EXPERIMENTAL STUDY

Expression of the cholecystokinin₂-receptor in normal human thyroid gland and medullary thyroid carcinoma

Michael Bläker, Andreas de Weerth, Mareike Tometten, Martina Schulz, Wolfgang Höppner¹, Dorit Arlt¹, Cuong Hoang-Vu², Henning Dralle², Harald Terpe³, Ludwig Jonas³ and Tammo von Schrenck

Medizinische Kernklinik und Poliklinik, Universitätsklinikum Hamburg-Eppendorf, Germany, ¹Institut für Hormon- und Fortpflanzungsforschung, Hamburg, Germany, ²Universitätsklinik für Allgemeinchirurgie, Halle, Germany and ³Institut für Pathologie, Universität Rostock, Germany

(Correspondence should be addressed to M Bläker, Medizinische Kernklinik und Poliklinik, Universitätsklinikum Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany; Email: blaeker@uke.uni-hamburg.de)

Abstract

Objective: The cholecystokinin₂-receptor (CCK₂R) promotes secretion and cell growth induced by its ligands cholecystokinin (CCK) and gastrin. The receptor has recently been shown to be expressed in human medullary thyroid carcinomas (MTCs). The objective of this study was to analyze CCK₂R expression in MTC samples of different tumor stages as well as in non-malignant thyroid tissues.

Design and Methods: Using RT-PCR we investigated 19 MTC samples and TT-cells (a human MTC cell line), as well as samples of normal thyroid. In addition, we performed immunohistochemistry using calcitonin- and CCK₂R-specific antibodies on MTCs and samples of C-cell hyperplasia.

Results: We demonstrate for the first time that CCK₂R is expressed not only in MTCs but in all samples of normal thyroid tissue. Using immunohistochemistry the receptor could be localized on calcitonin-secreting C-cells. The highest incidence of CCK₂R expression in MTCs was observed in early-tumor stages, whereas CCK₂R could not be detected in advanced or metastasized tumors.

Conclusions: The expression of CCK₂R in C-cells suggests a physiological function for gastrin and/or CCK in the regulation of calcitonin release, presumably related to bone and calcium metabolism. Moreover, these ligands might act as growth factors in MTCs. Efforts in the development of CCK₂R scintigraphy for the detection of MTC lesions might have to consider a lower incidence of the receptor in advanced tumor stages.

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Introduction

The G-protein coupled cholecystokinin₂-receptor (CCK₂R) mediates the functions of two enteroendocrine hormones, cholecystokinin (CCK) and gastrin. Stimulation of CCK₂R leads to acid secretion as well as cell proliferation within the gastric mucosa (1). In addition, gastrin has been shown to induce growth of a variety of normal as well as malignant gastrointestinal cell types *in vitro* (2). Furthermore, in several tumor systems an autocrine loop has been proposed between gastrin, secreted by tumor cells, and CCK₂R, expressed on tumor cells (3–6).

In recent years, CCK₂Rs have been detected in various extraintestinal cell types and tumors (7, 8). Based on the well-established proliferative effects of gastrin, CCK₂Rs might be important promoters of growth in these extraintestinal organs and tumors and therefore might serve as interesting diagnostic and therapeutic targets.

Gastrin pentapeptide (pentagastrin) is a well-known secretagogue in medullary thyroid carcinomas (MTCs),

which originate from calcitonin-producing C-cells (9). The increase in calcitonin serum levels after i.v. injection of pentagastrin is widely used as a diagnostic tool in MTCs. In 1996, Reubi & Waser (10) showed by autoradiography that MTCs express CCK₂Rs with an incidence of 92%. This report was the first to demonstrate CCK₂R expression in any kind of thyroid tissue. Subsequently, Amiri-Movasi *et al.* (11) detected CCK₂R mRNA in six out of six MTCs. However, it remained unclear whether malignant transformation of C-cells leads to aberrant CCK₂R expression or whether even in normal thyroid tissue CCK₂R is expressed. Moreover, there are no available data on the relationship between CCK₂R expression in MTCs and tumor stage, calcitonin levels and possible underlying mutations of the RET proto-oncogene (12).

The aim of the present study was to analyze CCK₂R expression in normal thyroid tissues as well as in MTCs by RT-PCR and immunohistochemistry using a CCK₂R-specific antibody. Furthermore, we compared receptor expression with clinical and pathological

data. In addition, to address the possibility of an auto-crine loop, we analyzed expression of the known CCK₂R ligands, CCK and gastrin, in normal thyroid tissue and MTCs.

