Virchows Arch [Pathol Anat] (1985) 407:407–417

Virchows Archiv A

Ultrastructural demonstration of calcitonin in osmium-fixed human medullary carcinoma of thyroid by the protein A-colloidal gold technique

Toshimitsu Suzuki[1], Seiki Ito[2], Yukio Yamada[3], Fumio Matsuzuka[4], Sunao Matsubayashi[4], and Akira Miyauchi[5]

[1] First Department of Pathology, Niigata University School of Medicine, 1-Asahi-machi-dori, Niigata, 951
[2] Internal Medicine, Niigata University School of Medicine, Niigata
[3] Shinrakuen Hospital, Niigata
[4] Kuma Hospital, Kobe
[5] Department of Surgery, Kagawa Medical School, Kagawa, Japan

Summary. In two medullary carcinomas of the thyroid gland two types of secretory granules were found electron microscopically in the cytoplasm of the tumour cells. The sizes of the granules in one case ranged 103–345 nm in diameter; they were round in shape, and they co-existed in the same tumour cell. They could not, therefore, be distinctively subdivided into two types. In another case, secretory granules in the cytoplasm closely resemble EC granule in morphology. Using the protein A-colloidal gold (PAG) technique the content of secretory granules could be identified as calcitonin irrespective of their sizes or morphology. Immunoreactivity at the ultrastructural level was fairly well preserved even in the osmium-fixed tumour cells. The labelling index, expressed as a mean number of gold particles per unit square area of the secretory granule, was higher in the non-osmium-fixed tumour cells than in the osmium-fixed. Non-osmium-fixed tumour cells embedded either in epoxy or methacryl resin were almost equally labelled with gold particles. The result indicates that the PAG method is practicable to demonstrate the ultrastructural localization of calcitonin even in the osmium-fixed, epoxy resin embedded material.

Key words: Thyroid medullary carcinoma – Calcitonin – EC granule – Serotonin – Protein A-colloidal gold

Introduction

Medullary carcinoma of the thyroid gland has been reported to be chiefly composed of calcitonin producing C-cells (Kameya et al. 1977; Capella et al. 1978; Huang and Goltzman 1978), although other endocrine cells are occasionally included. The peptide hormone calcitonin was demonstrated in C-cells by biochemical analysis (Iwanaga et al. 1978) and by immunocytochem-

ical methods at the light microscopic (De Lellis et al. 1977; Kameya et al. 1977; Capella et al. 1978) and electron microscopic level (Huang and Goltzman 1978; Zabel 1983). This hormone is localized in storage granules (Huang and Goltzman 1978; Zabel 1983), which are subdivided in two types according to size and electron density (De Lellis et al. 1977).

PAG technique has been developed recently by Romano and Romano (1977), actively introduced by Roth (1982), and widely recognized by many authors as an useful device in immunoelectron microscopy. In the present study PAG technique was used to demonstrate calcitonin in the human medullary carcinoma of the thyroid ultrastructurally. In the present investigation the calcitonin containing granules are identified. It is clear that a variety of morphological types of granule contains calcitonin.

Materials and methods

Cases. A 51-year-old female (case 1) and a 42-year-old female (case 2) with non-familial type of medullary carcinoma of thyroid were examined. Serum calcitonin level was 21,300 pg/ml and 20,000 pg/ml respectively (normal, less than 165 pg/ml).

Tissue preparation. Tissues of the thyroid medullary carcinoma removed surgically were fixed for light microscopic investigation in buffered 10% formaldehyde. For electron microscopy 1 mm thick cubes were cut and fixed by immersion in 2.5% glutaraldehyde buffered with 0.1 M phosphate buffer pH 7.4 or 4% paraformaldehyde plus 1% glutaraldehyde for 2 h. The tissue was postfixed for 2 h in 1% osmium tetroxide buffered with the phosphate buffer at pH 7.4. In some tissue blocks the last step was omitted. After dehydration through graded ethanol series, embedding was carried out in Epon 812 via propylene oxide or in stylene/methacryl resin (Nisshin EM Co. Ltd., Tokyo). Ultrathin sections were cut from selected blocks, stained with uranyl acetate and lead citrate, and examined in an HS-9 electron microscope (Hitachi Co Ltd. Tokyo).

