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Effect of octreotide on expression of calcitonin gene in cultured cells of medullary thyroid carcinoma

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Summary. The studies were performed on cultured TT cells, originating from medullary thyroid carcinoma and producing calcitonin (CT) and CT gene-related peptide (CGRP), synthesized on the template of the same gene. Addition of octreotide (a synthetic somatostatin analogue) to the cell culture augmented amounts of CGRP mRNA and of CGRP protein in the cells as well as increased levels of CGRP in the culture medium. In contrast, amounts of CT mRNA and of CT protein in the cells as well as levels of CT in the medium remained unchanged. The results showed that octreotide stimulates transcription and alternate splicing of the CT gene.

Key words: Octreotide, Calcitonin gene expression, Medullary thyroid carcinoma

Introduction

Medullary thyroid carcinoma develops from the parafollicular cells. The cells are known for their production of polypeptide hormones, calcitonin (CT) and calcitonin gene-related peptide (CGRP), synthesized on the template of the same gene (Benett and Amara, 1993). Alternate splicing of the CT gene may yield mRNA for CT or mRNA for CGRP. Mechanisms of the splicing control have not yet been fully clarified and the process is thought to be influenced by several factors, including hormones (Lou and Gagel, 1998, 1999, 2001; Coleman et al., 2003; Tran and Roesser, 2003).

Moreover, parafollicular cells as well as medullary thyroid carcinoma cells express somatostatin but the amounts of somatostatin produced exhibit extensive variability in various species and at various stages of development. For example, high amounts of somatostatin are produced in young but not in adult rats. In guinea pigs the reverse situation is true (Zabel et al., 1988). Human medullary carcinomas may contain very different amounts of somatostatin (Hinze et al., 2001). Medullary carcinomas have been demonstrated to carry all five known types of somatostatin receptors, which points to a significant role of the hormone in cell function (Papotti et al., 2001).

The TT cell line represents a convenient research model for human medullary thyroid carcinomas. In previous studies we have demonstrated that somatostatin remains specifically bound to the TT cell surface, which points to the presence of receptors for the hormone (Zabel et al., 2001). Studies with the use of specific somatostatin agonists and antagonists have confirmed the presence in the cells of at least two types of somatostatin receptors (Zatelli et al., 2001). Earlier we have also demonstrated that octreotide (a somatostatin analogue which binds with high affinity to type 2 and with low affinity to types 3 and 5 somatostatin receptors) inhibits proliferation of TT cells, decreases amounts of PCNA and Ki-67 in the cells and stimulates necrosis and apoptosis in the cells (Zabel et al., 2001). The application of specific agonists of some somatostatin receptor types has demonstrated distinct effects on proliferation of the cells (Zatelli et al., 2001).

In the present study we wished to examine whether octreotide affects the expression of the CT gene and its alternate splicing in the TT cell line. Such effects are probable since the cells carry somatostatin receptors and produce somatostatin themselves (Zabel et al., 1995, 2001), which may control not only cell proliferation but also expression of hormones in an autocrine way.

Material and methods

Cell cultures and proliferation experiments. The experiments were performed on the TT cell line, originating from human medullary thyroid carcinoma (Leong et al., 1981). The cells were purchased from the American Type Culture Collection (CRL-1803; Manassas, VA, USA) in a frozen form. All the experiments were conducted in the course of the third to fifth passage after thawing the cells.

TT cells have been shown to express CT, CGRP and somatostatin (Zabel et al., 1994, 1995). The cells were cultured in L-15 medium supplemented with 10% fetal

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bovine serum, 1 g/l glucose, 80 IU/l insulin, 2.5 mg/l transferrin and 6.25 mg/l fetuin. The cultures were set up in 12-well culture plates or on four-chamber microscope glasses, at $1x10^4$ cells/cm^2. The cells were cultured for 3 days in basal medium or in medium supplemented with octreotide (octreotide acetate, Novartis, Switzerland) at 10^{-12}-10^{-6} M. Three separate experiments were performed in duplicates for the control and for each of the octreotide supplementation levels. After terminating the experiment on culture plates, the used medium was collected for estimation of CT and CGRP levels. Following trypsin treatment, the total number of cells was established in each culture by scoring the cells in a haemocytometer.

Immunocytochemistry

In three independent experiments, the cells grown on microscope glasses were fixed in 4% buffered formalin for 30 min. The reactions were performed by using the biotin-streptavidin-alkaline phosphatase system (LASB-2 kit, DAKO, Glostrup, Denmark). Hormones were demonstrated by using specific polyclonal anti-CT antibodies (DAKO, Glostrup, Denmark) and polyclonal anti-CGRP antibodies (Amersham Corp. Arlington Heights, IL, USA). Details of the technique and control reactions have been described earlier (Zabel et al., 1994, 1995).