Materials and methods

Patients

All patients gave informed consent to the study of their tissue samples and clinical data prior to surgery. The study was approved by the local ethics committee.

Patient data corresponding to the investigated MTC samples are summarized in Table 1.

Tissue samples and cells

After surgical resection, aliquots of tissue samples were immediately frozen in liquid nitrogen and then stored at -80°C . Diagnosis of underlying disease was verified by histological examination. The tissue samples investigated were 19 MTCs and two samples of normal thyroid gland removed during surgery for non-toxic goiter ('goiter 1', 'goiter 2'). In addition, commercially available pooled thyroid cDNA (Clontech, Heidelberg, Germany) was studied for the presence of CCK₂R transcripts. Tissue samples of normal human stomach and normal human colon served as positive and negative controls respectively. TT cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in Ham's F12 medium, supplemented with 2 mmol/l glutamine, 1% non-essential amino acids, 1% sodium pyruvate and 10% fetal calf serum (Gibco

Life Technologies, Eggenstein, Germany) at 37°C . When grown to confluence, cells were trypsinized (trypsin 0.05%, EDTA 0.02%) and RNA was extracted as described below.

Pentagastrin stimulation test

Pentagastrin (Cambridge Laboratories, Cambridge, Cambs, UK) (0.5 U/kg body weight) was injected i.v. Blood samples were drawn 2 min before, immediately before, 2 min after and 5 min after pentagastrin injection.

RT-PCR

Total RNA was extracted from the tissue samples or TT cells using RNazol (WAK-Chemie, Bad Homburg, Germany) according to the protocol supplied by the manufacturer. RNA was then treated with DNase I for 15 min at 37°C . First-strand cDNA was transcribed from 2 μg total RNA. Three hundred nanograms of cDNA were used for PCR using the following primers: CCK₂R forward: 5'-CGT GTG CTG CAG TGC GTG CA-3', CCK₂R reverse: 5'-GGT GGT GTA GCT AAG CCT GG-3'; β -actin forward: 5'-ATC TGG CAC CAC ACC TTC TAC A-3', β -actin reverse: 5'-GCT CAT TGC CAA TGG TGA TGA C-3'; gastrin forward: 5'-CAG CGA CTA TGT GTG TAT GT-3', gastrin reverse: 5'-TTC TTG GAC GGG TCT GCC AC-3'; CCK forward: 5'-CCA AAA GCC ATG AAC AGC GGC G-3', CCK reverse: 5'-ATC CAT CCA GCC CAT GTA GTC C-3'. The PCR conditions were: 5 min at 95°C followed by 35 cycles for 1 min at 94°C , 1 min

Table 1 Patient data, tumor stage, calcitonin levels, RET mutation and CCK₂R status in the investigated MTC tumor population. Note that pathological data according to the T, N, M classification system were not available for all investigated tumor samples.

Sample	Gender	Age	T N M	Calcitonin (ng/l)	RET mutation (codon)	Remarks	CCK ₂ R expression
1	F	48	2 0 0	1000	918	—	Positive
2	M	56	2 0 —	—	—	—	Positive
3	M	67	4 1 1	1033	—	—	Negative
4	M	53	1 1 0	3330	—	—	Positive
5	M	60	4 — 1	1802	918	—	Negative
6	F	62	— — —	—	—	—	Positive
7	F	56	2 — —	483	—	—	Negative
8	M	56	— 1 —	3774	918	—	Positive
9	F	63	2 1 —	214	—	—	Positive
10	F	17	2 1 —	2810	—	MEN-2	Negative
11	F	33	3 1 —	2145	—	—	Negative
12	M	35	2 0 —	3262	—	—	Positive
13	F	47	1 1 0	807	918	—	Positive
14	F	65	— — —	1350	—	—	Positive
15	F	63	— 1 1	—	—	—	Negative
16	F	71	2 0 0	—	—	—	Positive
17	F	45	4 1 0	—	918	—	Negative
18	M	57	3 1 —	900	—	PGT negative	Negative
19	M	56	2 0 0	238	634	MEN-2; PGT positive	Positive

PGT, pentagastrin test.

at 60 °C and 2 min at 72 °C, followed by 10 min at 72 °C. Aliquots (5 µl) of the PCR products were analyzed on 1.2% agarose gels. Specificity of CCK₂R PCR products was confirmed by restriction endonuclease digestion.