Light microscopic immunocytochemistry. The peroxidase-anti-peroxidase complex method (Sternberger et al. 1970) was applied. Deparaffinized sections of 5 μm thickness were first treated with methanol containing 1% H_2O_2 to abolish endogenous peroxidase activity. After washing in phosphate buffered saline (PBS), sections were incubated for 12 h with rabbit antibodies against calcitonin (Yamada et al. 1979) or serotonin (a gift from Dr. J. Uwo, Shionogi Research Laboratories, Shionogi Co. Ltd., Osaka) in a humidified incubation chamber. The specific antibodies were diluted 1:1,000 (calcitonin) or 1:2,500 (serotonin). After brief washing in PBS the slides were incubated for 30 min with swine antirabbit immunoglobulin (DAKO Immunoglobulins Ltd. Copenhagen, Denmark) diluted 1:40 followed by peroxidase-antiperoxidase complex diluted 1:60 (DAKO) for 30 min. After rinsing with PBS the diaminobenzidine (Sigma Chemical Co. St Louis MO) reaction (Graham and Karnovsky 1966) was used to visualize the peroxidase activity. Control reactions were performed without specific antisera, with non-immune rabbit serum or preabsorbed antisera with the homologous antigens instead of specific antisera.

Electron microscopic immunocytochemistry. Ultrathin sections were collected on uncoated nickel grids and allowed to dry before staining. Protein A-colloidal gold was prepared principally according to the method by Slot and Geuze (1981) as described before (Suzuki et al. 1984). For the protein A-colloidal gold staining method the following steps were followed: osmium-fixed but not non-osmium-fixed sections on the grids were first placed to perform etching on a droplet of 10% sodium metaperiodate (Wako Pure Chem. Indust., Osaka, Japan) for 5 min according to Bendayan and Zollinger (1983). After rinsing with PBS containing 1% bovine serum albumin (BSA, Sigma) for 20 min the grids were transferred on to drops of rabbit anti-calcitonin or serotonin antibodies diluted as above for 60 min at room temperature.

