation at 500 × *g*. The enzymatic digestion was with a solution consisting of collagenase (CLSPA, Worthington) at 20 units/ml, trypsin (GIBCO 1:300) at 0.75 mg/ml, and 2% heat-inactivated

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Abbreviations: Tg, thyroglobulin; TSH, thyrotropin; PDL, population doubling level; FCS, fetal calf serum.

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dialyzed chicken serum (GIBCO) in Ca^{2+}- and Mg^{2+}-free HBSS (CTC solution) (15).

Two-hour digestion in a shaking water bath at 37°C reduced the tissue mostly to a cell suspension. Larger fragments were allowed to settle for 2 min at 1 × *g*; supernatants were then collected and cells and small fragments of tissue were seeded at a density of 10^5 per 100-mm plastic tissue culture dish (Falcon, Becton Dickinson).

Passage of Cells and Cloning. Secondary cultures were made by incubating monolayers in CTC solution for approximately 25 min at 37°C, after washing in Ca^{2+}- and Mg^{2+}-free HBSS. For cloning, single-cell suspensions were plated at a density of 10^2 to 10^3 cells per 100-mm dish. Cloning plates were fed with medium conditioned by incubating 12 ml of fresh medium for 24 h in crowded plates of the "parental" mass cell populations. Individual well-isolated epithelial colonies arisen from previously marked single cells were selectively trypsinized, using cloning cylinders. Clones were tested for their ability to produce Tg. One of them, clone HNTB-2K, chosen because of its high rate of Tg production, is described here.

Tg Production Assay. Twenty-four-hour conditioned medium was collected from individual plates of mass populations or clones and assayed for Tg secreted in the supernatants by a standard immunoradiometric assay method using a commercial kit (Henning, Berlin), according to manufacturer's instructions.

Chromosome Counts. Two hours after medium changing, cells were treated with demecolcine (Colcemid, Calbiochem) at 10 μg/ml for 3 h, released by enzymatic treatment with CTC solution, centrifuged, and resuspended in hypotonic solution (4 parts of 5.6 g of KCl per liter and 1 part of 7.3 g of $CaCl_2 \cdot 2H_2O$ per liter). Fifteen minutes later, cells were fixed by progressively adding 0.1 ml, 0.2 ml, and 0.5 ml of fixative (methanol/acetic acid, 3:1, vol/vol) to the cell suspension, eliminating the supernatant by 5-min centrifugation at 500 × *g*, and then adding up to 5 ml of fresh fixative, dropwise under gentle shaking. Three more fixing cycles were performed by repeating the above procedure. Fixed cells were then spread on microscope slides and 25 metaphases were counted, using phase-contrast optics and a drawing attachment.

Growth Assays. The ability of added bovine TSH, alone or in the presence of insulin, to stimulate DNA synthesis was tested by [^{3}H]thymidine incorporation. TSH-induced [^{3}H]thymidine incorporation was assayed as already described (16), with minor modifications. Human normal thyroid cells (HNTB-2K) and FRTL5 cells were seeded in 24-well plates at densities of 5×10^4 and 4×10^5 cells per well, respectively, in complete medium. Twenty-four hours later, after being washed three times in Ca^{2+}- and Mg^{2+}-free HBSS, cells were shifted to 0.5% FCS extract-free medium with the 5H mixture and no added TSH. After 7–10 days, cells were washed twice in Ca^{2+}- and Mg^{2+}-free HBSS and incubated 72 h at 37°C in 0.5 ml per well of medium with no thymidine, containing 0.1% bovine serum albumin (Janssen), [^{3}H]thymidine (Amersham) at 2.5 μCi/ml (1 μCi = 37 kBq), no insulin or insulin at 5 μg/ml, and no TSH or added bovine TSH at concentrations ranging from 10^{-8} M to 10^{-10} M. At the end of incubation, cells were washed twice with Ca^{2+}- and Mg^{2+}-free HBSS and twice with 0.5 ml of ice-cold 10% trichloroacetic acid per well. After removal of supernatants, 0.5 ml of 2% sodium dodecyl sulfate was added per well, and 10 min later supernatants were collected to be analyzed for incorporated [^{3}H]thymidine by liquid scintillation spectroscopy.

