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MALIGNANT TRANSFORMATION OF RAT THYROID CELLS TRANSFECTED WITH THE HUMAN TSH RECEPTOR cDNA

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Growth and function of well differentiated FRTL-5 thyroid cells depend on thyrotropin as its main regulatory hormone. We demonstrate here that stable transfection of FRTL-5 cells with the human thyrotropin receptor cDNA results in cellular transformation of these cells with altered cell shape and loss of contact inhibition. The transformed cells replicate in soft agar and form invasive tumors when cell suspensions are implanted onto nude mice. They have lost their thyrotropin dependent growth and their ability to concentrate iodide and synthesize thyroglobulin. But they still express the rat thyrotropin receptor mRNA and accumulate cAMP in response to thyrotropin stimulation. However, although the full length human thyrotropin receptor cDNA is integrated into their genome, transformed cells do not express the human thyrotropin receptor mRNA.

FRTL-5 cells are a continuous rat thyroid cell line (1, 2) that display many differentiated functions including active iodide transport, thyroglobulin synthesis (3), cAMP synthesis (4) and growth in response to thyrotropin (TSH) stimulation (1, 5). These cells are presently being used in more than 90 laboratories throughout the world in studies of intracellular pathways (5, 6), thyroid cell growth and function in vitro and in vivo (7-10), and cloning and expression of thyroid specific genes (11-14).

Furthermore, FRTL-5 cells provide an valuable model for studying thyroid cell transformation in vitro. Like other cells, FRTL-5 cells can be transfected with various oncogenes resulting in malignantly transformed cells that have lost TSH dependent growth, thyroglobulin expression and iodide trapping (15-17).

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We report here that FRTL-5 cells stably transfected with the human TSH receptor cDNA acquire a malignant phenotype with a fibroblast-like aspect, loss of contact inhibition, and TSH independent growth in vitro and in vivo when transplanted onto nude mice.

MATERIALS AND METHODS

Cell cultures: Cells were grown in Coon's modified Ham F12 medium supplemented with 5% calf serum, glycyl-histidyl-lysine (10 ng/ml), insulin (10 μg/ml), somatostatin (10 ng/ml), transferrin (5 μg/ml), hydrocortisone (3.2 ng/ml), and with 10 mU/ml (H6-medium) or without bovine TSH (H5-medium).
Assay of tumorigenicity: Transformation of FTSHr cells were analyzed by soft agar growth (18) and by transplantation of cell suspensions (1.5 x 10^6 cells per mouse) onto male dysthymic nu/nu mice (9).
Transfection of FRTL-5 cells: The human TSH receptor cDNA (hTSHR-cDNA) inserted into the eukaryotic expression plasmid pSV2-NEO-ECE (kindly provided by Dr. B.Rapoport, San Francisco; 19) was transfected into FRTL-5 cells using a modified lipofection method (20). 4 days after transfection 400 μg/ml geneticin (G418) was added to the medium to select transfected cells. Transformed cells of three independent transfections were further subcloned by limiting dilution. As negative control, FRTL-5 cells were transfected with the plasmid pSV2-NEO-ECE without the hTSHR-cDNA.
Southern blot analysis: Genomic DNA was isolated from FRTL-5 cells and FTSHr cells by standard methods (21) and digested with Eco RI to release in FTSHr cells the hTSHR-DNA ligated to Eco RI sites of the plasmid vector. Pre- and hybridization were performed under high stringency condition (21) using the full length hTSHR-cDNA as a probe (19) that had been labeled with digoxigenin-11-dUTP. Signal was detected by non-radioisotope technique (AMPPD amplification; Boehringer Mannheim) and visualized after appropriate exposure to a Kodak XAR film.
Reverse transcriptase reaction (RT) and polymerase chain reaction (PCR): Total RNA was isolated from FRTL-5 cells and FTSHr cells (22) and treated with RNase free DNase (Pharmacia) for 1 h at 37 °C to digest contaminating DNA . Then the first strand cDNA was synthesized from 2 μg of total RNA as described before (12).
For PCR four 20er primers were synthesized : hTSHr 1 (ATGAGGCCGGCGGACTTGCT) extending downstream from a position 100 bp and hTSHr 2 (ACATCAAGGACTCAAGGATT) extending upstream from a position 1000 bp in the human TSH receptor cDNA sequence (19) and rTSHr 1 (TTGACAGCCGCAACGATGA) extending downstream from a position 10 bp and rTSHr 2 (GGCTGGGGATTTGGTGGAGT) extending upstream from a position 202 bp of the rat TSH receptor cDNA (13). The homology of rTSHr 1 and rTSHr 2 with the hTSHR-cDNA is 40% and 65%, respectively. 1 μg cDNA was amplified in 50 μl reaction mixture containing 2 U Taq polymerase (Cetus), 1 μM of each primer, 1.25 mM dNTP, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0,001% (w/v) gelatine (35 cycles: 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min.) (23). PCR products were electrophoresed on a 1.0% or 1.6% agarose gel.
Measurement of cAMP accumulation: Cells maintained in H5 medium were incubated with bovine TSH and 1 mM 1-methyl-3-isobutylxanthol for 2 h at 37 °C. The intracellular cAMP concentrations were measured using a RIA kit (Amersham) and corrected to the protein content of the cells.

