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Invited Review

Characterisation of thyroid medullary carcinoma TT cell line

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Summary. TT cell line is the best known stabilized cell line derived from the human medullary thyroid carcinoma. The ultrastructural characteristics of these cells include well developed rough endoplasmic reticulum, a prominent Golgi apparatus and a considerable number of secretory granules. Numerous hormones were immunocytochemically demonstrated in TT cells of which calcitonin and calcitonin gene-related peptide (CGRP) are the products of the same gene but an alternative RNA processing. TT cells were found to produce some other hormones as well, namely ACTH, neurotensin, enkephalin, PTHrP, gastrin-releasing peptide (GRP), serotonin but also functional proteins of the chromogranin group, synaptophysin, NSE, calbindin and tyrosine hydroxylase. Some marker proteins have been detected in the cytosol (CEA) and in the cytoskeleton (alpha-tubulin, cytokeratin). The influence of numerous factors on the secretory activity of these cells has been demonstrated so far, including effects of 1,25-dihydroxycholecalciferol, glucocorticoids, sex steroids, cAMP, gastrin-releasing peptide, sodium butyrate, phorbol esters, ionomycin and forskolin. The investigators performed on the TT cell line demonstrate that this is the most reliable model system for the human parafollicular cells developed so far, in comparison to other cell lines derived from the medullary carcinoma of the thyroid.

Key words: TT cell line, Thyroid medullary carcinoma, Hormones, Functional proteins, Cell culture

Introduction

The designation «parafollicular» was first given to the specialized and supposedly endocrine, nonfollicular cells of the mammalian thyroid gland by Nonidez (1932). The successful demonstration by immunofluorescence (Bussolati and Pearse, 1967) of calcitonin in the parafollicular cells, together with the demonstration that they are not invariably parafollicular (Cavalheira and Pearse, 1967) or confined to the thyroid gland (Carvalheira and Pearse, 1967), and that they are derived (penultimately at least), from the ultimobranchial body (Pearse and Carvalheira, 1967) led to the proposal by Pearse (1966) that they should be called C cells (C for calcitonin). Therefore, the terms C cells and parafollicular cells are in use to describe the same type of thyroid gland cells, which produce calcitonin. In the human thyroid gland, C cells are normally restricted to the posterior part of the lateral lobes.

It is form the parafollicular cells that the medullary thyroid carcinoma (MTC) arises. Prior to 1959 it was not distinguished as a tumor separate from anaplastic carcinoma. Overall, it accounts for about 10% of all thyroid cancers and occurs in a younger age group. An estimated 25% of all MTC are familial and are associated with multiple endocrine neoplasia syndromes (MEN). Data from the German MTC Registry indicate that 16.6% MTC are associated with MEN 2A (Sipple's syndrome) accompanied by pheochromocytoma and abnormalities of the parathyroid. 5.3% MTC are associated with FMTC (familial MTC alone), and 2.7% with MEN 2B characterised by MTC, pheochromocytoma and ocular and oral neuromas with gastrointestinal ganglioneuromatosis. The syndrome is inherited as an autosomal dominant trait. 75% of MTCs are nonfamilial. This proportion, however, is likely to decrease as a result of detailed genetic analyses of patients with apparent sporadic tumors (DeLellis, 1995). A variety of somatic abnormalities are mostly associated with the effects of the secretory products of MTC on many tissues and organs (Thomson, 1981; Kohler, 1986). The MTC cells were found to produce calcitonin (CT), considered as a marker of this neoplasm. CT and other substances typical for parafollicular cells (Gagel et al., 1980; Bose et al., 1992) are valuable in early diagnosis of MTC especially after pentagastrin stimulation in the screening of potentially affected members of MEN 2 families (Raue and Grauer, 1994). Also, some substances non typical for parafollicular

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cells, such as ACTH, neurotensin, enkephalin, PTHrP and serotonin (Zabel, 1984; Oosterom et al., 1986; Zeytin and DeLellis, 1987; Zeytin et al., 1987; Ikeda et al., 1988; Bidard et al., 1993) are in use for the screening.