TSH-Induced cAMP Accumulation. TSH-induced cAMP accumulation was assayed as already described (16), with minor modifications. HNTB-2K and FRTL5 cells were seeded in complete medium at densities of 5×10^4 and 2×10^5 cells per well, respectively, in 96-well plates. Twenty-four hours later, after being washed three times in Ca^{2+}- and Mg^{2+}-free HBSS, cells were shifted to 0.5% FCS extract-free medium, with the 5H mixture and no added TSH. After 7–10 days, cells were washed twice in modified Krebs–Ringer buffer (NaCl, 7 g/liter; KCl, 374 mg/liter; $MgSO_4 \cdot 7H_2O$, 295 mg/liter; $NaHCO_3$, 850 mg/liter; $CaCl_2 \cdot 2H_2O$, 149 mg/liter; KH_2PO_4, 163 mg/liter) and incubated 1 h at 37°C in the same buffer at 0.1 ml per well, with 0.1% bovine serum albumin, glucose at 2 mg/ml, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma), and added bovine TSH at 10^{-7} to 10^{-13} M. The reaction was stopped by removing the incubation medium and adding 70% (vol/vol) ethanol (0.1 ml per well). After 20 min at room temperature, plates were centrifuged, supernatants were transferred to plastic tubes, and the ethanol was evaporated at 40°C. cAMP was determined by a commercial radioimmunoassay kit (Diagnostic Products, Los Angeles), according to manufacturer's instructions. cAMP values were normalized by counting cells in duplicate wells at the end of the stimulation.

Growth in Agar. The ability to grow and form colonies in soft agar was tested as already described (17).

Growth Efficiency. To calculate growth efficiency, cells were counted at selected passages. After being detached with CTC solution, as already described, cells were appropriately diluted and counted in a modified Neubauer hemocytometer. Population doubling time was determined, calculating the time (h) necessary for one cell doubling.

RESULTS

Characteristics of the Cultured Cells. We obtained cultures from several normal (organ donors) and diseased glands. The primary culture procedures and medium formulation used always yielded proliferating thyroid cell cultures from different human donors. Even in the presence of 6H and 5% FCS, pituitary or hypothalamus extracts, when tested separately, were unable to sustain human thyroid cell growth. Phase-contrast photomicrographs of cultures (Fig. 1) show the morphology of cells after 2 weeks in medium without extracts (Fig. 1*A*), with bovine pituitary extract alone (Fig. 1*B*), with bovine hypothalamus extract alone (Fig. 1*C*), or with both extracts (Fig. 1*D*). Even in the presence of serum and 6H, but no extracts or with either one of them, cells are unable to divide (at least not appreciably) and the cytoplasm appears enlarged and very pale. Each culture, independent of its origin (whether from a normal or diseased gland), showed a noticeable difference in its requirements for pituitary extract, the optimal concentration varying between 5 and 50 μg/ml; in most instances the latter concentration was evidently an excess. In this case, when observed under phase-contrast microscopy, cells appeared gradually larger, contained evident stress fibers, and ultimately died.

Cultures also showed variable levels of Tg release per cell in the culture medium. Individual variations among the different normal gland-derived noncloned populations and among the individual clones, even within the ones derived from the same gland, were found (Table 1).

The culture of the HNTB-2K cell strain was initiated approximately a year ago, and it has been in culture since then. The clone morphology is not homogeneous when observed under phase-contrast microscopy, and it is influenced by the proliferative state of the cells: nonconfluent, logarithmic-phase cultures show mostly elongated rather pale cells, while at confluence they become more like classical epithelial cells, showing darker cytoplasm and many granules (Fig. 2).

The clone HNTB-2K released more Tg than any of the others tested (Table 1).

