Establishment and Characterization of Continuous Cell Line MTC-SK Derived from a Human Medullary Thyroid Carcinoma

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ABSTRACT

Tumor cells of a human medullary thyroid carcinoma were isolated and propagated in tissue culture. Several cell lines with different morphology developed from the primary culture, among others a fibroblast-like growing cell line (MTC-F) and a cell line growing as a suspension of single cells and spherical cell clusters (MTC-SK). The MTC-SK cell line was serially propagated for 90 passages, over 3 years.

When examined at different times throughout the in vitro period, MTC-SK exhibited properties characteristic of medullary thyroid carcinomas: the cells maintained their epitheloid morphology; endocrine granules were demonstrated in the cytoplasm by electron microscopy; in situ hybridization confirmed the production of calcitonin- and bombesin-related peptide mRNA (gastrin releasing peptide); the cells revealed positive immunoreactivity with antibodies to calcitonin, calcitonin gene-related peptide, and bombesin. The in vitro properties of the MTC-SK cells corresponded to the results obtained from the tissue of origin.

Cytogenetic studies of the MTC-F cell line revealed a supernumerary metacentric chromosome (20?). In the MTC-SK cell line the predominant pairing of the homologous chromosomes 16.

INTRODUCTION

MTCs originate from C-cells of the thyroid gland, known to produce several peptides, among them CT, CGRP, GRP (bombesin), SRIF (somatostatin), and 5-HT (serotonin). The cyto-physiological characteristics of C-cells have been reviewed in detail by Nunez and Gershon (1). MTCs of the rat were studied concerning chromosome lip, i.e., the locus of the calcitonin and calcitonin gene-related peptide genes and the H-ras oncogene, and a characteristic instability of the centromeric region of chromosome 16 and somatic pairing of the homologous chromosomes 16.

Very few studies of human medullary thyroid carcinomas grown in tissue culture have been reported: in 1976 Tischler et al. (5) grew cells of two human medullary thyroid carcinomas in short-time culture and studied their electrical excitability with and without the addition of nerve growth factor. The production of calcitonin from these cells was proved by immunocytochemistry. In 1981 Leong et al. (6) established a continuous cell line, TT, from a human MTC, which retained essential physiological characteristics are reported.

MATERIALS AND METHODS

Origin of the Cell Lines. Tissue was explanted from the primary tumor of an apparently sporadic MTC of a 51-year-old central European woman. Histologically, the tumor tissue consisted of solid areas, as well as fusocellular parts, and contained amyloid. In some regions follicular differentiation appeared.

Cell Culture Procedure. Tissue handling and disaggregation has already been described (14). Briefly, fresh tumor tissue was cut into small pieces, minced, and resuspended in Ham’s F-12 containing 10% fetal calf serum, 100 U penicillin/ml, and 100 µg streptomycin/ml medium. Antibiotics were only added to the very first passages. Ficoll-Paque was used for removing cellular debris from the cell suspension. Initially, the cells formed monolayers of epithelial cells (MTC-F) which were maintained through 2 months undisturbed by subculture. Fibroblasts were eliminated by selective adhesion, selective detachment (15, 16), or simply by changing the medium for several weeks in order to avoid trypsin stimulation of fibroblast growth. At this stage many areas of increased cellular density piled up (Fig. 1a), and the piles increased in size, formed spheroids and finally detached (MTC-SK, Fig. 1b). The suspension cultures were routinely passed by centrifugation and resuspended in medium three times/week, and they were maintained in Nunc tissue culture flasks at 37°C in a 5% CO₂-enriched atmosphere without magnetic stirring. Cell counting and growth measurements were made with a Coulter Counter. Mycoplasma testing of cultures and controls was negative.

Morphological Studies. For transmission and scanning electron microscopy, the original tumor tissue and the cell lines were prepared following routine techniques.

IHC. Tumor tissue was formalin fixed, paraffin embedded, and cut at 5 µm. The ABC method was used as described in detail elsewhere (17).

ICC. For ICC regarding MTC-SK, about 10⁵ cells suspended in phosphate-buffered saline (pH 7.2) were applied to each slide, briefly dried, and immersion fixed in acetone at −20°C for 10 min, air dried, and stored at −20°C. After rehydration and washing in phosphate-buffered saline, the immunocytochemical reactions were performed using either the ABC technique (18) or the alkaline phosphatase-anti-alkaline phosphatase technique (19).

