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Ultrastructural and electron immunohistochemical features of medullary thyroid carcinoma

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Summary. An ultrastructural study, both morphological and immunohistochemical, has been carried out on eight thyroglobulin-positive and nine thyroglobulin-negative medullary carcinomas of the thyroid. The morphometric analysis of granule size showed that all tumours contained cells with small granules and cells with medium size granules, whereas eight tumours had additional cells with large granules. The small granules had an electron dense core, while the medium and large sized granules were both pale-cored and dense-cored. The cells with small, medium or large secretory granules were all immunoreactive for calcitonin and CGRP. No ultrastructural differences were observed between thyroglobulin-positive and thyroglobulin-negative cases of medullary carcinoma of the thyroid.

Key words: Thyroid neoplasms – Electron microscopy – Immunohistochemistry – Calcitonin

Introduction

In the fifties and sixties medullary carcinoma of the thyroid (MCT) was established as a distinct form of thyroid cancer originating from calcitonin secreting C-cells (Hazard et al. 1959; Williams 1966; Meyer and Abdel-Bari 1968; Tashjian and Melvin 1968), and the production and secretion of calcitonin from the tumour has been firmly established (Hazard 1977; Sundler et al. 1977; Mendelsohn et al. 1978; Deftos et al. 1980; Sano et al. 1980; Charpin et al. 1982; Marcus et al. 1982; Saad et al. 1984). It came as a surprise to many that some tumours produce both major thyroid hormones, calcitonin and thyroglobulin (Hales et al. 1982; DeLellis et al. 1983; Pfaltz et al. 1983; Uribe et al. 1985; Fenoglio-Preiser 1986; Holm et al. 1986; Holm et al. 1987).

A common feature of MCT is the presence of secretory granules (Albores-Saavedra et al. 1964; Meyer and Cochran 1967; Gonzalez-Licea et al. 1968; Meyer 1968; Braunstein et al. 1968; Tateishi et al. 1972) which have been reported to be of two different sizes (Kakudo et al. 1977; Tasca and Stefaneanu 1981). It has been suggested that some of the granule heterogeneity may be related to the production of various "ectopic" hormones (Gould and Benditt 1973; Tasca and Stefaneanu 1981). In previous studies the two common types of granules were shown to be immunoreactive for calcitonin (DeLellis et al. 1978; Huang and Goltzman 1978; Nesland et al. 1986). However, Dämmrich and coworkers (1984) identified calcitonin in the small sized granules only. These reports dealt with rather few cases and a careful morphometric study has only been performed on one case (Dämmrich et al. 1984).

This paper presents a comprehensive morphometric analysis of granule size in seventeen sporadic cases of MCT and identifies the hormone content in the secretory granules. It also compares the ultrastructural and immunoelectron microscopic features of two groups of sporadic MCT, one producing calcitonin and the other immunoreactive for both calcitonin and thyroglobulin.

Materials and methods

Seventeen cases of sporadic MCT were collected from the files of The Norwegian Radium Hospital. Nine cases, including 6 women and 3 men with a mean age of 61.9 (range 27–80 years), had tumours producing calcitonin only, whereas eight cases, including 4 women and 4 men with a mean age of 37.8

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(range 18–68 years), had tumours immunoreactive for both calcitonin and thyroglobulin.

Tumour tissue was fixed in 10% buffered formalin and embedded in paraffin. Sections were processed for conventional routine staining with hematoxylin-eosin and Congo red and for immunohistochemistry.

For electron microscopy small blocks of tissue were fixed in a cacodylate buffered solution of 4% formaldehyde and 1% glutaraldehyde (McDowell and Trump 1976). Half of the blocks in each tumour were then osmicated (1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.3). Following dehydration in a series of graded ethanols and infiltration with propylene oxide, the tissue was embedded in an Epon-Araldite mixture (Mollenhauer 1964) and polymerized at 60° C overnight. Semithin sections for light microscopic orientation were stained with toluidine blue. Ultrathin sections were collected on uncoated 200-mesh nickel grids and processed for conventional transmission electron microscopy with uranyl acetate and lead citrate and for immunohistochemistry.

One tissue block from each case was used for searching after cells with different granule size. In each case ten cells, with granules ranging from small to large, were selected for photography at a final magnification of 17600. Seven to 99 granules were measured in each cell using a Kontron SEM-IPS (Munich, West Germany). The diameters including the limiting membrane of the secretory granules were determined, and the largest diameter was used for those granules that deviated from a spherical shape. The average diameter of the secretory granules in each cell was calculated.