Detection of RET oncogene mutations

For the detection of mutations in codon 918 (exon 16) of the RET proto-oncogene, RNA was extracted from sporadic MTCs by the RNazol method and transcribed into cDNA (see above). PCR was performed using the following primers: exon 16 forward: 5'-AGG GAT AGG GCC TGG GCT TC-3', exon 16 reverse: 5'-TAA CCT CCA CCC CAA GAG AG-3'. PCR conditions: 1 min at 96 °C followed by 35 cycles for 1 min at 94 °C, 30 s at 65 °C and 45 s at 72 °C. PCR products were purified using a Qiagen purification kit (Qiagen, Hilden, Germany) and sequence analysis was performed using an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry

Paraffin sections of human C-cell hyperplasia or MTCs of about 5 µm thickness were deparaffinized in xylene, rehydrated and washed in PBS. The sections were incubated with 3% H₂O₂ for 20 min to block endogenous peroxidase activity followed by incubation in 5% swine normal serum. The sections were incubated with specific primary antibodies (anti-calcitonin IgG from rabbit (DAKO A0576; DAKO, Hamburg, Germany); anti-human CCK₂R IgY from chicken (13, 14)) for 48 h at 4 °C in a moist chamber at dilutions between 1:30 and 1:1280. After washing three times with PBS, the sections were incubated with anti-rabbit IgG from swine (DAKO) or anti-chicken IgY (Dianova, Hamburg, Germany) at a dilution of 1:50 for 1 h at 22 °C. After washing in PBS three times, the sections were incubated with peroxidase-anti-peroxidase complex and peroxidase-conjugated streptavidin (Dianova) respectively, for 1 h at a dilution of 1:50 at 22 °C. After washing, the horseradish peroxidase activity was detected by substrate solution, containing diaminobenzidine, 0.05 mol/l Tris-HCl buffer (pH 7.6) and 0.01% H₂O₂. Finally, the sections were counterstained with acidic haemalaun and, after embedding, were investigated by light microscopy.

Results

RT-PCR studies

Using primers specific for human β-actin cDNA, a PCR product of the expected size was amplified in all investigated samples. A 729 bp CCK₂R-specific PCR product was amplified from the following samples: normal human stomach cDNA, commercially available pooled cDNA from normal human thyroid, normal thyroid

tissue of two patients who underwent surgery for non-toxic goiter ('goiter 1', 'goiter 2'), TT-cells (a human MTC cell line), and 11 out of 19 MTC samples. No CCK₂R-specific PCR product could be detected in eight MTC samples (Figs 1 and 2; Table 1). Expression of gastrin was not detected in any of the investigated samples (data not shown). CCK expression was detected in normal thyroid cDNA as well as in one thyroid sample from a patient with non-toxic goiter but was not detected in any of the MTC samples (Fig. 1).

Immunohistochemistry

In addition to RT-PCR, CCK₂R expression was detected in human MTCs by immunohistochemical analysis. Using a CCK₂R-specific antibody, directed against the extracellular N-terminal portion of the receptor protein, receptor expression was visualized in three out of five investigated MTCs (Fig. 3). In addition to MTCs, CCK₂R immunoreactivity was observed in C-cell hyperplasia, which is defined as an increase in the number of non-malignant C-cells. Immunohistochemistry using antibodies against calcitonin and CCK₂R demonstrated that only C-cells express CCK₂R (Fig. 4).

Comparison of clinical, molecular and pathological data

Table 1 summarizes the available clinical and pathological data in the MTC patients. All patients had elevated baseline calcitonin levels. Only in two patients were results of pentagastrin tests available. One test was negative (patient 18) and the corresponding tumor sample did not show CCK₂R expression (Fig. 1a). The positive pentagastrin test (patient 19) is shown in Fig. 1b. In the corresponding tumor sample, CCK₂R was detected (Fig. 1a). This patient and patient 10 had MTC as part of the multiple endocrine neoplasia type 2 (MEN-2) syndrome. All other MTCs were sporadic tumors.

Activating mutations of the RET proto-oncogene have been shown to play a key role in the pathogenesis of MEN-2 and MTC (12). Consistent with previous reports (15), we found mutations of codon 918 within exon 16 of the RET gene in 30% of the sporadic MTCs investigated. No correlation was found between RET mutations and CCK₂R expression.

Comparison with pathological data revealed that detectable CCK₂R expression was restricted to T1- and T2-stage tumors which did not show metastases. In contrast, in none of the further advanced tumor stages (T3, T4, Mx) was CCK₂R expression detected (Fig. 2).