As model systems of the C cells various cell lines derived from the MTC have been developed. The first CT-secreting WAG/Rij transplantable rat MTC (rMTC) was described by Boorman et al. (1974). They showed that the transplanted neoplasm maintained some of the morphological and functional characteristics of the original tumor (Zeytinoglu, et al., 1980). Another well known stabilized cell line derived from the rMTC is the 44-2C cell line (Zeytin and DeLellis, 1987). The cells were shown to synthesise and secrete neurotensin, CT and somatostatin (Zeytin et al., 1987). The murine CA-77 cell line was also successfully used as a model system of the parafollicular cells (Muszynski et al., 1983). In the cells, expression of the CT/CGRP and cholecystokinin genes has been demonstrated (Odum and Rehfeld, 1990; Collignon et al., 1992).

The best known stabilized cell line derived from human MTC is the TT cell line. It was developed by Leong et al. in 1981 (subcultures 24 to 30) (Leong et al., 1981). It is very important to investigate the characteristics of these cells as they are the most reliable model system of the human parafollicular cells developed so far.

When investigating the characteristics of cultured endocrine cells it is important to remember that neoplastic endocrine cells, particularly when in culture, may decisively alter the expression of several proteins and their hormone expression, as indicated by numerous studies (Chaiwun et al., 1994).

Morphology and immunocytochemistry of TT cells

TT cells exhibit lower number of secretory granules than in normal thyroid parafollicular cells (Zabel and Schafer, 1988; Zabel et al., 1994). Some other ultrastructural characteristics of TT cells include well-developed rough endoplasmic reticulum present within a restricted space in the perinuclear region and a prominent Golgi apparatus (Zabel et al., 1994).

TT cells were found to produce numerous hormones as well as some functional proteins and markers although in man and in most mammalian species, including rat, the first immunocytochemically demonstrated hormones were CT, calcitonin gene-related peptide (CGRP), somatostatin, gastrin-releasing peptide (GRP) and ACTH (Gagel et al., 1986; Oosterom et al., 1986; Cote et al., 1987; Haller-Brem et al., 1988; Sunday et al., 1988).

The presence of the remaining hormones has indirectly been hinted at by hybridocytochemistry demonstrating the presence of the appropriate mRNA, by radioimmunological detection of the hormones in medium, or by immunocytochemical studies on the cultured TT cells (Raue, 1985; Bose et al., 1992).

In the cells the presence of proteins associated with secretory granules has also been demonstrated. The proteins include chromogranin A, SP-I and synaptophysin (Murray et al., 1988; Zabel et al., 1995). A very important finding involved the detection of NSE in TT cells, since the quantity of enolase is known to change with variations in CT secretions (Schafer and Zabel, 1983; Kameda, 1985; Zabel and Schafer, 1988). Intensity of NSE cellular staining has been noted to be inversely related to CT content of the cells and directly related to CT level in the medium. Therefore, NSE may be used to evaluate the functional status of TT cells (Zabel et al., 1995) (Fig. 2).

From amongst the enzymes participating in biogenic amine formation (Zabel, 1985), thyrosin hydroxylase has been demonstrated in TT cells (Zabel et al., 1995).

The diagnostic significance of the CEA and cytoskeleton proteins has been confirmed by immunocytochemical studies on medullary carcinomas (Raue, 1985; Miettinen, 1987).

As far as the localization of secretory products within TT is concerned, the secretory granules contain CT, CGRP, somatostatin, neurotensin, met-enkephalin, leu-enkephalin, GRP, parathyroid hormone-related protein, functional proteins of the chromogranin group and synaptophysin while in the cytosol NSE, calbindin and tyrosine hydroxylase can be found. Some marker proteins have been detected in the cytosol (CEA) and in the cytoskeleton (alpha-tubulin, cytokeratin) (Zabel et al., 1995).