The primary polyclonal antisera used were all raised in rabbits. Antisera to calcitonin (donated by Prof. K.M. Gautvik, Institute for Medical Biochemistry, University of Oslo, Norway), thyroglobulin (donated by Dr. E. Paus, The Norwegian Radium Hospital, Norway), calcitonin gene related peptide (CGRP) (Amersham International, England), leu-enkephalin, substance P, vasoactive intestinal peptide (VIP), serotonin and bombesin (Immuno Nuclear Corp. Stillwater, MN), and somatostatin, adrenocorticotropic hormone and gastrin (Dako Corp., Santa Barbara CA) were used at dilutions varying from 1:700 to 1:30000. Calcitonin (Sigma Chemical Corp., St. Louis MO), thyroglobulin (donated by Dr. E. Paus, The Norwegian Radium Hospital) and CGRP (Peninsula Lab. Inc., Belmont, CA) were applied in concentrations of 25–100 µg/ml diluted antisera for testing the specificity of the antibodies. Further characterization of the antibodies has been given in previous reports (Holm et al. 1985 and 1986). Antisera raised against somatostatin, leu-enkephalin and bombesin also contained antibody against thyroglobulin and were therefore absorbed with 400 µg thyroglobulin/ml diluted antiserum to abolish this reaction. The avidin-biotin-peroxidase complex (ABC) method of Hsu and coworkers (1981) was used for light microscopic immunohistochemistry as described previously (Holm et al. 1985).

The indirect single immunogold staining method according to Varndell et al. (1982) was employed for immunoelectron microscopy. The grid-mounted sections of unosmicated tissue were etched in 10% hydrogen peroxide for 10 min, while osmicated tissues were treated with a saturated solution of sodium meta-periodate (Bendayan and Zollinger 1983) for 1 h. After washing in distilled water the sections were placed in normal goat serum (NGS) for 30 min. The NGS was drained from the grids and sections were then incubated with the primary antisera for 18–22 h at 4° C. After washing in phosphate buffered saline (PBS), pH 7.4, and treatment with 1% bovine serum albumin (BSA) diluted in PBS for 10 min, the sections were incubated with 5 nm gold labelled goat anti-rabbit IgG (GAR-5 nm) (Janssen Life Sciences Products, Belgium) for 1 h. The grids were then thoroughly washed in PBS and finally rinsed in distilled water. Silver enhancement of the 5 nm gold particles was performed by incubating the sections for 2 min with an IntenSEM silver enhancement kit (Janssen, Life Sciences Products, Belgium). The grids were then counterstained with 5% uranyl acetate before examination in a Philips 201 transmission electron microscope.

The formaldehyde blockade protein A-gold double staining method modified from Wang and Larsson (1985) has been reported in detail elsewhere (Holm et al. 1988a). In this method, the sections are exposed to the first primary antibody before incubation with 5 nm gold-protein A (Janssen Life Sciences Products, Belgium). After treatment with paraformaldehyde vapour the grids are incubated sequentially with the second primary antibody and 15 nm gold-protein A.

All the dilutions of NGS, antisera and gold-conjugates were made with PBS containing 1% BSA as the diluent.

Calcitonin was identified in tissue post-fixed in osmium tetroxide, whereas the other hormones and serotonin were tested on nonosmicated tissue. Nonosmicated tissue was only available in 13 cases.

The following controls were used for the single gold labelling methods: (1) substituting normal rabbit serum for the primary antiserum and (2) incubating the sections with primary antiserum preabsorbed with homologous antigens. For the double labelling method two controls were used in every experiment. These controls include: (1) substitution of the first primary antiserum with normal rabbit serum and (2) substitution of the second primary antiserum with normal rabbit serum.

Results

Histologically, 16 cases showed a solid growth pattern (Fig. 1). In one of the cases which was not immunoreactive for thyroglobulin a follicular growth pattern dominated (Fig. 1). All tumours exhibited round or polygonal cells, and small areas with spindle-shaped cells were present in five cases. In two thyroglobulin-positive and three thyroglobulin-negative cases follicle-like structures were observed within the solid masses of the tumours. Amyloid was seen in 13 cases.

Calcitonin and CGRP were present in all tumours. In most cases the vast majority of tumour cells stained positively for calcitonin, while a few to many cells were immunoreactive for CGRP. Positive staining for thyroglobulin was seen in 8 cases, including one lymph node metastasis (Fig. 2). Thyroglobulin-positive cells were few in number, unevenly distributed and present in solid areas. In two cases, one primary and one metastatic tumour, thyroglobulin immunoreactivity was also observed in tumour cells that lined follicles and contained a colloid-like material.

Immunoreactivity for bombesin (70% of the cases), somatostatin (29%), serotonin (29%), leu-enkephalin (17%), gastrin (17%), substance P (12%) and VIP (12%) was found in both thyroglobulin-negative and thyroglobulin-positive tumours. The cells that were positive for these peptides and serotonin were few in number. A ques-