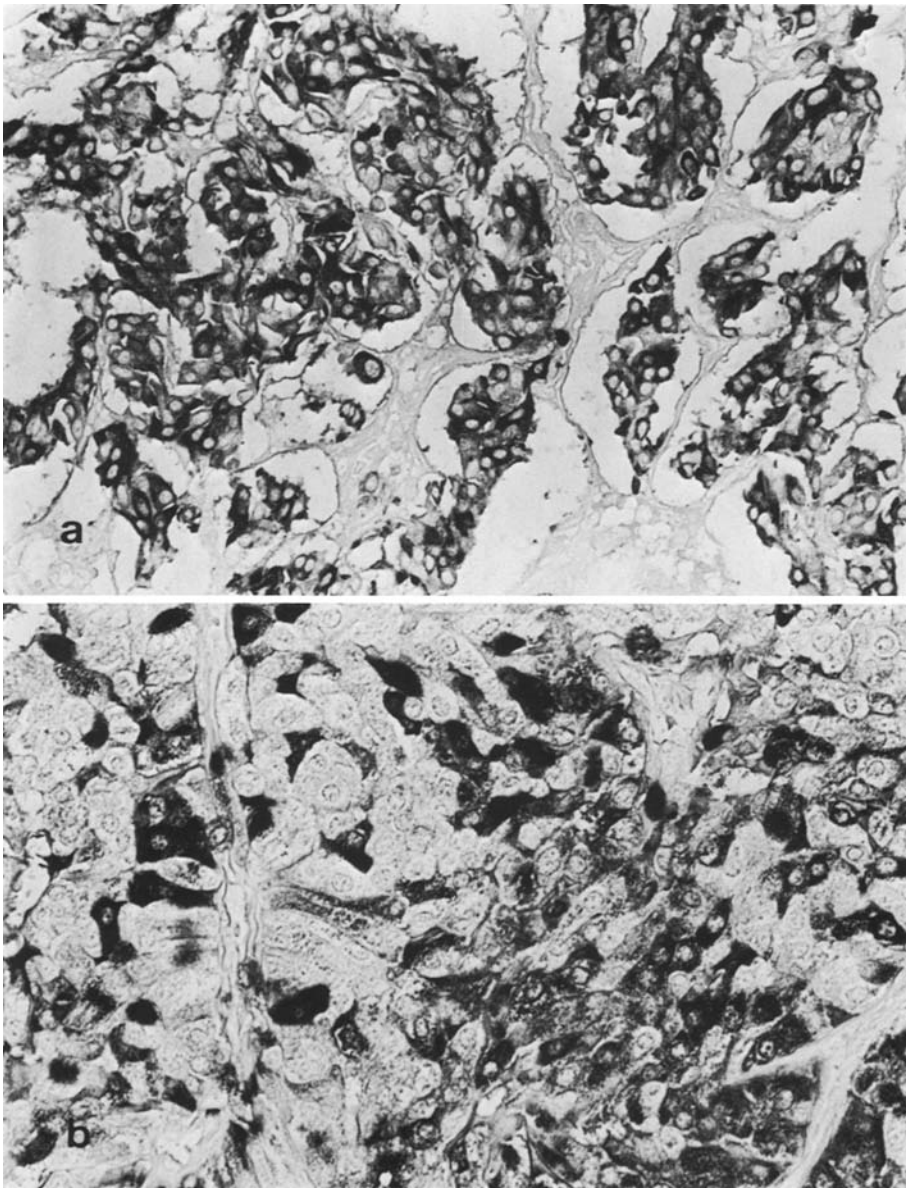

Fig. 1 a, b. Medullary thyroid carcinoma in light microscopic immunocytochemistry with positive immunoreactivity of tumour cells to calcitonin (**a**: case 1, **b**: case 2). PAP, × 200

After washing in PBS containing 1% BSA for 10 min they were placed on a solution of protein A-colloidal gold with a mean particle size of 12 nm (range 8–15 nm). After rinsing with PBS for 10 min the grids were jet-washed with distilled water. The sections were briefly counterstained with uranyl acetate and lead citrate. Controls for the specificity of reaction were performed using protein A-colloidal gold with the absence of primary antisera, or the presence of non-immune rabbit serum or preabsorbed antisera with the homologous antigen.