ISH. ISH was done with the following probes: h-CT (0.298 kilobases), h-CGRP (0.3 kilobases), h-GRP (0.7 kilobases), and r-SRIF (0.38 kilobases). A detailed protocol for in situ hybridization utilizing antisense RNA probes was published previously (20). The specificity of the calcitonin probe was controlled by Northern blot analysis (technical details see Ref. 21).

Cytogenetic Analyses. Cytogenetic analyses were performed in the MTC-F cell line at passages 6 through 8 and in the MTC-SK cell line at passages 12 and 13. The cells were exposed to colcemid (0.4 µg/ml) for 30 min and 10-15 min, respectively, exposed to a hypotonic solution for 30 min and 10-15 min, respectively, and stored at −20°C. After rehydration and washing in phosphate-buffered saline, the immunocytochemical reactions were performed using either the ABC technique (18) or the alkaline phosphatase-anti-alkaline phosphatase technique (19).
**RESULTS**

Establishment and Growth Characteristics of the MTC-SK Cell Line. Tumor tissue of a human MTC initially formed monolayers of epithelioid cells (MTC-F cell line). From these adherent cells, single cells and cell spheroids detached spontaneously (MTC-SK cell line), as has been previously reported (22). This cell line was established in suspension culture and has been maintained for 3 years. The tumor cell spheroids contained morphologically uniform nonnecrotic cells, apparently due to the loose attachment of the cells and the small diameter of the spheroids (up to 30 μm). Before cell counting, the spheroids were easily dissociated by aspirating with a pipette. An inoculum of $5 \times 10^5$ cells/ml has a doubling time of 2.4 days. (Fig. 2). The addition of nerve growth factor, epidermal growth factor, and dexamethasone to the medium did not influence the growth characteristics of the cells (23); addition of bombesin (GRP) supported the formation of tumor cell spheroids.

Ultrastructure. Ultrastructural studies proved the preservation of the characteristic tumor cell morphology in the MTC-SK cell line. The original tissue (Fig. 3a) contained only a moderate number of neuroendocrine granules. In the cultured cells (Fig. 3b) even fewer granules were found. These granules showed relatively uniform electron-dense cores and slender halos. The nuclei were large with one or two prominent nucleoli. Their profile was irregular. The number of Golgi complexes was increased (Fig. 3c). In many cytoplasmic areas a rough endoplasmic reticulum was well developed. The cisternas were wide with dilated regions. No cellular junctions were found between the cells of the spheroids.

IHC. By immunohistochemistry CT and GRP were localized in almost all cells of tissue sections of the primary MTC. GRP immunoreactivity was only weakly demonstrable in most of the tumor cells, 5-HT was occasionally found, and SRIF gene expression was not detectable (data not shown). Incubations of MTC-SK cells grown in tissue culture revealed an identical pattern of immunoreactivity as compared to primary tumor tissue, except that a stronger CGRP immunoreactivity was noticed in MTC-SK cells and 5-HT could not be detected in these cells (Figs. 4 and 5).

ISH. In situ hybridization of tumor tissue sections allowed, in addition to the intense reactions with CT and GRP probes, the unequivocal visualization of CGRP mRNA in tumor cells. The experiments to localize SRIF mRNA revealed negative results (data not shown). Hybridization of the CT, CGRP, and GRP probes to MTC-SK cells (Fig. 6) allowed the visualization of specific mRNAs, whereas SRIF mRNA was not detectable. The results of the IHC, ICC, and ISH experiments are summarized in Table 1. Studies on oncogene expression of Ha-ras, c-myc, and N-myc in both primary tumor and its lymph node metastases by in situ hybridization and Northern blot analyses are published elsewhere (24). Similar studies in the MTC-SK cell line are in progress.

Chromosomal Findings. In the MTC-F cell line, 25 of 30 metaphases had a supernumerary metacentric chromosome (chromosome 20?) (see karyotype in Fig. 7), and 5 metaphases had a normal chromosome number.

In the MTC-SK cell line, 42 of 125 metaphases contained TA. Thirty-two of these rearrangements concerned 11p (Fig. 8). Five of 125 metaphases had different translocations, four involving 11p. In 3 of 125 metaphases a supernumerary chromosome was present. Seventy-five of 125 (60%) of the metaphases had a normal chromosomal constitution. Ten % of the metaphases showed a centromeric instability of chromosome
with various degrees of despiralization; somatic pairing of the two homologous chromosomes 16 was present in 2% of the metaphases (Fig. 9).