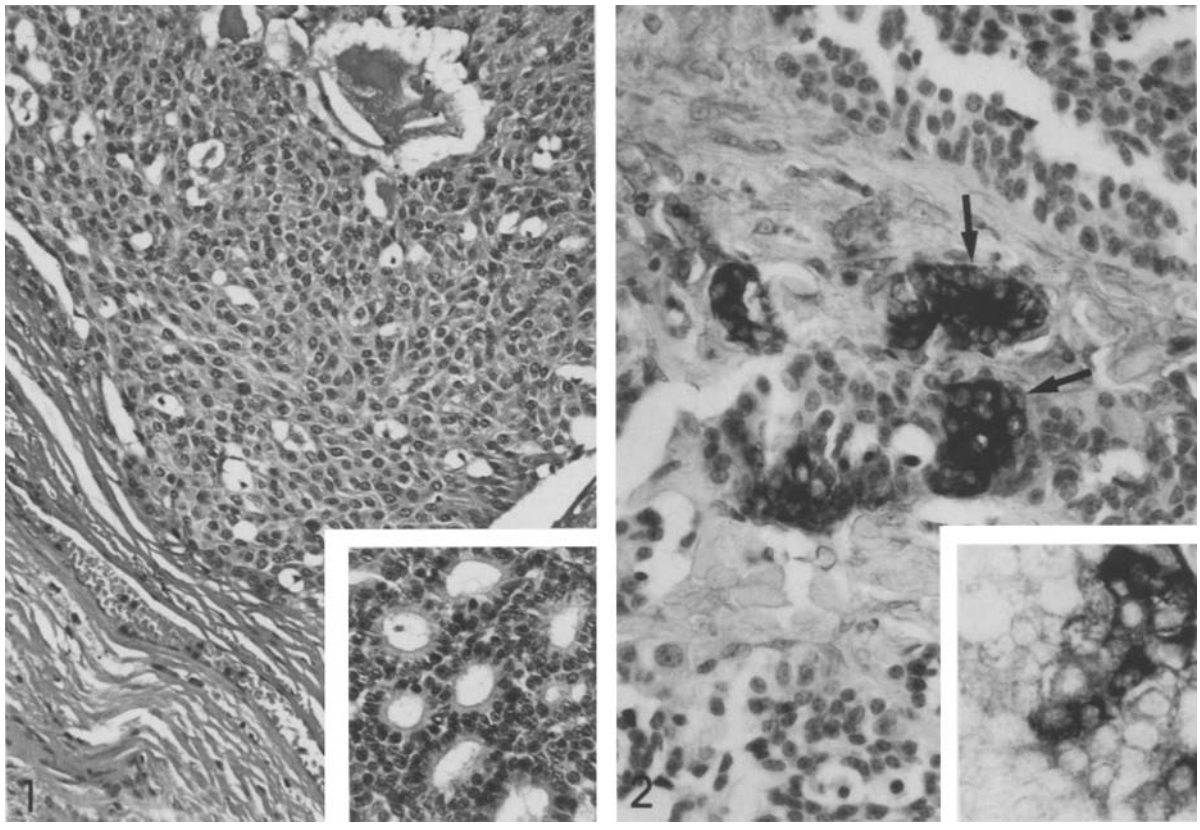


Fig. 1. Solid area with polygonal tumour cells (Case 2). *Inset:* Follicular growth pattern (Case 14). H&E, × 300

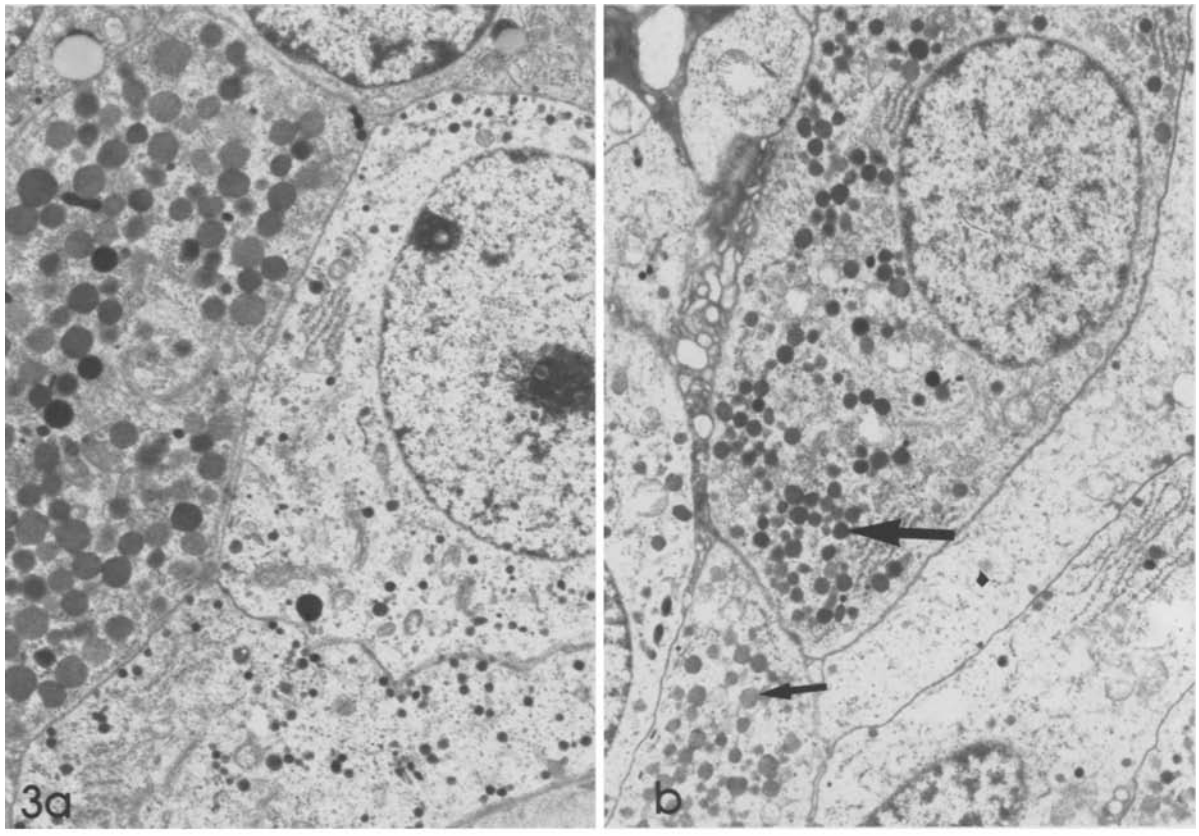
Fig. 2. Primary tumour immunoreactive for thyroglobulin (*arrows*) (Case 2). *Inset:* Lymph node metastasis immunoreactive for thyroglobulin (Case 6). × 580