In situ hybridisation

In three independent experiments, the cells grown on microscope glasses were fixed in 4% buffered formalin for 30 min. For detection of CT mRNA an exon 4-specific probe for was used and for detection of CGRP mRNA the probe was specific for exon 6. The probes were detected by using the biotin-streptavidin-alkaline phosphatase system (LASB-2 kit, DAKO, Glostrup, Denmark). Details of the reaction as well as appropriate controls and hybridisation variables have been described earlier (Zabel and Schafer, 1988; Zabel et al., 1994).

Evaluation of the amount of reaction product. Immunocytochemically-stained preparations and preparations with *in situ* hybridisation reactions were subjected to morphometric analysis using a x20 objective in an Alfaphot-2 (YS-2) microscope (Nikon, Japan), an industrial Colour CCD Camera (Panasonic GP-KR222, Japan), and an IBM microcomputer and Screen Measurement M. Version 3.52a Lucia software (Laboratory Imaging, Ltd, Prague, Czech Republic). In the final analysis, the number of positive cells, area fraction and integral optical density were estimated, which permitted us to calculate reaction intensity per cell. In each group 50 cells were analyzed (Zabel et al., 1999b).

Radioimmunological determination of hormone levels in medium

Following three-day culture, the used medium was collected and frozen at -20 °C. Using commercial diagnostic kits, levels of CGRP (DRG-Instruments GmbH, Marburg, Germany) and of CT (Medgenix Diagnostic, Berlin Germany) were estimated in the medium. The final result was calculated per 10^3 cells.

Statistics

Calculations and statistical comparisons involved the t–test. Differences were taken as significant at $p \leq 0.05$.

Results

Following three days of TT cell culture in the octreotide-containing medium an inhibited cell proliferation was disclosed, which was particularly

Table 1. Effect of octreotide on TT cell proliferation after 3-day culture.

TT CELLS	OCTREOTIDE CONCENTRATION IN MEDIUM (mean±SD)							
	control	10^{-12} M	10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
Number of cells ($x10^3$)	15.5±0.5	15.2±0.4	15.3±0.5	14.2±0.5[a]	12.2±0.7[a]	10.1±0.6[a]	9.7±0.6[a]	7.8±0.9[a]

[a]: significant difference as compared to control conditions (at $p<0.001$).

Table 2. Effect of octreotide on CT-mRNA and CGRP-mRNA contents in TT cells after 3-day culture. Contents of both mRNAs were quantified by densitometry and expressed by an arbitrary score per cell.

TT CELLS	OCTREOTIDE CONCENTRATION IN MEDIUM (mean±SD)							
	control	10^{-12} M	10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
CT-mRNA	16.7±10.3	17.2±11.3	15.9±9.7	16.2±10.5	16.2±12.3	15.8±10.8	15.8±11.4	15.6±10.3
CGRP-mRNA	22.3±14.4	23.2±16.1	23.0±14.3	24.6±17.0	25.2±12.8	26.0±16.6	27.7±13.8[a]	27.4±14.3[a]

[a]: significant difference as compared to control conditions (at $p<0.01$).

evident beginning at the 10^{-8} M concentration of the drug (Table 1).

All TT cells, whether cultured in control cultures or in octreotide-supplemented cultures, demonstrated the presence of both CT mRNA and CGRP mRNA. However, the intensity of the reaction was variable not only in individual cells but also in various portions of the same cell (Fig. 1a,b). In general, the reaction was clearly more pronounced in the case of CGRP mRNA, as compared to CT mRNA. Quantitation of the CT mRNA reaction documented its slight decrease with the increasing concentration of octreotide in the medium, but in none of the cases this involved significant differences when compared to the control. On the other hand, the intensity of the reaction employed to detect CGRP mRNA grew with the increasing octreotide level and, beginning at 10^{-7} M of octreotide, this increase was significant (Table 2).