The structure and expression of CT/CGRP gene

The structure and expression of the CT gene were of special interest as much of it is known to yield two distinct mRNAs, which provide templates for production of either CT or CGRP (Amara et al., 1982; Nelkin et al., 1984; Edbrooke et al., 1985; Jonas et al., 1985; Sabate et al., 1985; Steenbergh et al., 1986; Emerson et al., 1989). This has been worked out in detail using biochemical techniques (Rosenfeld et al., 1984; Jonas et al., 1985; Steenbergh et al., 1986). It has been demonstrated that this gene located on the short arm of chromosome 11 consists of six exons, of which exons 1, 2 and 3 are present in each of the mRNAs. Moreover, CT mRNA contains sequences complementary to exon 4 while CGRP mRNA contains sequences for exons 5 and 6 (Amara et al., 1982; Jonas et al., 1985). The expression of the CT gene is frequently quoted as an example of alternate RNA processing. Thus, the CT gene yields a single hnRNA (primary transcript), which is processed in alternate ways to provide two distinct mRNAs, i..e. CT mRNA and CGRP mRNA (Steenbergh et al., 1986) (Fig. 1). Some authors, however, suggest that control of production of the two mRNAs may take place at the transcription level (Emerson et al., 1989). If that would be the case, two distinct hnRNAs would be formed to yield two mRNAs. Recent hybridocytochemical and

immunoultrastructural studies confirm that all TT cells produce both mRNAs and both hormones (they were always expressed together in the same secretory granule) in parallel (Zabel et al., 1994, 1995). The decision to produce CT mRNA or CGRP mRNA appears to be regulated in a tissue-specific manner. In the thyroid C cells, CT mRNA predominates whereas CGRP mRNA is produced in neural tissue (Amara et al., 1982; Rosenfeld et al., 1984). However, this splicing decision is not absolute. The normal thyroid C cells produce small amounts of CGRP in addition to CT (Sabate et al., 1985). Several tumor types, including MTC and carcinoma of the lung as well as cell lines derived from these tumors, have been shown to produce both peptides (Morris et al., 1984; Nelkin et al., 1984; Steenbergh et al., 1984; Edbrooke et al., 1985).

TT cell secretion regulatory factors

Regulatory mechanisms of TT cell secretory activity always were of special interest to those investigating their characteristics. Since the ultrastructure and immunocytochemistry of TT cells indicate that the cells resemble more closely normal parafollicular cells of the thyroid and cells of most medullary carcinomas analyzed in histological sections (Chaiwun et al., 1994) than any other cell line, it seems reasonable to use them as a model system for further studies on endocrine functions and their regulation as well as for clinical studies.

Most studies on regulation of TT cell secretory function refer to CT and CGRP, both generated by alternative RNA processing from the same primary RNA transcript. It has been shown that TT cells reversibly alter alternative RNA processing patterns depending