tionable immunoreaction for calcitonin in ten cases and CGRP in one case was seen in the amyloid.

The ultrastructural features of thyroglobulin-negative and thyroglobulin-positive tumours were similar. Nuclei tended to be regularly shaped and to display a peripheral concentration of chromatin. Rough endoplasmic reticulum and Golgi complexes were well developed and amyloid occurred as small extracellular fibrils. A follicular growth pattern dominated in one of the thyroglobulin-negative tumours (case 14) and in this case the granules preferred the apical cytoplasm. Occasional small lumina without or with microvilli were observed in 5 thyroglobulin-positive and in 3 thyroglobulin-negative MCT. Colloid-like material was not seen in any of these lumina.

Both groups of MCT contained secretory granules of varying sizes. The granules exhibited round, uniform cores surrounded by a tight or loosely fitted membrane. The distribution profile of the size of secretory granules in each cell showed no cells with two or more granule populations. The average diameter of the granules in each cell varied from 106 nm to 406 nm in thyroglobulin-positive and from 131 nm to 405 nm in thyroglobulin-negative tumours. No particular size dominated within these ranges. Cells with small granules (average granule size less than 200 nm in diameter) (Fig. 3a) and cells with medium size granules (average diameter between 200 nm and 300 nm) (Fig. 3b) were seen in all cases. In each group four tumours (case 5–8 and 14–17) had additional cells containing larger granules with an average diameter of more than 300 nm (Fig. 3a). There were no significant differences in the average diameter of the granules between thyroglobulin-positive and thyroglobulin-negative tumours (Tables 1 and 2). The small granules had an electron dense core. The medium and large size granules were pale-cored or dense-cored, and most frequently found in distinct cells (Fig. 3b). However, in a few cells both dense-cored and pale-cored medium or large size granules were present. The tumour cells with small granules were often sparsely granulated, while cells with larger granules were usually densely granulated.

On the ultrastructural level a similar antigenic distribution of calcitonin and CGRP was observed in thyroglobulin-positive and thyroglobulin-nega-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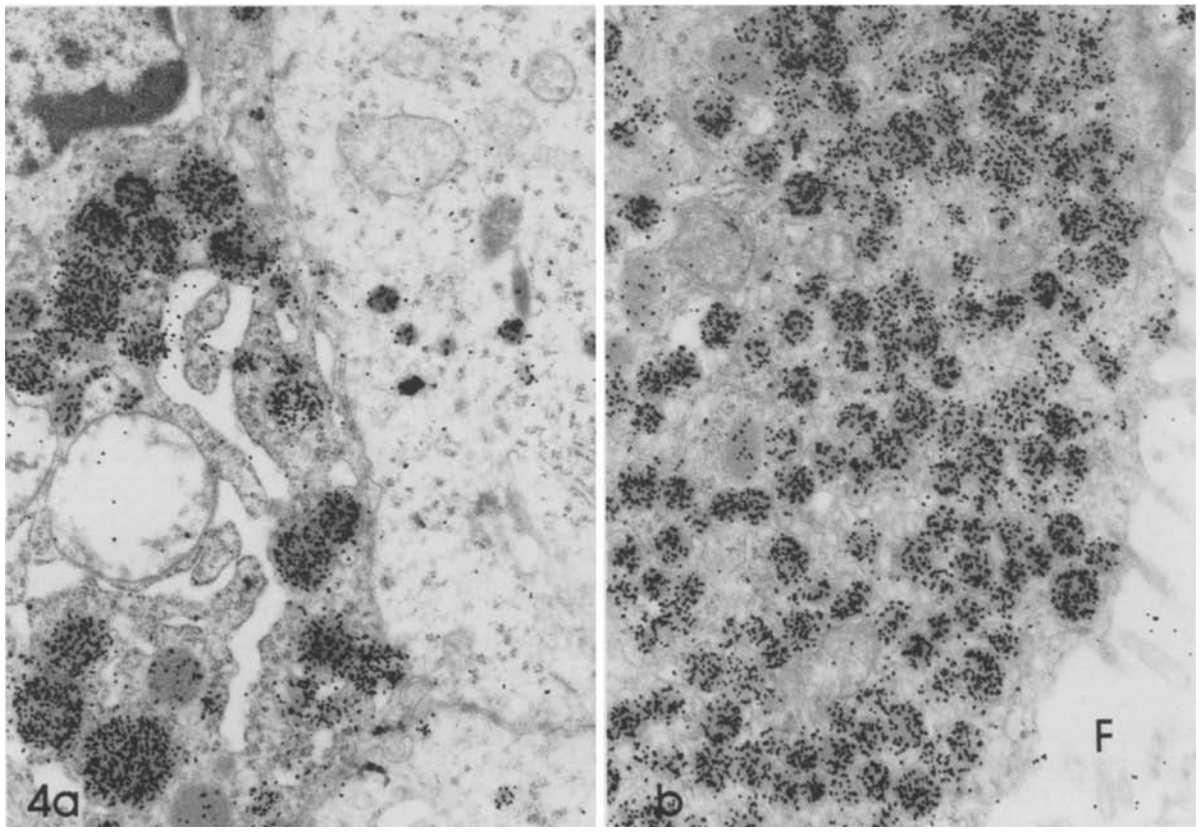
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Table 1. Size of secretory granules in thyroglobulin-positive MCT