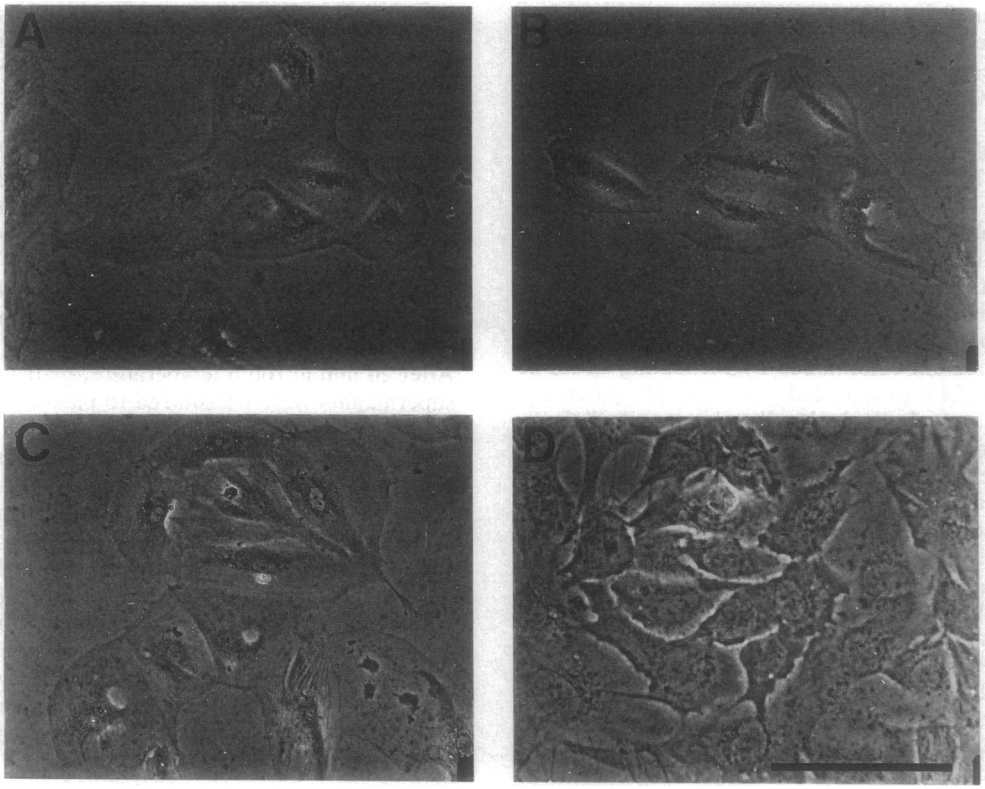

FIG. 1. The primary culture procedures and the medium formulation described yielded a good outcome in all attempts to culture proliferating thyroid epithelial cells from different normal human donors. Even in the presence of serum and 6H, but without any extract or with either one of them alone, cells are unable to grow (at least not appreciably) and the cytoplasm is enlarged and very pale, as opposed to complete medium. (*A–D*) Cell morphology of the HNTB-2K clone after 2 weeks in medium without extracts (*A*) or with bovine pituitary extract alone (*B*) or bovine hypothalamus extract alone (*C*) or with both extracts (*D*). (Phase-contrast photomicrograph. Scale bar = 100 μm.)

Chromosome Count. Only clone HNTB-2K was tested. It consistently showed a normal diploid number in all metaphases counted at all PDLs tested (up to PDL 40).

Growth Assays. Values of cpm were divided by the mean of unstimulated FRTL5 (no insulin, no TSH) and expressed as times control (mean ± SEM). Baseline values were 4000–5000 (FRTL5) or 2000–3500 (HNTB-2K) cpm per well in the various experiments performed. Under the conditions described, at all concentrations tested, added bovine TSH, alone or in the presence of insulin, was unable to stimulate [^{3}H]thymidine incorporation acutely in HNTB-2K cells, in contrast to the rat system (FRTL5), where added bovine TSH is a mitogenic factor (Fig. 3).

Table 1. Tg production

Cell population	PDL	Tg, fg per cell per day
HNTU-1	15	780
HNTU-2	15	880
HNTU-3	15	522
HNTB-1	20	1157
HNTB-1A	20	18
HNTB-1D	20	25
HNTB-1F	20	224
HNTB-1G	18	456
HNTB-2	18	1267
HNTB-2A	15	26
HNTB-2C	15	77
HNTB-2F	15	420
HNTB-2K	15	914
HNTB-2K	20	862
HNTB-2K	30	1062

Tg was measured in supernatants from some nonclonal (HTNU-1, HTNU-2, HTNU-3, HNTB-1, and HNTB-2) and clonal follicular thyroid cell populations in cultures derived from normal human donors. One of the clones, HNTB-2K, has been tested at various generations (PDL). Twenty-four-hour conditioned medium was collected from individual plates and assayed for Tg.

TSH-Induced cAMP Accumulation. Total cAMP values were normalized by counting the cells in duplicate wells for each experimental point at the end of the stimulation time and are expressed as fM cAMP per 10^3 cells (mean ± SEM).

Added bovine TSH was able to induce a dose-dependent, up to 10-fold, increase of cAMP production in HNTB-2K cells. The stimulation was evident within 1 h and at very low concentrations (10^{-10} M). The data reported in Fig. 4 reflect assays run on cells at PDL 35. Passage number (at least up until PDL 35) did not significantly affect TSH-induced cAMP response in any population tested (data not shown).

Growth in Agar. Cells were unable to grow in soft agar.