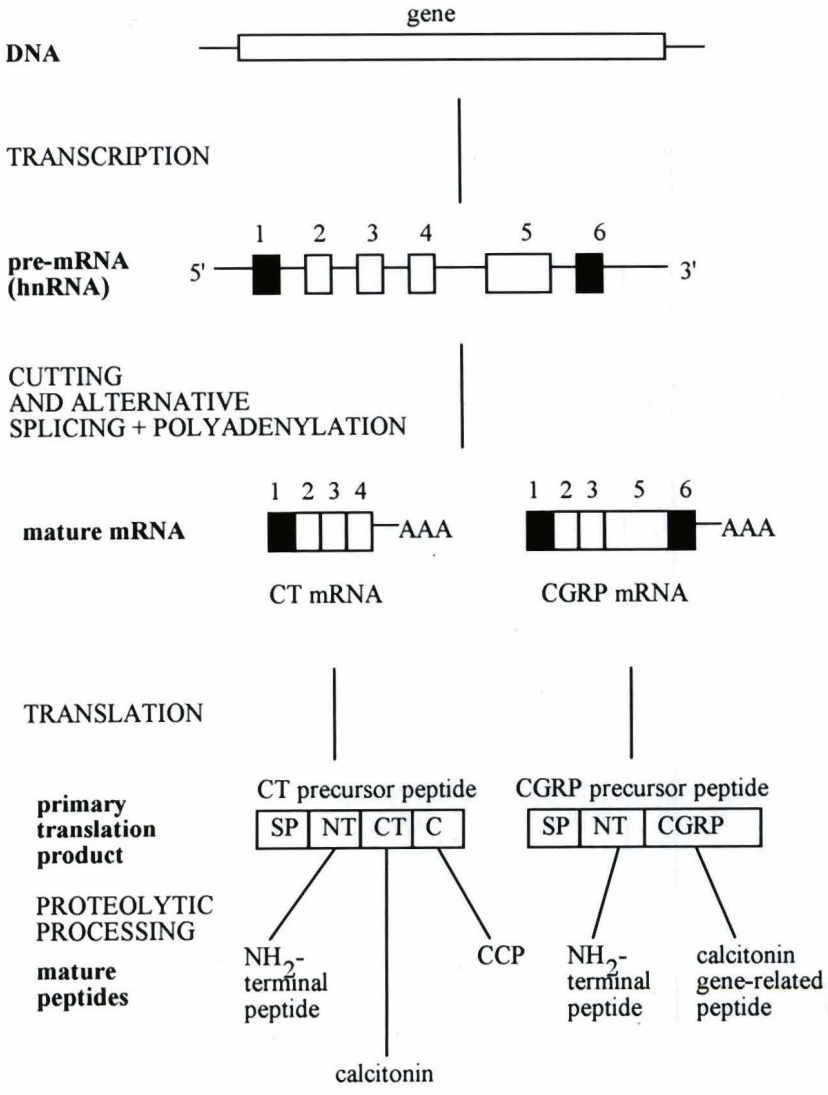

Fig. 1. A model to describe alternative RNA processing in CT/CGRP gene expression. The light boxes indicate sequences complementary to exons, the lines between them are the sequences complementary to introns and the black boxes indicate the noncoding sequences of RNA. SP: signal peptide; NT: NH_2-terminal peptide; CT: calcitonin; CGRP: calcitonin gene-related peptide; C: catacalcin; CCP: COOH-terminal polypeptides.

upon growth conditions in vitro, such that CT mRNA is lowest and CGRP mRNA is highest during rapid growth (Berger et al., 1984). the mechanisms underlying this RNA-processing may play an important role in patients with aggressive forms of MTC, in whom a decrease or loss of CT heralds a poor prognosis (Nelkin et al., 1989).

In 1987 Cote et al. reported calcitriol to decrease expression of the CT gene in TT cells. This observation was confirmed in rats by Naveh-Many and Silver (1988). In 1991 for the first time the calcitriol (1,25-dihydroxycholecalciferol) receptors have been demonstrated immunocytochemically by Zabel and Dietel (1991) in TT cell nuclei and in small amounts in the cytosol. They found receptor levels increased when the cells were cultured at physiological or somewhat higher concentrations of calcitriol. In parallel, the same doses of calcitriol markedly inhibited secretion of CT into the medium indicating a feedback loop between calcitriol and CT. They postulated the following regulatory mechanism of calcium (considered the main regulator of CT release) and calcitriol action on TT cells: calcium ions modify CT release (no functional Ca^{2+} channel has been detected in membranes of TT cells so far) (Krautwurst et al., 1993) by parafollicular cells in a matter of minutes without primarily affecting CT mRNA synthesis, thus indicating a direct effect on the secretory process. Calcitriol, however, influences CT secretion only after longer periods, apparently using the time-consuming pathway of DNA transcription (Zabel and Dietel, 1991).

Calcitriol is not the only steroid which influences CT gene expression in the TT cells. The effect of glucocorticoids on the regulation of gene expression has been extensively studied. It was shown that glucocorticoids predominantly act to stimulate gene transcription and increase mRNA and protein levels (Cote et al., 1986). Dexamethasone was found to affect the splicing mechanism of RNA causing a dosage-dependent increase in CT mRNA levels and a decrease in CGRP mRNA levels. Its effect was reversible after dexa-