Discussion

CCK₂R was originally cloned from parietal cells (16, 17) and later found to be identical to the CCK receptor in

Figure 1 Detection of CCK₂R expression. (a) CCK₂R is expressed in human stomach as well as in all samples of normal thyroid tissue (lanes 2–4, upper panel). CCK₂R expression is also detectable in TT-cells and MTCs. Lane 6 represents the tumor sample from patient 19 (see Table 1). The asterisk indicates that the pentagastrin test shown in (b) corresponds to the result shown in this lane. Not all MTCs express CCK₂R; lane 7 represents the tumor sample from patient 18 (see Table 1) in which no CCK₂R expression is detected. As expected, CCK₂R is not expressed in the colon (lane 8). The housekeeping gene β-actin is expressed in all investigated samples (lower panel of (a)). (b) Positive pentagastrin stimulation test in patient 19 (a) (see Table 1). Pentagastrin was injected i.v. (arrow). Calcitonin serum levels rose from 238 to 1717 ng/l after 2 min and to 3523 ng/l after 5 min. The pentagastrin test is considered positive if calcitonin levels rise >200 ng/l after pentagastrin injection. (c) Detection of CCK expression. CCK mRNA is detectable in samples of small intestine and normal thyroid but not in MTC.

Figure 2 Comparison of MTCs which do express (white bars) and do not express (gray bars) CCK₂R. In the investigated tumor population for which pathological data according to the T, N, M classification were available, CCK₂R-positive samples are smaller in size and have not metastasized to other organs, whereas the group of CCK₂R-negative tumors includes large and metastasized tumors.

brain (18). In recent years, it has become apparent that expression of this receptor subtype is more widespread than initially expected. Using molecular detection techniques as well as immunohistochemistry and autoradiography, expression of CCK₂R has been detected in pancreatic acinar cells, monocytes, T lymphocytes, kidney, and in a wide variety of human tumors (7, 8, 19). In most of these tumors CCK₂R expression occurs aberrantly, possibly related to dedifferentiation of the tumor cells.

Reubi & Waser (10) reported the expression of CCK₂Rs with very high incidence in human MTCs but did not find expression of the receptor in normal thyroid tissue using autoradiography. We now demonstrate by RT-PCR that CCK₂R is expressed not only in MTCs but also in all investigated samples of non-malignant thyroid tissues. This difference from the report by Reubi & Waser is probably explained by the higher sensitivity of the RT-PCR method. Moreover, in normal thyroid glands, C-cells are found singularly or in groups of

Figure 3 Immunohistochemical detection of CCK₂R in MTC. (a) Staining of tumor tissue with antibodies against calcitonin (magnification $\times 3.2$). (b) Adjacent section showing immunoreactivity using a CCK₂R-specific antibody (magnification $\times 3.2$). (c) Adjacent section after incubation with IgY antibodies which were preabsorbed using the antigen (magnification $\times 3.2$). (d) High power magnification ($\times 40$) of CCK₂R-positive tumor cells.

Figure 4 Immunohistochemical detection of CCK₂R in C-cells. (a) Immunoreactivity against calcitonin in a sample of benign C-cell hyperplasia (magnification $\times 25$). (b) Immunohistochemical detection of CCK₂R in C-cells (magnification $\times 25$). (c) High power magnification ($\times 80$) of calcitonin reactivity in C-cells. (d) High power magnification ($\times 80$) of CCK₂R-reactivity in C-cells.

two to five cells and are located primarily in the dorsolateral part of the organ. Therefore, in microscopic studies these cells might be missed. Based on these considerations we used samples of non-malignant C-cell hyperplasia for immunohistochemical studies and were able to show for the first time that CCK₂R is expressed in normal C-cells.

The detection of CCK₂R expression supports the concept of a physiological function of gastrin and/or CCK in C-cells. Thirty years ago, it was shown for the first time that both hormones induce calcitonin secretion (20, 21). The physiological and pathophysiological relevance of these early findings has been discussed controversially (22, 23). It is well known that gastrectomy, including the removal of the antrum, where gastrin-producing G-cells are located, leads to a significant reduction in bone density and alterations in calcium metabolism (24, 25). In fact, it has been shown in gastrectomized patients that in response to a meal, increases in calcitonin levels were significantly smaller than in control groups, most likely based on impaired gastrin secretion (23). Animal models have confirmed

the importance of gastrin for calcium and bone homeostasis; in rats, antrectomy and total gastrectomy, both resulting in hypogastrinemia, lead to osteopenia and reduction in bone mineral content (26, 27). However, a direct positive correlation between gastrin levels and calcitonin levels could be established neither in these models nor in rats after fundectomy and reflexory hypergastrinemia (28). One explanation might be that gastrin leads to pulsatile calcitonin release in response to enteral stimulation rather than influencing basal secretion of calcitonin from C-cells. In addition, CCK might compensate in part for the lack of gastrin after gastrectomy, albeit not to a degree that prevents osteopenia. The detection of CCK₂R on C-cells reported in this study clearly supports the concept of an interrelationship among calcemic hormones and gastrointestinal hormones (29).