Lymphocyte cultures were first done 1 year after the patient underwent surgical irradiation and, therefore, are of limited significance. Almost 100% of the metaphases contained structural chromosomal abnormalities including dicentric and ring chromosomes, translocations, chromatid and isochromatid breaks, quadriradial figures, and double minutes. In 2 of 20 metaphases, however, terminal chromosomal associations of chromosome 11, ter rea (11p') and ter rea (11q;16q), were present, as seen in the MTC-SK cell line. At that time the patient had developed bone metastases and these two cells may

| Table 1 Neuroendocrine gene expression in tumor tissue and MTC-SK cells |
|-----------------|-----|-------|-----|-----|-----|
| MTC (tissue)    | CT  | CGRP | GRP | SRIF | 5-HT |
| IHC             | +++ | +    | +++ | -   | +   |
| ISH             | +++ | ++   | +++ | -   | ND* |
| Northern blot   | +++ | +++  | +++ | -   | ND  |
| MTC-SK cells    | ++  | +++  | +++ | -   | -   |
| ICC             | +   | +    | ++  | -   | ND  |
| ISH             | +   | +    | ++  | -   | ND  |

* ND, not done.
have been tumor cells. Skin fibroblasts had a normal chromosome constitution.

DISCUSSION

In this paper we describe the successful establishment of a continuous cell line in suspension culture derived from a human medullary thyroid carcinoma (MTC-SK) maintaining the peptide expression pattern of the primary tumor. In contrast to the TT cell line which grows as a monolayer, the MTC-SK cell line forms tumor cell spheroids. The loose attachment of the MTC-SK cells corresponds to the lack of cellular junctions.

In their small cell lung carcinoma cell lines (26), previously shown to reveal, like MTCs, amine precursor uptake and decarboxylation cell properties (27), Gazdar et al. (25) described a so-called “classic” small cell lung carcinoma line retaining
typical morphology and typical biochemical profile and growth characteristics. A "variant" cell line had discordant expression of the biochemical markers and either typical or altered morphology and growth characteristics (growth as loosely attached floating aggregates and relatively short doubling times). These variant growth characteristics resemble those seen in our MTC-SK cell line, but by contrast, our cells retained the typical morphology and biochemical profile.

The maintenance of the neuroendocrine features (peptide expression pattern) in vitro was confirmed by different methods. Ultrastructural studies indicated secretory activity by a remarkable hypertrophy of the Golgi areas and by the dilated endoplasmic reticulum. Immunohistochemistry and in situ hybridization confirmed the production of CT, CGRP, and GRP in the original tumor and cell lines; 5-HT, which present in the original tumor, could not be detected in the cell lines.

Absence of somatostatin production in the tumor tissue and cell lines of our apparently sporadic MTC may support the findings of Capella et al. (28) who reported high amounts of somatostatin immunoreactivity in familial cases only. Sikri et al. (29), however, could not find a difference in their familial and nonfamilial tumors. These inconsistent findings may be based on the fact that calcitonin, CGRP, and GRP do not coexist with somatostatin in MTC cells (28, 29) and, along with the calcitonin-, CGRP-, and GRP-producing C-cells, typical D-cells can also be present in this tumor (28).

Among the cellular products, bombesin (GRP) is of special interest. Moody et al. (30, 31) have suggested that the presence of bombesin might be an essential property for the continued growth in small cell lung cancer, bombesin acting as an autocrine growth factor. This is supported by the fact that our original tumor tissue and MTC cell lines produced bombesin spontaneously and addition of bombesin to the cell cultures increased the formation of cell spheroids.

Chromosomes in MTC were first studied by Wurster-Hill et al. (32) in two primary MTC tumors and one metastasis, by direct short-term culture methods. They found in their specimens hypodiploid modal chromosome numbers of 42–44. In patient 1, five consistent markers were present in the tumor and the lymph node metastases as well, with breakpoints at 1p11, 1q11, 4q27, 11p15, 20q11, or 20q13. In the tumor cells of the second patient, two consistent markers were present: (t(3;7)(3pter-3pl1::7pl1-7pter) and t(17;22) (17qter-17ql1::22p11-22qter). The only common findings were the hypodiploid modes and loss or rearrangements of at least one chromosome 22. Leong et al. (6) did chromosome studies in their TT cell line and found a hypodiploid modal range of 37–44 and several unidentifiable marker chromosomes. Other chromosome studies in early (6–7th) and late (68–70th) passages of this TT cell line (12) revealed that most of the cells were hypodiploid with a modal number of 43. The karyotypic pattern was relatively stable; consistent alterations involved the following chromosomal breakpoints: 1p36, 3p12, 5q33, 7q22, and q31, 8q24, 9p13, 10q25, 11p13, 12p13, and 20q13. The TT cell line and the tumor cells shared the hypodiploid chromosome

Fig. 9. MTC-SK, chromosome 16. a, centromeric instability; b, somatic pairing of the homologous chromosomes in a diploid (left) and tetraploid (right) metaphase.