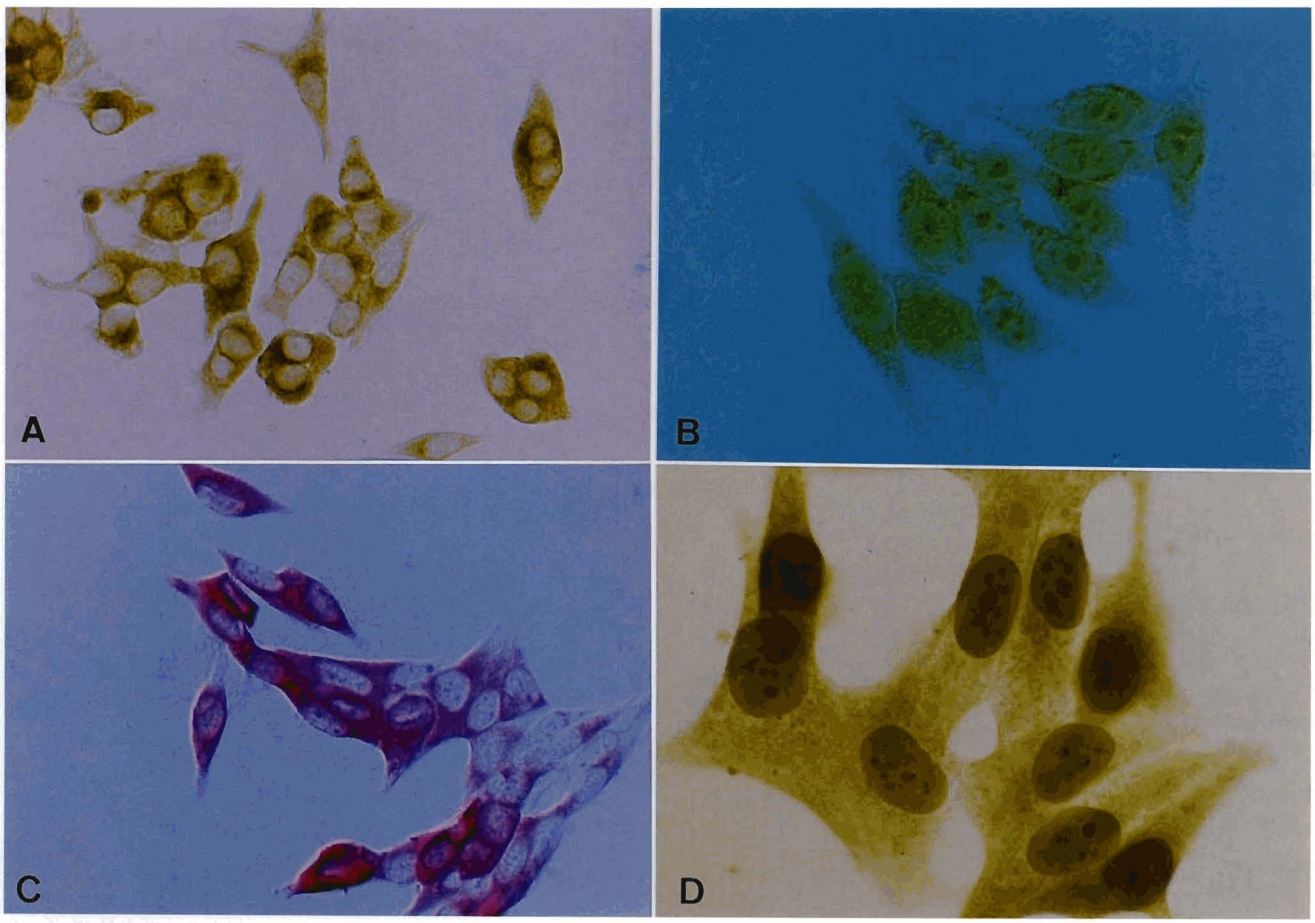

Fig. 2. TT cells derived from medullary thyroid carcinoma. All cells contain considerable amounts of calcitonin. (**a,** ABC-peroxidase immunocytochemical method, x 400) and calcitonin mRNA (**b,** in situ hybridisation using biotin-labelled RNA probes detected subsequently by streptavidin-peroxidase complex x 500). Cells vary in their content of neurone-specific enolase (**c,** ABC-alkaline phosphatase immunocytochemical method. x 400). In cells nuclei the calcitriol receptor has been visualised (**d,** ABC-peroxidase immunocytochemical method. x 620).

methasone withdrawal (Cote and Gagel, 1986). It was also demonstrated that TT cells treated with dexamethasone showed an almost complete inhibition of somatostatin peptide production at 48 h of treatment. In the same study, analysis of mRNA content by hybridization revealed that dexamethasone also caused a decrease in detectable somatostatin mRNA (Cote et al., 1986).