Given the physiological expression of CCK₂R in C-cells we speculate that the pentagastrin stimulation test, which is used as a diagnostic tool, reflects the vast increase in the number of C-cells in MTCs rather than a specific expression of the receptor in malignant vs

non-malignant cells. This view is supported by the fact that the pentagastrin test has been shown to be positive in cases of benign C-cell hyperplasia (30, 31). Furthermore, pentagastrin injection has been shown to cause a slight yet significant increase in calcitonin serum levels in normal subjects (32). All patients investigated in our study already had substantially elevated baseline calcitonin levels (Table 1). Therefore, in most cases pentagastrin stimulation was not performed. Only for two patients were the results of pentagastrin tests available. As expected, CCK₂R expression was detectable in the tumor sample corresponding to the positive result, whereas CCK₂R expression was not detected in the sample from the patient with the negative pentagastrin test (Table 1, Fig. 1). Although performed only in a limited number of patients, the pentagastrin test appears to correlate with CCK₂R expression in MTCs.

In contrast to multiple other tumors, in which CCK₂R expression most likely occurs due to loss of differentiation of the tumor cells, the expression of CCK₂R in MTCs appears to be related to maintained differentiation of the malignant C-cell. Thus, CCK₂Rs are found in almost all early-stage tumors (T1 and T2 stages) while further growth and potential loss of cell differentiation, i.e. in T3 and T4 tumors and metastasized tumors, might be associated with a loss of receptor expression. This phenomenon has also been reported for somatostatin receptors in MTCs (33, 34). The conclusion that CCK₂R expression in MTCs depends on the degree of tumor differentiation is further supported by a recent report by Kwekkebom *et al.* (35). In that study, aimed at visualization of CCK₂Rs on MTCs *in vivo* using radio-labeled CCK-8 (which has high affinity for CCK₂R), scintigraphic detection of known tumor metastases could not be achieved in one of two patients. Autoradiographic analysis of one of the respective liver lesions revealed the absence of CCK₂R expression in the investigated sample; in addition, the pentagastrin test in the patient remained negative (35). Therefore, in the light of encouraging technical results (35–37), CCK₂R scintigraphy for visualization of MTC lesions might be more useful in earlier stages of the disease than in far-advanced metastasized tumors. However, studies with larger numbers of patients comparing clinical, scintigraphic and molecular data are required to reach a final conclusion.

Gastrin is a well-known major growth factor for enterochromaffin-like (ECL) cells in the stomach (38). In mouse models, lack of gastrin or its receptor leads to marked reduction in the number of ECL cells (39, 40). In addition, differentiation and maturation of ECL cells appears to be highly dependent on gastrin (39). In conditions associated with hypergastrinemia ECL cells become hyperplastic and may ultimately transform into malignant carcinoids (38). Like ECL cells, C-cells are neuroendocrine cells and both cell types share several histomorphological and biological features. Therefore, in analogy to ECL cells, C-cells might

become hyperplastic in states of hypergastrinemia either due to disease (pernicious anemia, Zollinger–Ellison syndrome) or due to pharmacotherapy (e.g. proton pump inhibitors). Moreover, gastrin might act as a growth factor in MTCs.

In several tumor models, an autocrine loop involving gastrin and CCK₂R has been proposed, based on the coexpression of both the ligand and the receptor in these tumors (3–6). Our data do not indicate that such an autocrine loop exists in MTCs. Neither of the two CCK₂R ligands, gastrin or CCK, is expressed in MTCs. In contrast, CCK mRNA, together with CCK₂R mRNA, is detectable in normal thyroid tissue and in one sample of goiter (Fig. 1c). CCK has been identified as a secretory product of rat MTC cells (41, 42). It is not known whether CCK is expressed in normal rat thyroid. Given the fact that we found CCK expression only in two samples of non-malignant human thyroid tissue while in none of the tumor samples was CCK detectable, one might speculate that CCK and CCK₂R are involved in an autocrine loop which contributes to normal C-cell function.

In summary, we have shown for the first time that CCK₂R is expressed in normal thyroid tissue, specifically in C-cells. These findings support previous reports which suggest that CCK₂R ligands might play a physiological role in calcitonin-related bone and calcium metabolism. It appears that in MTC the incidence of CCK₂R is particularly high in small primary tumors. CCK₂R might therefore serve as a marker of differentiation in MTCs and might be an interesting diagnostic and therapeutic target.

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