Fig. 10. MTC-SK1 with three marker chromosomes: 10p+ resulting from a 3;10 translocation (t(3;10)(q12;p11)), 11p+, 22p++; centromeric instability of chromosome 1 (arrows).
number and the breakpoints at 11p13 and 20q11 or 20q13. The marker i(1q) seen in the TT cell line was similar to the marker t(1q1q) described in the tumor cells.

In contrast to these findings, our MTC-F cell line had an isolated trisomy 20 (?) in 25 of 30 metaphases. Trisomy 20 has been found in various hematological disorders, in most types of lymphomas, and in solid tumors (33). In these malignancies, trisomy 20 was usually combined with other numerical and structural chromosomal abnormalities. Unfortunately, our MTC-F cell line stopped growing prior to our obtaining better banding of the chromosomes and before molecular studies could be done.

A deletion of chromosome 20p12.2 has been reported in the germ line of patients with the hereditary form of MTC (MEN2A and 2B syndrome) by two groups of investigators (34–36). These findings were not confirmed by other investigators, by either cytogenetic studies (37–42) or linkage analyses (42, 43). We did not find the deletion in lymphocytes and fibroblasts of our patient.

Again, in contrast to the hypodiploid chromosome number found in tumor cells and in the TT cell line, most MTC-SK cells had an euploid chromosome number (diploid or tetraploid). The frequent terminal rearrangements at 11p are of special interest because the genes for calcitonin/CGRP (11p15.4) as well as the oncogene Ha-ras-1 (11p15.5) and serum amyloid A (11pter-p12) map within this region.

TAs were observed previously in a B-cell prolymphocytic leukemia (44), in solid tumors (45–47), and in tumor cell lines (48) but were also present in nontumorous material as in lymphocyte cultures of patients with ataxia telangiectasia (49–51) and in esoteric and SV40-transformed human fibroblast cell lines (52, 53). In these reports chromosomes were randomly involved in the terminal rearrangements in contrast to the nonrandom involvement of 11pter in our MTC-SK cell line.

The significance of TAs is unclear. The possibilities are: an alteration of telomeric DNA sequences is the molecular basis of TA (46), they are nonspecific dysfunctions of telomeric replication in genetically unstable cells without direct role in carcinogenesis (47), or TAs are indeed characteristic of cancer cells (48).

Centromeric instability of chromosomes 1, 9, 16, and rarely 2 has been seen in lymphocyte cultures of patients suffering from a probable autosomal recessively inherited syndrome with variable immune deficiency, facial dysmorphism, and mental retardation (54–58). In our phenotypically normal patient, the centromeric instability was present in tumor cells only.

Chromosome instability has been reported in some patients with MTC (37, 38, 59) but was not present in others (60). In our patient the chromosome instability seen in the lymphocyte cultures seems to have been promoted by the surgical irradiation.

Recently, we studied later passages of the MTC-SK cell line (55 through 70) and found two consistent markers: one marker (10p+) apparently resulted from a (3;10) translocation leading to a partial trisomy 3q and monosomy 10p; in the second marker (11p+) the extra band on 11p is of unknown origin. One chromosome 22 was either missing or structurally abnormal. This marker chromosome 22 resembled the extra chromosome seen in the MTC-F cell line from which the MTC-SK cell lines had detached spontaneously. At these passages 50% of the metaphases exhibited a centromeric instability of chromosome 1 and rarely of chromosomes 2, 9, and 16 (see karyotype in Fig. 10).

These markers are of interest because a linked genetic marker for multiple endocrine neoplasia type 2A (MEN2A syndrome) has been reported on chromosome 10p11.2-q11.2 (61, 62) as well as allele loss in the tumor tissue at 1p (63) and 22q (64).

The aberrations present in our MTC cell lines concern chromosomal regions that are the subject of ongoing molecular studies: these may contribute to a better understanding of the as yet unknown neoplastic process in the sporadic MTC and MEN2 syndromes.

REFERENCES


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