Case no.	Mean diameter (nm) ±SD	Number of measured granules
1	221±71	454
2	184±59	571
3	171±67	388
4	189±58	621
5	252±65	440
6	255±63	345
7	269±99	431
8	235±68	362
Average of mean diameter	222	

Table 2. Size of secretory granules in thyroglobulin-negative MCT

Case no.	Mean diameter (nm) ±SD	Number of measured granules
9	221± 59	210
10	197± 61	843
11	193± 55	404
12	215± 71	707
13	196± 53	821
14	247±104	407
15	315± 96	397
16	249± 83	491
17	310±104	538
Average of mean diameter	238	

tive tumours. In all cases calcitonin immunoreactivity was identified in cells with small granules (Fig. 4a) as well as in cells with medium size granules (Fig. 4b). The additional cells containing large granules were also found to express calcitonin (Fig. 4a). Cells with medium or large size granules showed positive and similarly intense staining for calcitonin both in dense-cored (Fig. 5a) and in pale-cored granules (Fig. 5b). However, when pale and dense-cored granules were found in the same cell the immunostaining was weaker in the dense-cored granules than in the pale-cored ones (Fig. 6).

CGRP was identified in small, medium and large granules (Fig. 7), but because of the poor structure of nonosmicated tissue it was difficult to separate the pale and the dense-cored granules from each other. In all cases CGRP immunoreactivity showed an uneven pattern of staining. In three tumours (cases 2, 4 and 14), CGRP staining was observed in cells with small and medium size granules, and in case 14, even the additional cells with large granules showed CGRP immunoreactivity. In the other cases at least one cell population was CGRP-negative.

In cases 4 and 14 the double immunoelectron microscopic staining method demonstrated the coexistence of calcitonin and CGRP within the same secretory granule (Fig. 8). Amyloid, which was seen in five cases in both thyroglobulin-positive and thyroglobulin-negative tumours showed calcitonin positivity (Fig. 9). The amyloid in one of the cases was also immunoreactive for CGRP (Fig. 9). No immunoreactivity for thyroglobulin, bombesin, serotonin, somatostatin, leu-enkephalin, gastrin, substance P and VIP was observed on the ultrastructural level.

The control studies were satisfactory. No specific staining was seen in sections incubated with normal rabbit serum instead of specific antiserum or in sections incubated with antisera inactivated by their homologous antigens.

