Description of a Human Papillary Thyroid Carcinoma Cell Line

Morphologic Study and Expression of Tumoral Markers

Nicole Fabien, Ph.D.,* Alfredo Fusco, M.D.,† Massimo Santoro, M.D.,†
Yves Barbier, Ph.D.,‡ Paul-Marie Dubois, M.D.,*
and Christian Paulin, M.D.*

Background. The establishment of cell lines from thyroid carcinomas can provide an in vitro model of oncogenesis. B-CPAP is a new cell line that has been obtained from a differentiated papillary thyroid carcinoma. The data presented give a broader characterization and expression of tumoral markers of this cell line and identify the differentiated functions that are preserved.

Methods. An ultrastructural study was performed to confirm the thyroid nature of the new cell line. The cellular markers (thyroglobulin, S100, neuron-specific enolase [NSE]) and the oncogenes (mutated p53, H-ras, c-myc, PTC, trk) were studied by immunohistochemistry, Southern blot, or in situ hybridization.

Results. The cells were of a differentiated ultrastructural thyroid type. All of the cells proved immunoreactive with antibodies specific to thyroglobulin, S100 proteins, NSE, and mutant p53 protein. Mutations of H-ras, PTC, and trk were not observed. The c-myc gene was not amplified.

Conclusions. The cell line described in these data provides a suitable model for the study of thyroid carcinogenesis, given that the cells present thyroid characteristics, and metabolic disorders not previously found in such cell lines. In addition, the coexpression of S100 proteins and mutant p53 proteins in the cells should permit the study of the interaction between these two proteins. Cancer 1994; 73:2206-12.

Key words: thyroid cancer, papillary carcinoma of the thyroid, thyroid cell line, electron microscopic study, mutated p53, S100, neuron-specific enolase, human chorionic gonadotropin hormone, transforming growth factor β, oncogene.

Thyroid carcinomas arising from thyrocytes are classified as follicular, papillary, or undifferentiated, with various subtypes also being classified. The papillary type is the most common, either in its pure papillary form or in mixed follicular and papillary form. Most of the cell lines previously described were obtained from undifferentiated carcinomas; those obtained from differentiated carcinomas are rare.

A new cell line, B-CPAP, using a differentiated papillary thyroid carcinoma, has been established. Previous studies have demonstrated the synthesis of thyroglobulin in all of the cells of this line, and the synthesis of human chorionic gonadotropin hormone (HCG) by many of these cells.

The current study gives a broader characterization and expression of the tumoral markers of this cell line and attempts to identify the differentiated functions that are preserved. An ultrastructural study was performed to evaluate the differentiated thyroid aspect of the cells. The expression of some cellular markers (S100, neuron-specific enolase [NSE]) and of the transforming growth factor (TGF β) known to be involved in carcinogenesis also were studied. Some oncogenes involved in thyroid carcinogenesis, e.g., mutated p53, H-ras, c-myc, PTC, and trk, also were investigated.
Human Papillary Thyroid Cancer Cell Line/Fabien et al.

Materials and Methods

Thyroid Tumor

A thyroid tumor with local and lymph node metastasis was surgically resected from a 76-year-old woman at the Edouard Herriot Hospital, in Lyon, France. The tissue was cut up and immediately frozen in liquid nitrogen, immersed in a sterile culture medium, or fixed in glutaraldehyde or Bouin's fluid.

Culture

The tissue fragments were washed several times with RPMI 1640, placed in 25-cm² culture dishes, and grown in RPMI 1640 supplemented with glutamine (10 mg/ml), penicillin (100 IU/ml), streptomycin (25 mg/ml), 10% fetal calf serum, vitamin A (100 ng/ml), thyroxine (80 ng/ml), selenium (5 ng/ml), and insulin bovine (5 mg/ml). The culture medium was changed twice a week. When the culture reached confluence, the cells were detached from the support with a solution of 0.5% trypsin-0.02% ethylenediamine tetraacetic acid and re-distributed among other dishes or eight-compartment tissue culture with 10⁵ cells/compartment for immunohistochemistry studies or for a longer period of culture.

Electron Microscopic Study

The cells cultured in the chamber slides were washed with phosphate buffer saline (PBS) and fixed in 3.5% glutaraldehyde in monosodic-dipotassic phosphate buffer (Na/2K) 0.2 M, pH 7.4 with osmotic pressure of 380 mOsm for 1 hour. After three 10-minute washes in this buffer (Na/2K), the cells were postfixed with 1% osmium tetroxide and rinsed briefly with distilled water. They were dehydrated in 70°C, 95°C and 100°C ethanol (3X10 minutes), and embedded in Araldite-M. Gelatin capsules filled with this preparation were inverted over the cells. Polymerization of the resin was performed at 60°C for 72 hours. Ultrathin sections were cut with an ultramicrotome and collected on formvar-coated copper grids.

They were counterstained with uranyl acetate and lead citrate and examined under a Philips CM 10 electron microscope.