RESULTS AND DISCUSSION

FRTL-5 cells stably transfected with the human TSH receptor cDNA (FTSHr cells) acquire a malignant phenotype. In soft agar they display an anchorage-independent growth and form malignant tumors when they are transplanted onto nude mice (Fig. 1).

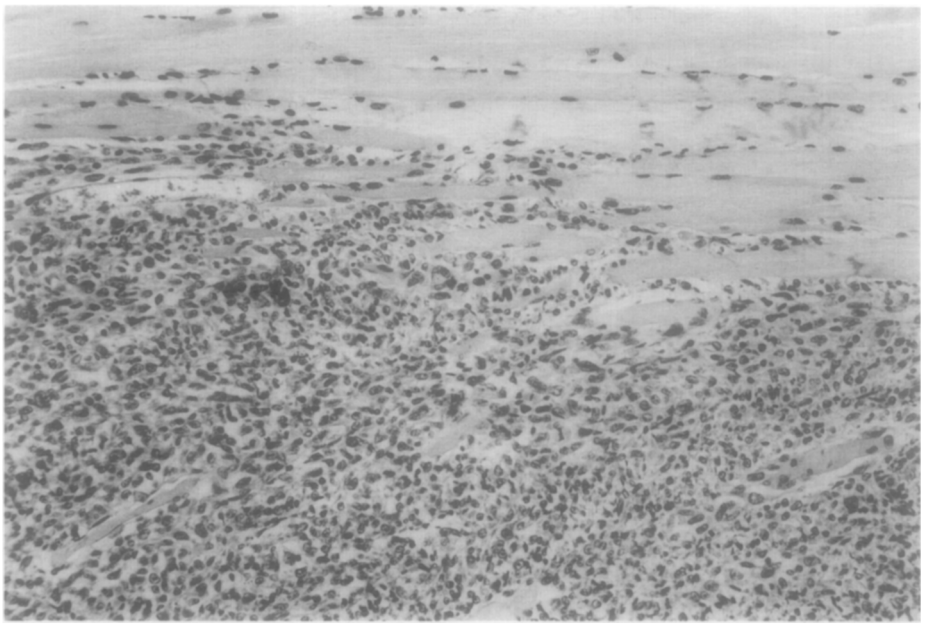

Figure 1. In vivo growth of FTSHr cells transplanted onto male dysthymic nu/nu mice. Note the malignant growth of the FTSHr cell tumor invading the surrounding muscle. Magnification, x 160.

In sharp contrast to nontransfected cells , FTSHr cells also grow in mice not pretreated with a TSH enhancing regimen. Thus, the transformed cells have not only lost the TSH dependency of normal FRTL-5 cells in vitro, but also in vivo, as it is known from FRTL-5 cells transformed by retroviruses carrying v-ras oncogenes (15-17). FTSHr 1 and 2 cells grow faster than FRTL-5 wild type cells with a doubling time of 18 and 20h, respectively (Fig. 2). In addition, transformed cells have also lost their ability to synthesize thyroglobulin (data not shown) and to concentrate iodide (data not shown).

To verify that the complete human TSH receptor cDNA was incorporated in the genome of the transformed cells, rather than only truncated forms, we analyzed genomic DNA of FTSHr cells. Both cell lines contained a full length human TSH receptor cDNA (Fig. 3). However, this does not exclude other mutations that might occur when DNA is transferred into mammalian cells (24).

A similar ligand-independent transformation of mammalian cells has very recently been described. Gaben and Mester stably transfected 3T3 fibroblasts with the human estrogen receptor cDNA and found one subline to be malignantly transformed (25). But these authors did not analyze the integration of the receptor DNA into the genome of the host cells.