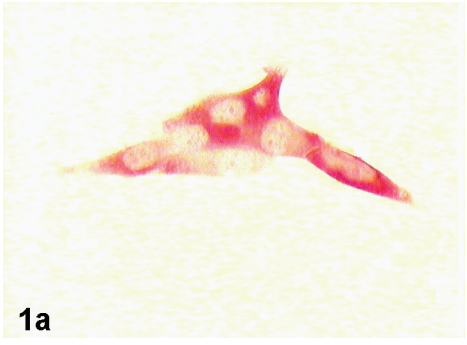
Immunocytochemical tests demonstrated presence of both hormones in each TT cell and their distribution in individual cells was more uniform as compared to distribution of respective mRNAs. The intensity of CGRP-detecting reaction was clearly higher than that of the CT-detecting reaction (Fig. 2a,b). The mean intensity of the CT-detecting reaction was very similar in the control and in cultures with octreotide and did not differ significantly in the two types of cultures. On the other hand, the mean intensity of the CGRP-detecting reaction was evidently higher and, beginning at 10^{-9} M octreotide, significantly greater than in the control (Table 3).

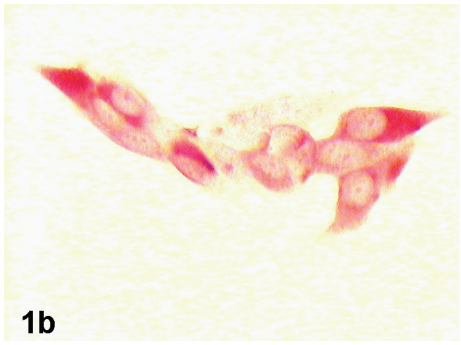
The amount of CT in the medium of control cultures was almost four times lower than the amount of CGRP. In octreotide-supplemented cultures the CT level manifested a slight, insignificant variation. The CGRP concentration in octreotide-supplemented cultures clearly increased and was significantly elevated starting at 10^{-9} M octreotide supplementation (Table 4).

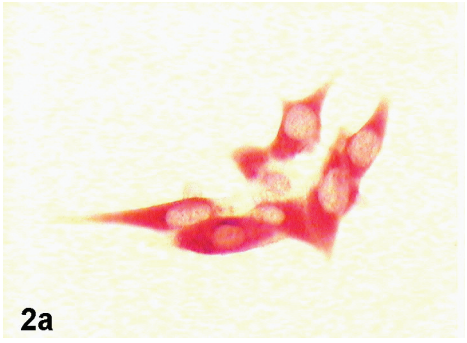
Table 3. Effect of octreotide on CT and CGRP contents in TT cells after 3-day culture. Contents of both hormones were quantified by densitometry and expressed by an arbitrary score per cell.

TT CELLS	OCTREOTIDE CONCENTRATION IN MEDIUM (mean ± SD)							
	control	10^{-12} M	10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
CT	18.2±11.3	17.6±12.3	17.9±15.7	19.2±15.6	17.9±14.6	17.2±15.8	17.8±12.4	17.9±11.4
CGRP	32.4±12.3	34.5±9.8	33.7±10.8	34.4±14.1	36.8±16.2[a]	38.5±14.8[b]	38.4±13.5[b]	38.6±14.5[b]

[a, b]: denote a significant difference as compared to control conditions (at $p< 0.05$ or $p< 0.001$, respectively).







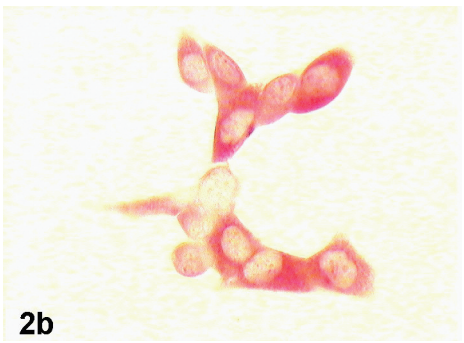


Fig. 1. CGRP mRNA **(a)** and CT mRNA **(b)** localised by *in situ* hybridisation in TT cells cultured in control conditions. Reaction product is uniformly distributed in individual cells and in various portions of the cytoplasm within each cell. x 450

Fig. 2. Immunocytochemical localisation of CGRP **(a)** and CT **(b)** in TT cells cultured in control conditions. The immune reaction is more pronounced in the case of CGRP detection, as compared to CT detection. x 450

Discussion

Our results have shown that octreotide evidently inhibits TT cell proliferation, as we had demonstrated previously. We have also documented that the inhibited proliferation is accompanied by a decreased expression of proliferation antigens and by more frequent signs of necrosis and of apoptosis at higher octreotide concentrations in the medium (Zabel et al., 2001). In the latter study, we also demonstrated the presence of somatostatin receptors in TT cells, which explains the effects of octreotide on the cells.