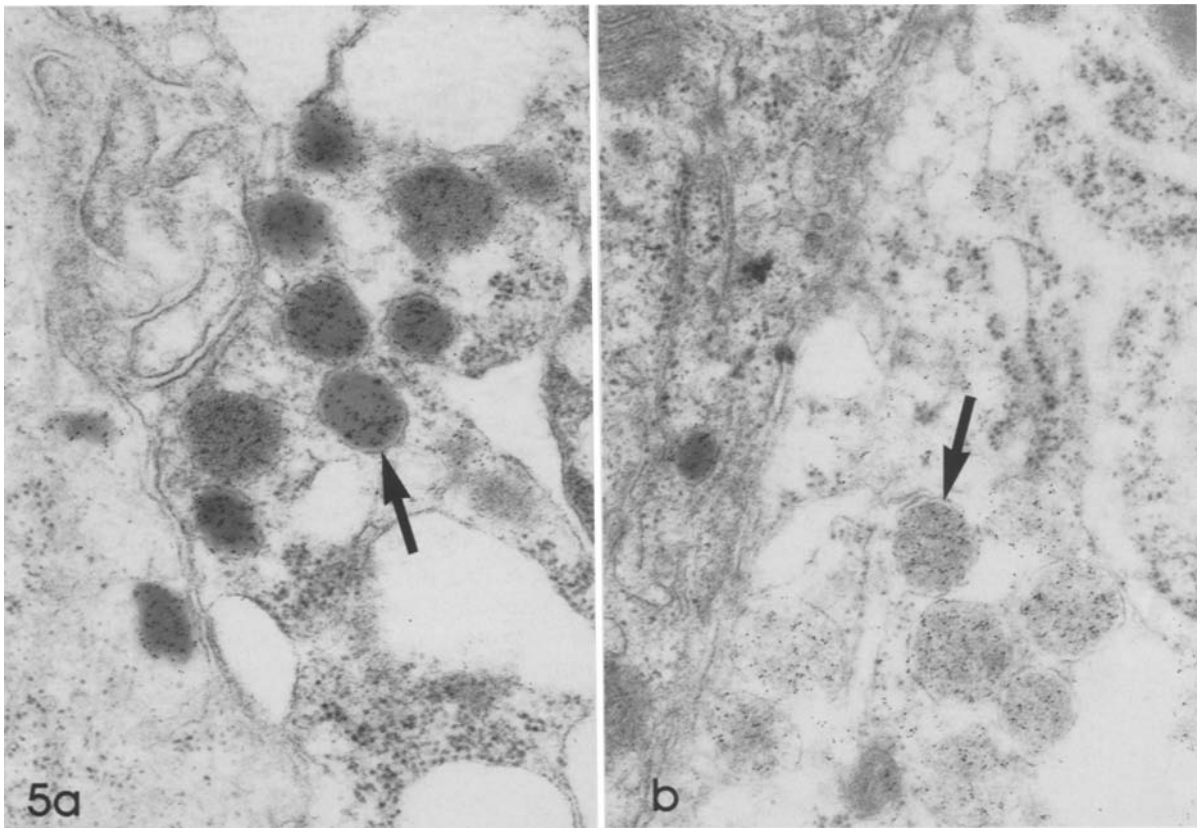
Discussion

Cells containing different types of secretory granules are seen in several endocrine tumours including carcinoids (Weichert et al. 1971), gastrinomas (Berger et al. 1985), glucagonomas (Bordi and Tardini 1980), insulinomas (Creutzfeldt et al. 1973; Bordi et al. 1975; Holm et al. 1988b), pituitary adenomas (Bassetti et al. 1986; Holm et al. 1989) and medullary thyroid carcinomas (Bordi et al. 1972; Kakudo et al. 1977; DeLellis et al. 1978; Capella et al. 1978; Huang and Goltzman 1978; Dämmrich et al. 1984; Nesland et al. 1986).

We found, as did DeLellis et al. (1978), Huang and Goltzman (1978) and Dämmrich et al. (1984), that the small granules, similar to type II granules of the aforementioned authors, have an electron dense core, while the medium size granules, similar in size to the moderately electron dense-cored type I granules of the same authors, might be either dense-cored or pale-cored. Within the groups of cells with small granules and the cells with medium size granules a considerable variation in the aver-

Fig. 3. (**a**) Cells with small granules and a cell with large granules and (**b**) cells with medium sized granules, one containing pale-cored granules (*small arrow*) and the other with dense-cored granules (*large arrow*). (Case 7). ×6200

Fig. 4. Calcitonin immunoreactivity is localized (**a**) in the cell with small granules, and the cell with large granules, and (**b**) in a cell with medium sized granules. The neoplastic cells form a follicle (F). (Case 14). ×17600