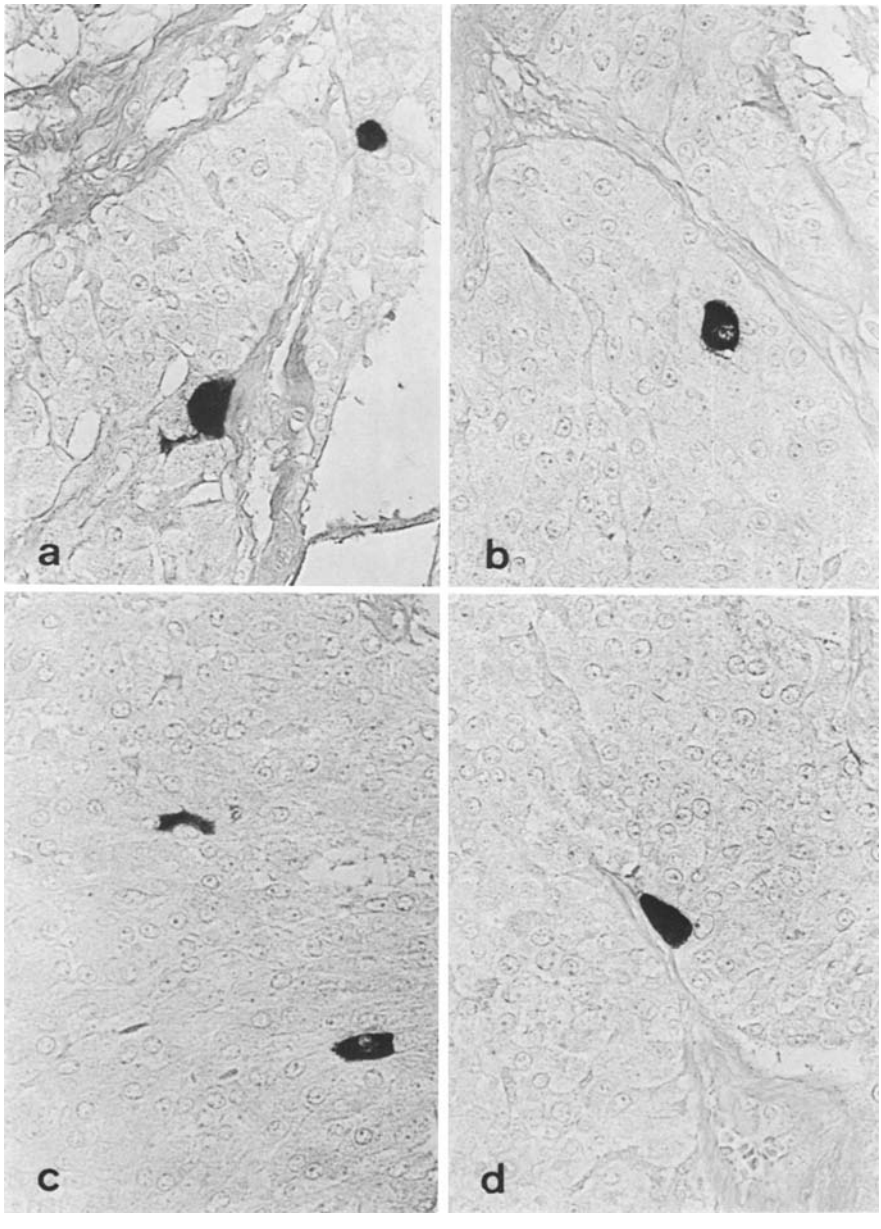

Fig. 2. A few tumour cells with positive immunoreactivity to serotonin in case 2 (**a–d**). PAP, ×200

To determine the mean diameter 100 secretory granules in conventional electron microscopy and 100 gold labelled granules were measured and classified on photographs of a magnification of 24,000, then mean values and standard deviations were evaluated. The calculated diameters are approximate values, because tangential sections of the spherical granules could not be excluded. Mean number and standard deviations of gold particles per an unit area of 100 granules labeled were also calculated.