This study aimed at the evaluation of the octreotide effect on the expression of the CT gene. The evaluation was based on RIA estimations of CT and CGRP content in media and on analysis of the content of the two hormones and their mRNAs in cells. By employing the techniques successfully used in other studies (Wang et al., 2000; Machaalani and Waters, 2002) we have quantitated results from immunocytochemical reactions and *in situ* hybridizations. Quantitation of the reaction product has previously been documented to reflect the amounts of molecule detected by the reaction (Rieux et al., 2002). The analysis employed here may provide an alternative to biochemical techniques, such as e.g., Western- and Northern-blot, or real time PCR.

In the present investigations we have shown that octreotide alters the expression of the CT gene in TT cells. In the cells, transcription was stimulated, as indicated by the augmented total content of mRNAs for CT and CGRP. In fact, the amount of CT mRNA remained unchanged but the amount of CGRP mRNA clearly increased. The change has proved that alteration took place in the alternate splicing. The augmented CGRP production was confirmed upon evaluation of the cellular content of the two hormones. Levels of the two hormones in the medium paralleled the intracellular content of the hormones. This indicates that octreotide does not directly alter secretion of the two hormones and that the observed changes in the medium levels rather reflect an augmented cellular content of CGRP.

Alternate splicing of the CT gene pre-mRNA results in formation of CT mRNA or CGRP mRNA, depending upon inclusion of the exon 4 into the mature mRNA. Exons 1, 2, and 3 are common for both mRNAs, exon 4 is present only in CT mRNA while exons 5 and 6 are noted only in CGRP mRNA. If the splicing site beyond exon 4 is not recognized, the exon will be spliced off together with introns and the mature mRNA will incorporate exons 5 and 6 (Lou and Gagel, 1998). Thus, the splicing site beyond exon 4 is of key importance for mRNA processing of the gene. Studies performed till now have shown that the site tends to be by-passed in the CT gene (Niwa et al., 1992; Benett and Amara, 1993) and that in parallel with typical splicing factors (i.e., snRNAs, PTB, ASF/SF2) other factors are indispensable for recognition of the splicing site beyond exon 4 (Lou and Gagel, 1998, 2001). The factors remain unidentified till now but circumstances have been documented in which the splicing changes. This indicates that in certain situations such splicing-affecting factors are produced in the cells. TT cells and other cell lines originating from medullary thyroid carcinoma demonstrate changes in transcription and/or in splicing of the CT gene product following culture with components of the extracellular matrix (laminin-1 and merosin), during proliferation or co-culture with other cells, including co-culture with follicular cells of the thyroid (Lausson et al., 1995; Lou and Gagel, 1999; Lekmine et al., 1999; Zabel et al., 1999a). Moreover, expression of the CT gene in TT cells can be altered by steroid hormones, calcitriol and dexamethasone (Cote and Gagel, 1986; Cote at al., 1987). The studies performed till now on the effects of somatostatin on TT cells have been restricted to the appraisal of CT secretion (Zabel, 1995; Endo et al., 1988; Zatelli et al., 2002a,b). The observed differences in effects induced by somatostatin and octreotide probably reflect the presence of a few receptor types for somatostatin on TT cells: octreotide has been demonstrated to affect TT cells through no more than three types of receptor (Patel et al., 1996). Moreover, the effects obtained following stimulation of individual receptor subtypes may reciprocally amplify their effects or inhibit them, which makes interpretation of the action of somatostatin and its analogues much more difficult (Zatelli et al., 2001). Moreover, apart from the receptors for somatostatin, TT cells also carry receptors for CT. Both hormones are produced in TT cells, are secreted to the medium in an autocrine way, and may affect expression of the CT gene. Therefore, the octreotide effect noted in our experiments may reflect its direct and/or indirect action on the cells. Results obtained in the present study have demonstrated that octreotide stimulates the transcription of the CT gene and affects alternate splicing, augmenting CGRP mRNA production.

Table 4. Effect of octreotide on CT and CGRP levels in medium after 3-day culture of TT cells. The level is expressed in pg/ml per 10^3 cells.

TT CELLS	OCTREOTIDE CONCENTRATION IN MEDIUM (mean ± SD)							
	control	10^{-12} M	10^{-11} M	10^{-10} M	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
CT	212±22	228±21	236±32	225±28	239±41	205±34	222±18	194±24
CGRP	845±134	808±145	922±183	867±104	1055±148[a]	995±114[b]	1109±144[b]	1188±114[b]

[a, b]: denote a significant difference as compared to control conditions (at $p<0.01$ or $p<0.001$, respectively).

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