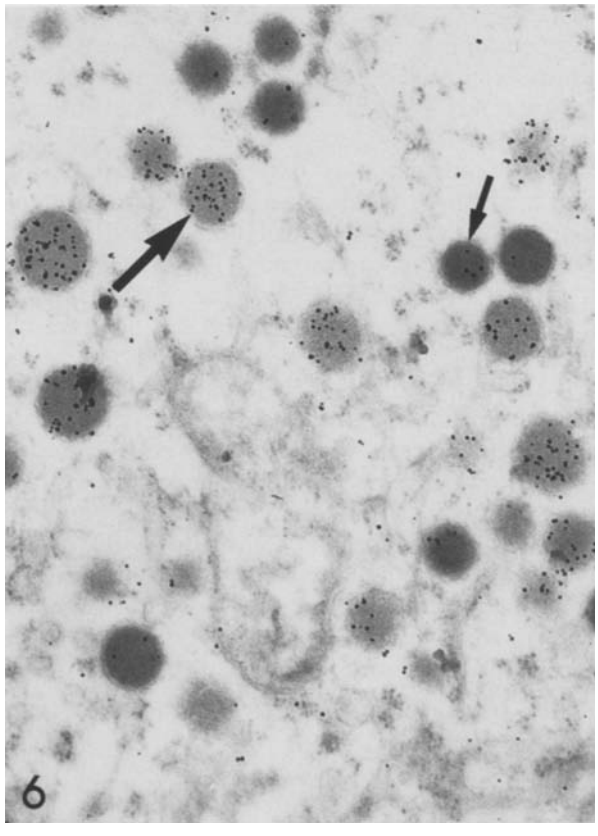
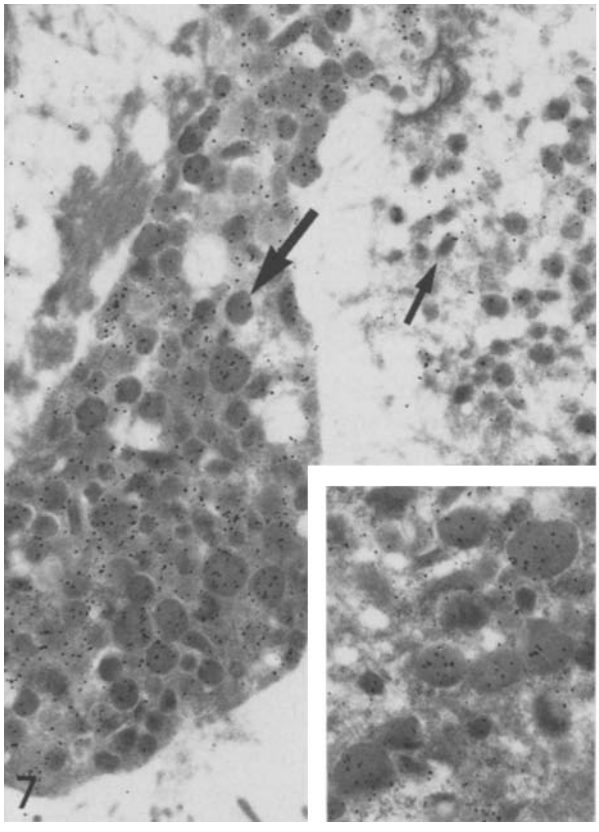
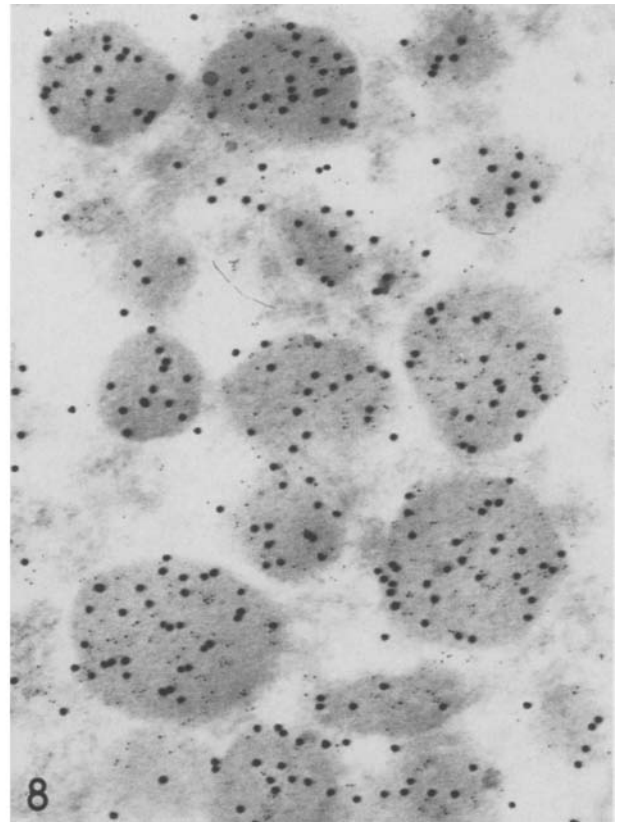


Fig. 5. Calcitonin immunoreactivity is localized (**a**) in the cell with dense-cored medium size granules (*arrow*), and (**b**) in the cell with pale-cored medium size granules (*arrow*). Silver enhancement has not been performed. (Case 12). × 39600

Fig. 6. Calcitonin immunoreactivity is localized in a cell containing both dense-cored and pale-cored medium sized granules. Immunostaining is weaker over dense-cored granules (*small arrow*) than over pale-cored granules (*large arrow*). (Case 6). × 32000





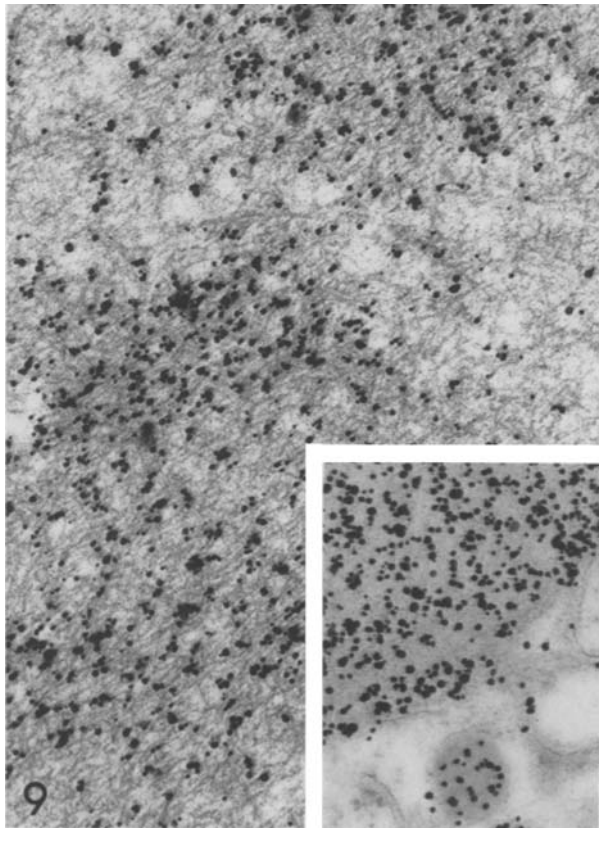


Fig. 7. CGRP immunoreactivity is localized in the cell with small granules (*small arrow*), in the cell with medium size granules (*large arrow*) and in the cell with large granules (*inset*). (Case 14). × 17600