Growth Efficiency. In the complete medium, the population doubling time (PDT) of the HNTB-2K clone was 58 h up to PDL 40. At about 45 generations, PDT increased to 69 h.

DISCUSSION

We report here the long-term *in vitro* culture, presently at about PDL 45, and the characterization of a clonal strain of thyroid follicular cells, HNTB-2K, from a normal human donor. We attribute this success to the development of our formulation of the culture medium, which features the presence of bovine pituitary and hypothalamus extracts. The identification of specifically active growth-promoting factors within the two extracts, a problem not yet fully solved even with animal sera, still universally added as such to cell cultures *in vitro*, has not been explored, and it may prove to be a difficult task because of the complexity of the crude material.

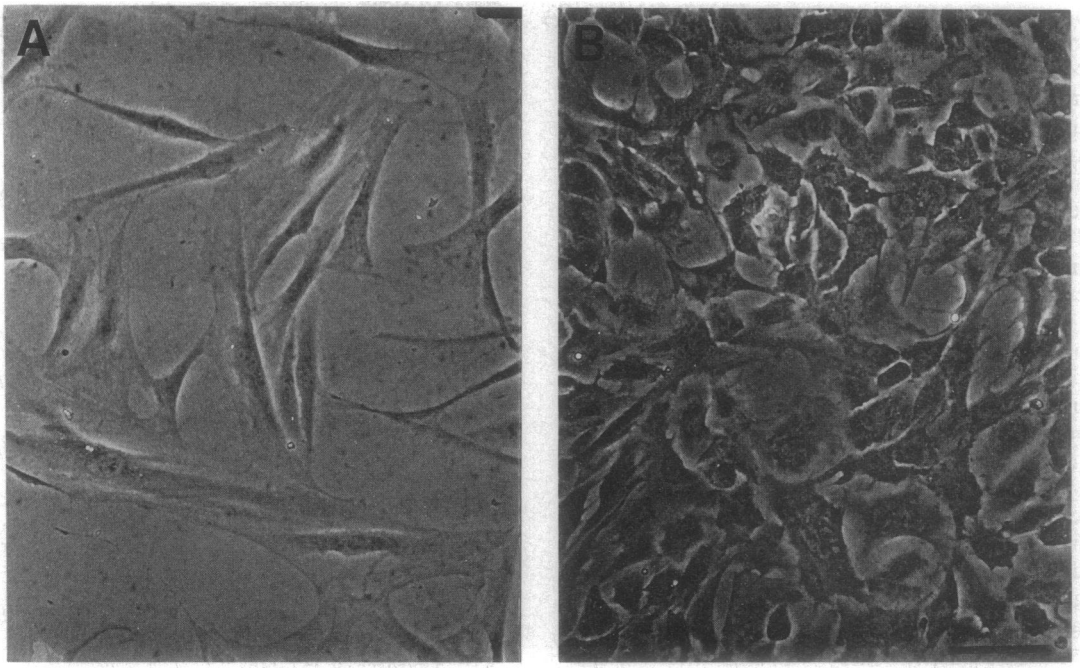

FIG. 2. HNTB-2K clone shows heterogeneous morphology, influenced by the proliferation state of the cells. (*A*) Nonconfluent logarithmic-phase cultures contain mostly elongated, rather clear, cells. (*B*) At confluence cells become more epithelial, showing a darker cytoplasm and many granules. (Phase-contrast photomicrograph. Scale bar = 100 μm.)

Cultures from different donors showed individual behavior and characteristics. The presence of both extracts is always required, while medium with added FCS and 6H alone is clearly unable to promote and sustain HNTB-2K or human thyroid cell proliferation in general. In contrast with earlier studies, even though our medium is significantly rich in proteins, we observe few fibroblasts (18). We think that the medium composition, the cloning procedures adopted, and the use of conditioned medium made it possible to overcome the *in vitro* proliferative problems of the human thyroid follicular cells and to avoid fibroblast overgrowth. Like FRTL5 thyroid cells, HNTB-2K cells are able to express typical thyroid marker proteins and functions *in vitro*, such as Tg production and TSH-stimulated cAMP production. In contrast to what has been reported for FRTL5 cells (1–3), the presence of added bovine TSH did not stimulate the DNA synthesis of HNTB-2K cells. Acutely added bovine TSH, alone or in the presence of insulin, did not act as a growth factor for human cells under the conditions described here, confirming earlier observations on human cultures (8, 19).