Our failure to detect expression neither of rat TSH receptor mRNA nor of human TSH receptor mRNA by Northern blot analysis in FTSHr cells (data not shown) is in ac-

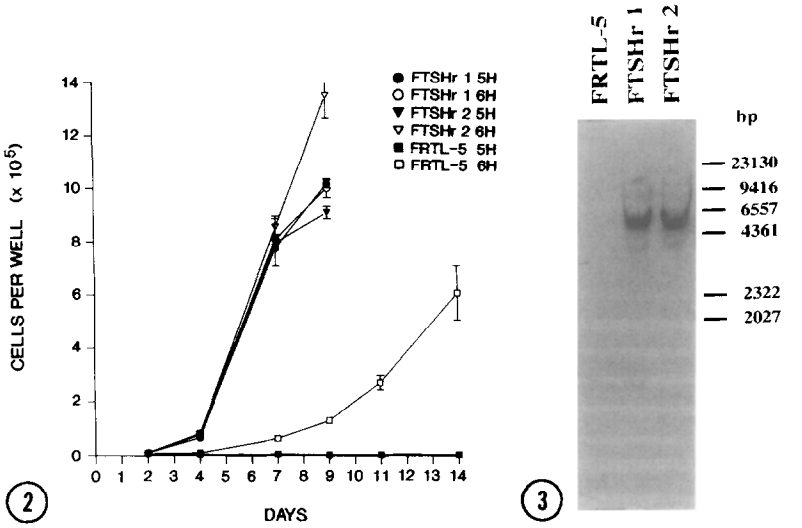

Figure 2. Growth of FTSHr 1, FTSHr 2 and FRTL-5 cells in vitro. Cells were cultured in complete Coon's Ham F12 medium supplemented with (H6 medium) or without TSH (H5 medium). While FRTL-5 cells do not grow in absence of TSH, FTSHr cells display a TSH-independent proliferation. Mean ± SEM of three separate experiments.

Figure 3. Southern blot analysis of the human TSH receptor cDNA integrated into the genome of FTSHr cells. DNA was digested with Eco RI to release in FTSHr cells the human TSH receptor cDNA originally ligated to the Eco RI sites of the plasmid vector. Under high stringency conditions using the full length hTSHR-cDNA as a probe a clear signal is visible at 4.3 kb that corresponds to the size of the full length human TSH receptor cDNA (19), but there are no detectable signals in FRTL-5 cells corresponding to the rat TSH receptor.

cordance with the recent demonstration that in transformed FRTL-5 cells loss of TSH-dependent growth is associated with lack of TSH receptor mRNA expression (26). However, using the highly sensitive RT-PCR method there was detectable rat but not human TSH receptor mRNA in both FTSHr 1 and 2 cells (Fig. 4).

In addition, although to a lower extent than FRTL-5 cells FTSHr cells synthesize cAMP in response to TSH stimulation (Fig. 5), indicating an functional TSH receptor-adenylate cyclase system.

At the first glance it seems puzzling that stably transfected FTSHr cells are transformed with low expression of their own receptor but lack of the human TSH receptor mRNA. The molecular mechanisms that diminish or inhibit the expression of TSH receptor mRNA in transformed thyroid cells are still unknown. Since in oncogene-transformed thyroid cells a thyroid-specific transcription factor 1 (TTF-1) that regulates cell type specific expression of the thyroglobulin gene is not expressed (27), it is conceivable that a similar mechanism may decrease TSH receptor expression in FTSHr cells.

Very recently, Belur et al. (28) reported that aged FRTL-5 cells grow more rapidly, while still remaining TSH-dependent. The TSH independence of FTSHr cells in vitro and