Tumorigenicity

To obtain tumors, 3X10⁶ cells in 0.2 ml of RPMI were injected subcutaneously into three 6-week-old athymic nude mice (IFFA CREDO). The tumors were removed and immersed in liquid nitrogen. Frozen fragment sections were counterstained with hematoxylin and eosin for a histologic examination.

Antisera

The materials used were monoclonal (MoAb) and polyclonal (PoAb) antithyroglobulin antibodies at a dilution of 1/200; MoAb antikeratin (1/100); PoAb anticalcitonin (1/100); MoAb anti-p53 protein (240 recognizing mutant forms, 1/10) and PoAb (CM1 reacting with wild and most mutant forms, 1/500); PoAb specific to S100 proteins (1/500) and (1/100); PoAb anti-NSE (1/100); MoAb antivimentin (1/100); and PoAb anti-TGF β1 (1/500) (a gift of Dr. Saez, Debrousse Hospital, Lyon, France).

Also used was rabbit anti-mouse or sheep anti-rabbit immunoglobulin G (IgG) (H + L) antibody coupled with fluorescein isothiocyanate or peroxidase (1/500). For the peroxidase technique, cells were treated with H₂O₂ (1% in PBS) for 1 hour to eliminate endogenous peroxidase.

Immunohistochemistry

Immunostaining was performed on cytocentrifuged cells or directly on cells in the chamber slides. The cells were washed twice with PBS containing calcium to avoid the detachment of the cells from the support. They were fixed in 4% paraformaldehyde for 30 minutes, washed three times (5 minutes each time) in PBS, and incubated with normal sheep serum for 30 minutes before incubation with the different antibodies mentioned for 1 hour at room temperature. For the p53 immunohistochemistry procedure, 0.3% of triton X100 was added to the antibody solution. After three 5-minute washes in PBS, the fixed antibody was labeled using the corresponding specific conjugates for 1 hour at room temperature. The cells were washed three times for 10 minutes in PBS. Those labeled by the indirect immunofluorescence technique were mounted in PBS-glycerin. To test for peroxidase activity, the conjugate was revealed using a solution of diaminobenzidine. The reactions were observed with a Zeiss microscope equipped for epifluorescence with specific filters (BP450–490) and flat APO lenses. Micrographs were taken on Kodak Ektachrome (England) film with a Zeiss photo system.
Titration of NSE

The amount of NSE was determined by radioimmunoassay in the cellular pellet after lysis of $10^6$ cells.

Detection of Estradiol Receptors

The estradiol receptors were detected by a radioligand receptor assay with tritiated estradiol in dissociated cells.

Expression of PTC

The use of in situ hybridization to detect PTC in the cells of the new cell line is described elsewhere. The sequence of the synthesized oligodeoxynucleotide used as a probe is: 5'-CCA GAT ACT GCA TCC CCT GTG AGA TCT GCC AGG CAA ATG AGA TGA GGT CGC-3' (Laboratory of Molecular and Cellular Biology, ENS, Lyon, France). This sequence corresponds to the 51-bp antisense probe for the tyrosine-kinase domain of the human ret proto-oncogene.

An NIH/3T3 cell clone transfected with the PTC oncogene (NIH/3T3-PTC) and a PTC-positive papillary thyroid carcinoma were used as positive controls.

Results

Establishment and Morphologic Characteristics of the Cell Line

The histologic type of the primary tumor and lymph node metastasis was found to be that of a differentiated papillary carcinoma with squamous metaplasia (Fig. 1). The cells grew as an adherent monolayer with characteristic epithelial features but with no papillary arrangement. The confluent monolayer showed numerous “holes.” All of the cells had the same morphologic aspect: they were oval or spindle-shaped and possessed one or several nuclei and refringent cytoplasm. The stability of the cell line was assessed by a 2-year culture.

The electron microscopic study showed irregular nuclei containing one or few prominent nucleoli and cytoplasmic invaginations. The endoplasmic reticulum was well developed; many mitochondria, free ribosomes, numerous dense secretory vesicles, and cytososonal bodies were observed (Figs. 2 and 3). Many cytosolic filaments and desmosome-like junctions confirmed the epithelial nature of the cells. The membranes showed some short microvilli.

Tumorigenicity

Eight weeks after the inoculation of the tumor cells, the nude mice had tumors 0.5 mm in diameter develop in the region of the right flank. Histologic examination of these tumors showed an epithelial organization with cellular strands but no papillary arrangement.

Immunohistologic Studies

All of the cells were labeled homogeneously using the antithyroglobulin antibody (Fig. 4) and with a reticular aspect using the antivimentin antibody (Fig. 5) and the antikeratin antibody.

The anticalcitonin P0Ab gave no positive immunoreaction.

A strong homogeneous labeling was obtained in all the cells, with antibody specific to NSE (Fig. 6), antibody specific to S100 proteins (Fig. 7), and antibody specific to TGF β1 (data not shown).

All of the nuclei were stained with anti-p53 MoAb recognizing mutant forms of the p53 protein and with P0Ab reacting with wild and most mutant forms (Fig. 8).