The sex steorid, estradiol had no inhibitory effect on somatostatin production by TT cells. However, studies on CT secretion in ovariectomized rats revealed that progesterone stimulated the basal secretion of CT and the treatment with estradiol stimulated CT secretion in response to calcium infusion (Tsai et al., 1992; Lazaretti-Castro, et al., 1991).

CT gene transcription in TT cells has also been shown to be controlled by cAMP. Results of numerous investigations indicate that transcription of human CT gene is markedly increased by cAMP in these cells. The cell response to cAMP is complex, requiring multiple elements acting in concert. In transfection experiments in TT cells, the downstream cAMP response element of DNA (CRE), combined with CT promoter sequences, generated 70% of the maximal cAMP response. The upstream CRE and the C-rich elements conferred 10 and 30% of this response respectively. The specific TT cell proteins were found to bind to each of these sequences (De Bustros et al., 1992). The cAMP was shown to regulate cystatin (cysteine proteinase inhibitor) in TT cells by cAMP -calcium-protein kinase C mechanisms that appear to be similar to those that regulate secretion of CT from these cells. However, in contrast to the CT gene, the expression of the cystatin C gene in these cells is not regulated by cAMP (Barka et al., 1992).

Some investigations of the effects of GRP on TT cells showed that GRP regulates TT cell function through modulation of $[Ca^{2+}]_i$ and thus stimulates CT release in a concentration-dependent manner at 0.1-100 nmol/l (Abe et al., 1992). It was also shown that CT secretion to the culture medium is stimulated by glucagon and pentagastrin and inhibited by somatostatin (Zabel, 1995). In the same study regulatory effects of biogenic amines and their precursors on CT secretion were demonstrated. Dihydroxy-1-phenylalanine and serotonin augmented while 5-hydroxy-1-tryptophan and dopamine inhibited CT secretion.

Some other studies demonstrated that CT gene transcription in the TT cell line is also regulated by sodium butyrate (Nakagawa et al., 1988), phorbol esters, ionomycin and forskolin. Ionomycin (10umol/l) was reported to raise the concentration of $[Ca^{2+}]_i$, concomitant with a transient stimulation of the secretion of CGRP and CT (Haller-Brem et al., 1988). 12-O-tetradecanoylphorbol-13-acetate (TPA) in concentration of 16nmol/l did not affect the concentration of $[Ca^{2+}]_i$, but caused a gradual rise in the secretion of CGRP and CT (Haller-Brem et al., 1988). Forskolin (10umol/l) alone did not change the concentration of $[Ca^{2+}]_i$, marginally enhanced the release of CGRP and CT and caused 23-fold increase in the cellular levels of cAMP (Haller-Brem et al., 1988).

Nelkin et al. (1990) demonstrated that in the TT cells the viral Harvey ras (v-rasH) oncogene induced differentiation, marked by morphological changes, diminution of growth, and increased expression of the CT gene.

The results of genetic studies on the TT cell line contribute towards progress in human MTC therapy. Knowledge of the role various oncogenes play in human malignancies creates perspectives for the future genetic treatment of many of them. Recently, TT cells were used in the studies resulting in demonstrating the ret oncogene, which is expressed in a number of human tumors. It was shown that ret mRNA levels were increased following (Bu)2-cAMP-induced differentiation of TT cells. Since the ret gene has been mapped on chromosome 10, close to the gene which predisposes patients to the MEN2A syndrome, this region of chromosome 10 might be involved in the proliferative and differentiative patterns of the MTC (Santoro et al., 1990).

As the studies on the structure and function of the TT cells continue, there is more and more material available supporting the thesis that this particular cell line is still the most reliable model system for studies on the human parafollicular cells.

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