The role of TSH in regulating the growth of thyroid follicular cells, alone or in the presence of cofactors, is the subject of a vast literature, which recently was extensively reviewed (20). The apparently contradictory data available seem to depend very much upon the animal species the cultures and the TSH were derived from, or upon the pathological situation *in vivo*. It is conceivable that there are important interspecific differences among the ways growth signals are delivered, a concept that has recently received new support (21, 22). Also, it is highly probable that thyroid diseases may affect the way follicular cells respond to normal signals (23). Last, the *in vitro* conditions may select subpopulations of cells that show unique behaviors.

Human normal thyroid follicular cells kept in long-term proliferative and differentiated cultures under the conditions described here apparently are not subject to "spontaneous" transformation *in vitro*, as they do not exhibit any of the usual signs of *in vitro* transformed cells: (*i*) they do not grow in soft agar; (*ii*) they have retained a diploid genotype; (*iii*) they have shown no modifications in serum or extract requirement for

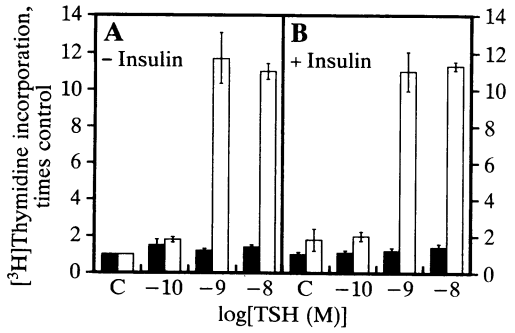

FIG. 3. Ability of added bovine TSH, alone or in the presence of insulin, to stimulate DNA synthesis. Data from a representative experiment are shown: TSH (*A*) or TSH and insulin (*B*) stimulation of [^{3}H]thymidine incorporation in HNTB-2K (solid bars) and FRTL5 (empty bars). C, control without TSH.

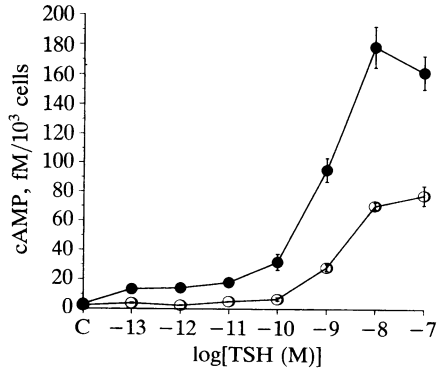

FIG. 4. Ability of added bovine TSH to stimulate cAMP production. Data from a representative experiment, a dose–response curve of 1-h TSH-stimulated cAMP total production, are shown. Added bovine TSH was able to induce a dose-dependent cAMP accumulation in HNTB-2K cells (●) [compared with the rat thyroid cells FRTL5 (○)], even at very low concentration (10^{-10} M). C, control without TSH.

growth; (*iv*) as part of another experiment, HNTB-2K did not produce tumors in 45 days after two SCID mice received grafts of 5×10^6 and 10^7 cells; and (*v*) the growth efficiency did not increase with successive generations in culture (in fact, we found that proliferation slowed down after the cells reached about PDL 45).

Preliminary results suggest that, by using the tissue culture approach described here, it will be possible to obtain proliferating and differentiated cell populations or clones from normal human donors and cultures from human thyroid glands affected by various diseases. Up to now we succeeded with many tissue samples from thyroid biopsy samples or surgical procedures. Detailed analysis of such material may help to clarify (*i*) if TSH alone may ever be a growth factor in humans under physiological conditions, as in other animal species, namely in rats, or if this may occur in pathological conditions; (*ii*) if TSH needs other cofactor(s), such as insulin or other factors, as, again, in rats or dogs; (*iii*) if TSH-dependent cAMP is involved as a second messenger, and if so by what mechanism(s); and (*iv*) if other second messengers are involved, and if this is the case, what may be the hierarchy in physiological or pathological conditions. We expect that all this information will be very helpful in comprehending the mechanisms underlying thyroid proliferative disorders. Thyroid diseases represent about 50% of all endocrine pathologies, among which most are proliferative disorders. Some of them may be due to dietary or intrinsic defects, but for a large number of them we do not have a clear understanding of the responsible molecular mechanism(s). A large body of data derived from animal cell cultures suggests a very complex regulation of cell proliferation, based upon the activation (or deactivation) of molecules in various transducing pathways in a sequential cascade and that proliferative disorders may be due to an interruption of the ordinate sequence of events. The availability of cell cultures, which, while proliferating, still resemble the tissue of origin, may give us the key to clarify such molecular mechanism(s).

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