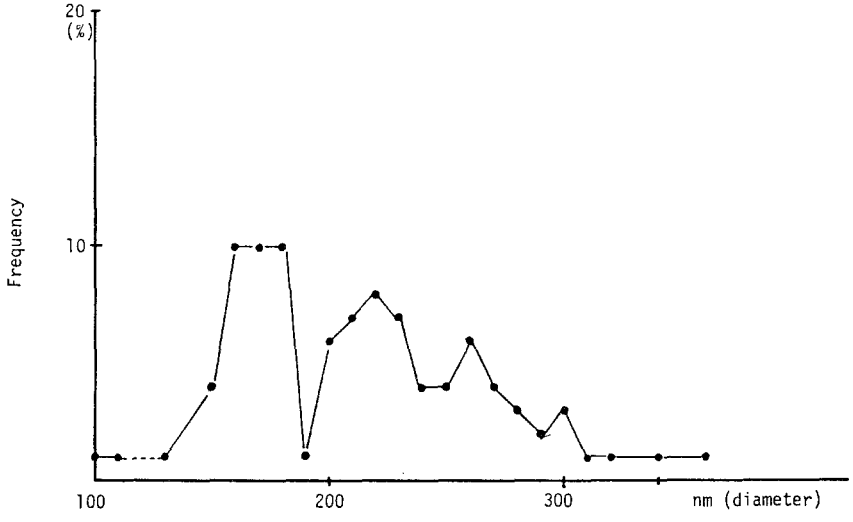

Fig. 3. Size distribution of secretory granules in case 1 tumour cells

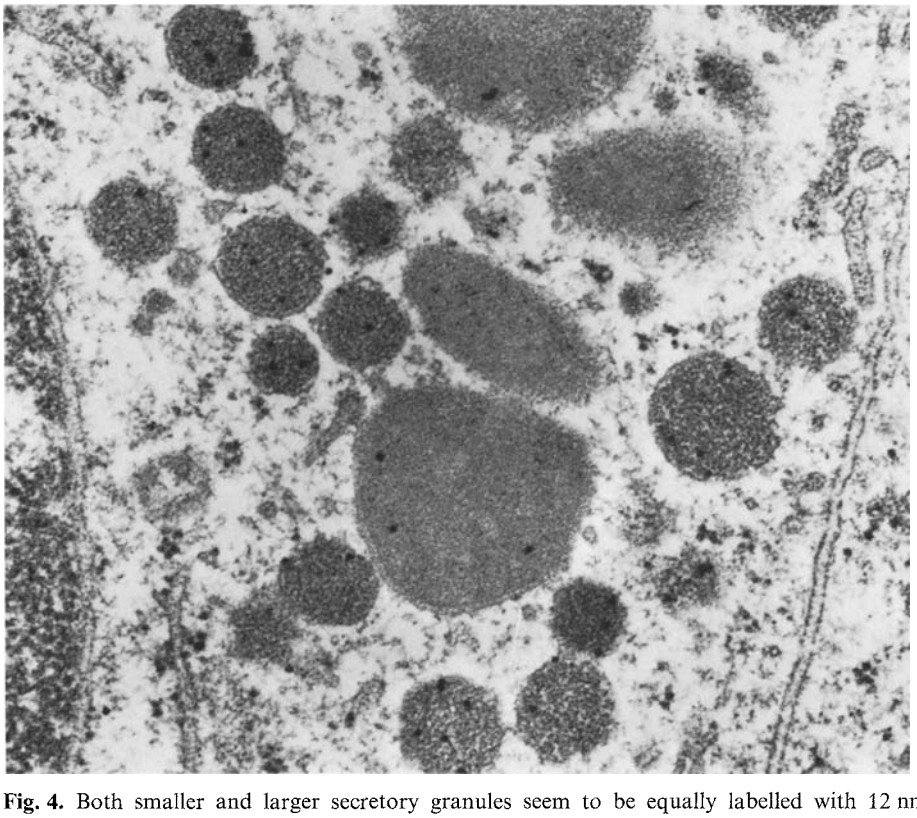

Fig. 4. Both smaller and larger secretory granules seem to be equally labelled with 12 nm gold particles. Osmium-fixed specimen of case 1, × 46,000

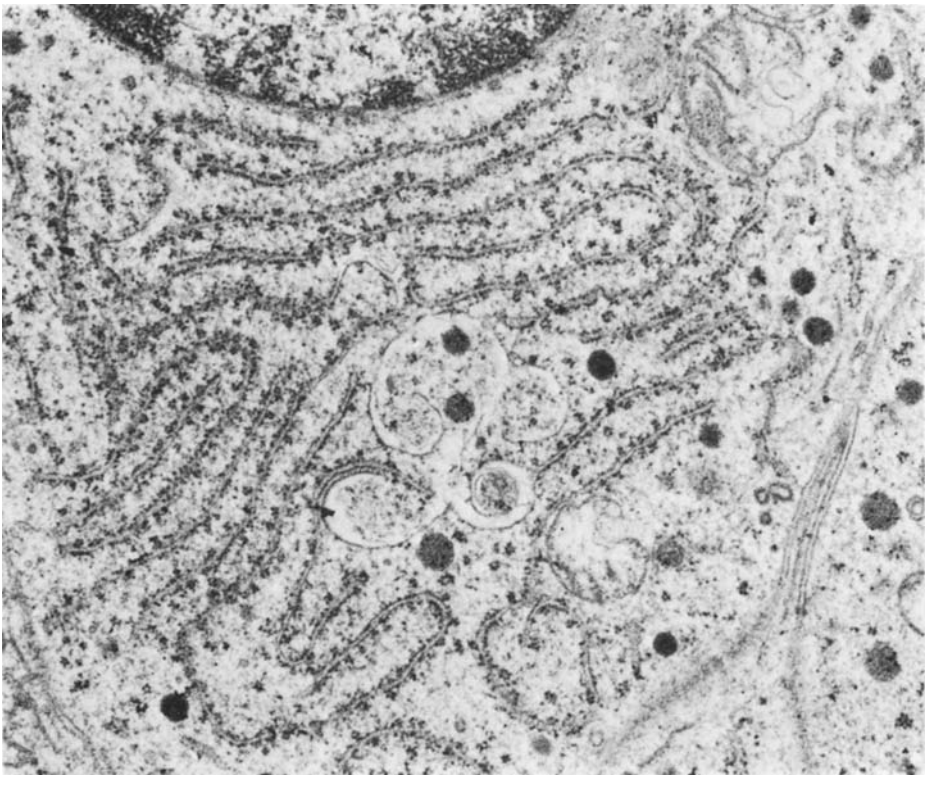

Fig. 5. Secretory granules are specifically labelled with gold particles, but not the rough endoplasmic reticulum. Osmium-fixed specimen of case 1, ×23,000

Results

In light microscopy the thyroid medullary carcinoma showed the characteristic appearance but there are slight differences in histological features between the two tumour tissues. In case 1 the round tumour cells were arranged in cords or trabecular strands but in case 2 the polygonal cells mainly proliferated in solid cell nests. Birefringent amyloid deposits were easily detected in polarized light after staining with Congo red in both cases.