Titration of NSE

The amount of NSE in the cellular pellet was 2250 μg/l.
Detection of Estradiol Receptors

No estradiol receptors were detected.

Expression of PTC

The cells of the NIH/3T3-PTC cell clone, like the thyreoïdes of the PTC-positive papillary carcinoma (data not shown), were homogeneously labeled. However, no in situ hybridization signal was observed in the B-CPAP cells.

Discussion

We used these data to better define the characterization of the new human papillary thyroid carcinoma cell line (B-CPAP). The malignant nature of this cell line had been confirmed by its growth in vivo in nude mice. The characteristics of thyroid papillary carcinoma cells, such as the synthesis of thyroglobulin, keratin of high molecular weight, and vimentin, were assessed using immunohistochemical techniques with the specific corresponding antibody. We also showed, by ultrastructural studies, that the cells behaved like oncocytic cells and had a secretory function corresponding to the ultrastructural description of thyroid carcinomas, e.g., abundant endoplasmic reticulum, well-developed Golgi complexes, and numerous secretory vesicles.
Figure 5. Reticular labeling using the antivimentin antibody is seen in all of the cultured cells (culture in chamber slides, immunofluorescence staining, original magnification ×500).

To assess an additional thyroid feature of the cell line, a study was performed on the expression of the synthetic gene composed of the thyroglobulin promoter and the tissue-specific enhancer transfected in the cells; this gene was found not to be expressed by the B-CPAP cells (Vassart G, personal communication, 1993). The cells synthesized the HCG hormone (α and β subunits) often secreted by the endocrine tumors. This hormone could play an autostimulating effect on the thyroid cells. B-CPAP also expressed NSE (EC 4.2.1.11), which is a marker of neurons and neuroendocrine cells. This enolase of the glycolytic pathway is detected in the thyroid gland in the C-cells and in the medullary thyroid carcinomas, but not in the normal thyroid follicular cells or in the follicular or papillary carcinomas. The search for calcitonin in B-CPAP was negative, thus eliminating the slim possibility that these are tumors that synthesize thyroglobulin and calcitonin.

We demonstrated by immunohistochemistry that all of the cells synthesize S100 proteins. S100 proteins (α and β isoforms) are present in numerous non-nerve tissues, including some follicular thyroid cells, where they function as calcium-modulated proteins. These proteins recently have been characterized as a substrate for the p53 protein, and they are strongly homologous with the product of the "ms11" gene involved in the metastatic processes.

Because 58% of the thyroid tumors expressed the TGF β-1, which seemed to be involved in the malignant stage of tumor development, we used an immunohistochemical technique on the cell line with an antibody specific to TGF β-1. We also showed that all of the cells contained this growth factor. This result was confirmed.

Figure 6. Homogeneous strong cytoplasmic labeling was obtained in all the cells with the antibody specific to NSE. Note the oval morphology of the cells (cytospin, immunofluorescence staining, original magnification ×500).

Figure 7. Diffuse immunostaining of the cytoplasm with the S100 proteins antibody is observed in all of the cells (cytospin, immunoperoxidase staining, original magnification ×500).
by a Northern blot analysis (Saez J, personal communication, 1993).

Estrogen receptors are found in 76% of thyroid papillary carcinomas, so their presence was investigated in the cell line. However, no such receptors were found.

We also looked for the expression of the oncogenes most frequently involved in thyroid carcinogenesis. No expression of the oncogene PTC was detected by Southern blot or in situ hybridization. Southern blots were used to look for mutations of trk and H-ras, competitive polymerase chain reaction for amplification of c-myc, but none was observed (Martin-Zanca D, Revol A, Vindimian M, personal communications, 1993). The only abnormal expression found in the cell line was a p53 mutation detected by antibody recognizing the most mutant forms of p53 protein. Thus, this mutation, which is a common event in undifferentiated thyroid carcinomas, also can occur in differentiated papillary carcinoma cells and can confer on thyroid tumor the ability to grow in vitro, which has been demonstrated.

The B-CPAP cell line could be used for studying thyroid carcinogenesis because it displays many interesting characteristics: the cells have ultrastructural and functional thyroid features; however, they display an important chromosomal aberration as a hyperploidy (65–70 chromosomes), and show many hitherto-unknown metabolic disorders in the normal follicular cells, such as NSE and HCG secretions. In addition, the co-expression of the tumor-suppressor protein p53 and S100 proteins should make possible an extensive study of the interaction between these two proteins because Baudier et al. have characterized p53 as an S100-binding protein. This interaction may be involved in the control of the cell cycle at the G0-G1/S boundary, thus leading to the neoplastic transformation of the thyroid cells.

In conclusion, the B-CPAP cell line provides a useful tool for research on thyroid carcinogenesis because it represents one of the steps between normal cells and undifferentiated malignant cells. Additional studies on this cell line will provide a better understanding of the genetic defect leading to tumorigenesis and may lead to the discovery of the chemical factors inhibiting pathologic growth in vitro.

References