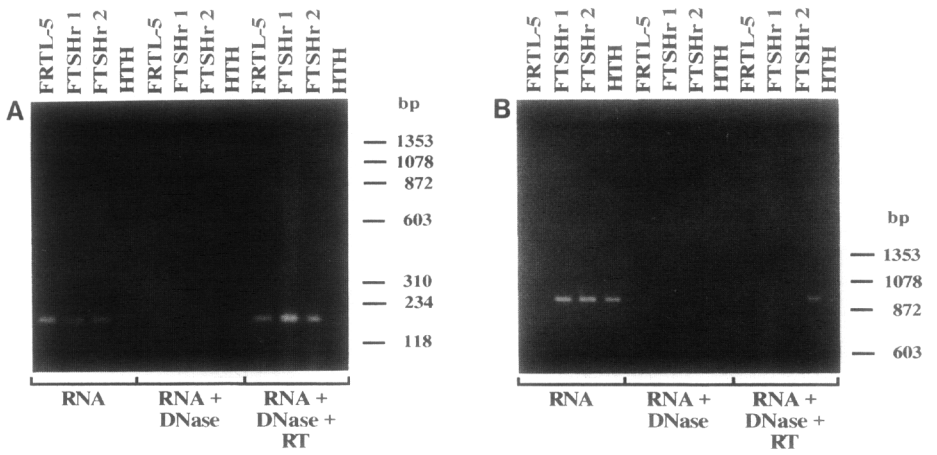

Figure 4. Detection of rat TSH receptor mRNA and absence of human TSH receptor mRNA in FTSHr 1 and 2 cells. PCR was performed using as template single stranded cDNA generated by reverse transcription from RNA of FRTL-5 cells, FTSHr 1 and 2 cells, and as control, from CHO-TSHr cells (19) expressing the human TSH receptor (HTH). To exclude false positive amplifications caused by contaminating DNA, total RNA preparations were treated with RNase-free DNase prior to reverse transcription. A, Rat specific primers. Lane 1-4: Analysis of PCR products using total RNA as template (contaminated with DNA); Lane 5-8: PCR products of DNase-treated RNA; Lane 9 - 12: PCR products of reverse transcribed, DNase-treated RNA. B, Human specific primers. Lane 1-4: Analysis of PCR products using total RNA as template ; Lane 5-8: PCR products of DNase-treated RNA; Lane 9-12: PCR products of reverse transcribed, DNase-treated RNA.

in vivo and their malignant phenotype exclude in our mind the speculation that FTSHr cells may be generated by preferential transfection of aged FRTL-5 cells with higher intrinsic proliferation rate.

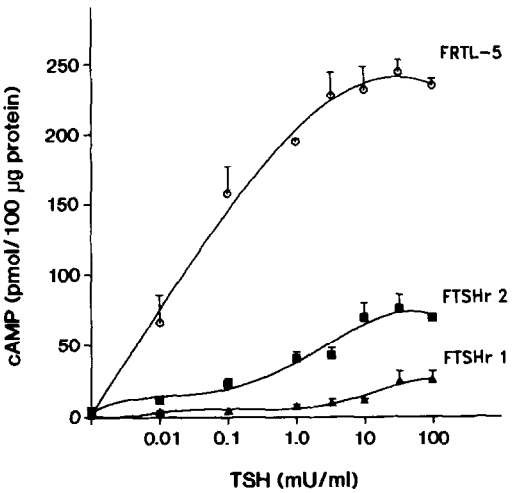

Figure 5. TSH-dependent cAMP synthesis in FTSHr 1, FTSHr 2 and FRTL-5 cells. cAMP accumulation was determined by a cAMP RIA kit after stimulation with the indicated TSH concentrations. Mean ± SEM per 100 µg protein (n = 3).

A marked heterogeneity of clonal growth and TSH dependency has not only been found in aged cells (7, 29), but is even more impressive in FRTL-5 cells grown as tumor-like organoid on nude mice (8). But these tumors are still fully TSH-dependent and do not display any signs of malignant transformation.

Last, our results have to be confronted with a recent report of spontaneously transformed FRTL-5 cells with a large, flattened and epithelium-like cell shape (30). Based on this observation it could be argued that we have transfected malignantly transformed cells with a higher growth rate overgrowing during the G418 selection process. Although we cannot absolutely exclude this, there are solid arguments against this hypothesis. First, transfections of FRTL-5 cells with the plasmid vector alone did not result in malignant transformation. Second, transformation of FRTL-5 cells with the human TSH receptor cDNA were obtained in three separate experiments. After selection in medium containing G418 all clones displayed a malignant phenotype. Furthermore, several other investigators using similar plasmid vectors for transfection of FRTL-5 cells did not observe any transformation of their cells (31, 32).

At this point in time the molecular mechanisms that led to transformation of FTSHr cells remain unknown. However, our findings should cast a note of caution on the prevailing assumption that insertion of a foreign receptor DNA into a cell cannot cause events other than overexpression and/or altered sensitivity of the host cell toward the specific ligand.

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