Fig. 8. Coexistence of CGRP (5 nm gold particles) and calcitonin (15 nm gold particles) within the same secretory granules. (Case 14). × 54000

Fig. 9. Amyloid immunoreactivity for CGRP and calcitonin (*inset*). (Case 6). × 41000

age granules size was seen, and we think that the classification in so-called type II and type I granules represents an oversimplification.

In eight of our seventeen cases, cells with even larger granules were seen. These cells also showed a considerable variation in size and in granule core density.

It has been questioned whether the various granule populations contain calcitonin and/or other hormones. Different staining patterns for calcitonin have been reported. DeLellis et al. (1978) and Huang and Goltzman (1978) observed calcitonin immunoreactivity in both type I and type II granules, whereas Dämmrich and coworkers (1984) showed calcitonin in type II granules, while type I granules did not stain. We found calcitonin immunoreactivity not only in cells with small granules and cells with medium size granules, but also in cells with large granules. The apparent discrepancy in staining patterns may be due to different calcitonin affinities of the antisera used.

When dense-cored and pale-cored medium or large size granules were found in the same cells the dense-cored granules showed weaker immunoreactivity with anti-calcitonin than did the pale-cored ones. These two granule types may be in different maturation stages, one storing procalcitonin and the other calcitonin, or they may contain different molecular forms of calcitonin.

CGRP was present in cells with small, medium or large granules. Calcitonin and CGRP were usually localized in the same granules. This is in agreement with the work of Sikri et al. (1985). Since the calcitonin gene is known to encode for CGRP and this peptide is produced as a result of differential RNA processing (Rosenfeld et al. 1983), the localization of calcitonin and CGRP in the same granules is not surprising. Even hybridization histochemistry studies have demonstrated that calcitonin and CGRP are synthesized within the same cells (Zajac et al. 1986).

The cause of the heterogeneity in secretory granules is not obvious. The variation in granule size within each cell may reflect the maturation stage or functional activity, granules may increase in size by fusion (Smith and Farquhar 1966; Farquhar et al. 1978) or may be produced in a range of fixed sizes in the Golgi area. If granules of the same size are produced in the Golgi area, the above mentioned variation might merely reflect various profiles of similar granules cut at different levels. Our results do not provide a definite answer to this question since we have mainly observed major differences in granule size from cell to cell and not within each cell.

It is also not obvious why each MCT contains different cell types producing calcitonin. The cells may be in different stages of maturation or performing different functional activities (Nakagami 1965; Roth and Capen 1974), but it is also possible that some cells synthesize and store other peptides and hormones in addition to calcitonin and CGRP. Despite the fact that we failed to reveal other hormones at the ultrastructural level, we do not exclude the possibility that unidentified peptides or hormones are present in some of the granules.

Previous immunoelectron microscopical studies have demonstrated, as we did, calcitonin immunoreactivity in amyloid (Butler and Kahn 1986; Berger et al. 1988). However, Huang and Goltzman (1978) were unable to identify calcitonin in extracellular amyloid. This discrepancy may be due to differences in the immunoelectron microscopical methods used. Calcitonin positivity is certainly not surprising since a part of the major protein in MCT amyloid consists of an amino acid sequence corresponding to residues 9 to 19 of the human calcitonin (Sletten et al. 1976). Our study and that of Williams and coworkers (1987) show that the amyloid in most tumours is not immunoreactive for CGRP, which may indicate that neither CGRP nor a part of the amino acid sequence of CGRP is incorporated into the amyloid to any great extent.

On the ultrastructural level we did not recognize any morphological or immunohistochemical differences between thyroglobulin-positive and thyroglobulin-negative medullary carcinomas of the thyroid. The few thyroglobulin-positive cells recognized by light microscopy were not seen ultrastructurally, possibly due to the tiny size of the tissue blocks used for electron microscopy. This may also explain our inability to demonstrate by immunoelectron microscopy the several "ectopic" hormones we recognized in a few neoplastic cells at the light microscopical level.

In conclusion, our ultrastructural studies have established that the granules in MCT are more heterogenous than previously described. Based on a comprehensive morphometric and immunoelectron microscopic analysis we have identified cells with small, medium or large secretory granules, which were all immunoreactive for calcitonin and CGRP. No ultrastructural (morphological and immunohistochemical) differences were observed between thyroglobulin-positive and thyroglobulin-negative MCT.

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