Immunohistochemically calcitonin was demonstrated in almost all of the tumour cells in case 1 but limited number of cells showed calcitonin immunoreactivities in some tumour cell nests in case 2 (Fig. 1), and reaction for serotonin was also noticed in very few cells only in case 2 (Fig. 2).

In electron microscopy many cells in case 1 contained abundant round granules which had a more-electron dense homogenous content separated by a narrow electron-lucent rim from the limiting membrane. The distribution pattern of the sizes of the granules is shown in Fig. 3. The pattern

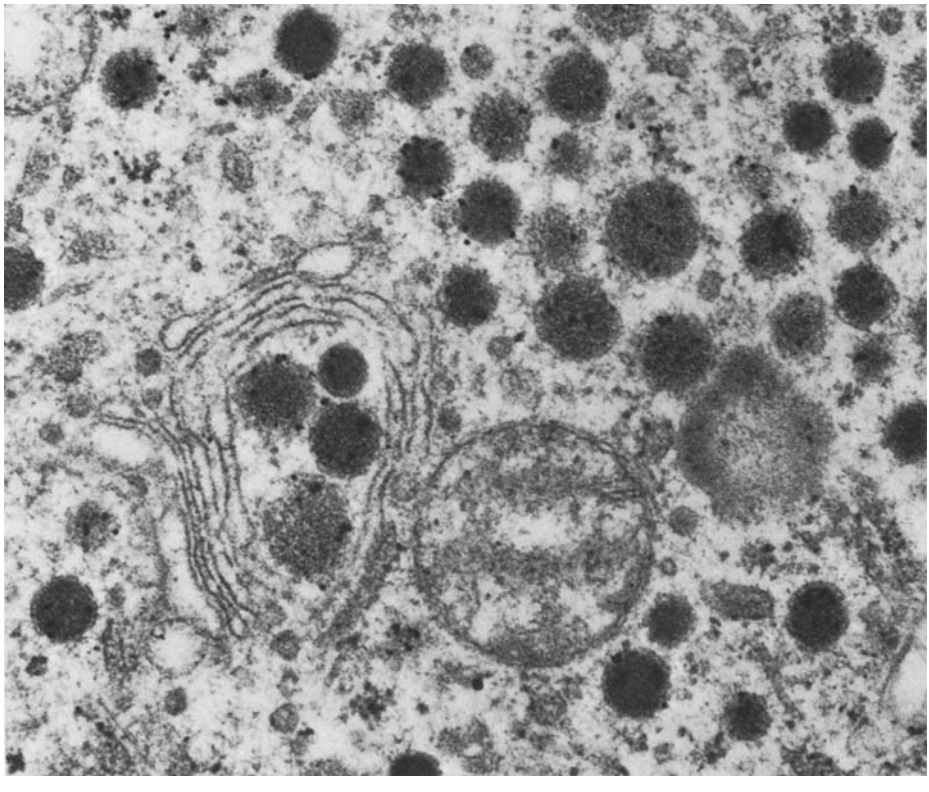

Fig. 6. Golgi complex is not labelled with gold particles but secretory granules closely associated with the complex are clearly labelled. Osmium-fixed specimen of case 1, × 46,000

seems to have two peaks; a smaller, sharper peak the size of which had a mean value of 162 ± 19 nm and a larger, broader peak the size of which had a mean value of 245 ± 38 nm in diameter. These values, however, did not correspond with so-called type II and type I granules described by DeLellis et al. (1977). Moreover, electron density of the smaller and larger granules seems to be identical, and these two kinds of granules co-existed in the same tumour cell. Therefore, secretory granules could not be subdivided in case 1. In case 2, rod-like, elliptical or occasionally vacuolated electron dense granules without distinct limiting membrane which resemble EC granules in endocrine cells of gastrointestinal tract were very frequently observed. Because of the paucity of round granules, the diameter of the secretory granules was not measured in this case. Amyloid fibrils were found in the interstitium with ease in both cases.

Immunocytochemically, calcitonin could be detected by the PAG technique in almost all of the secretory granules in both cases. The diameter of these calcitonin-containing, gold labelled granules ranged from 104 to

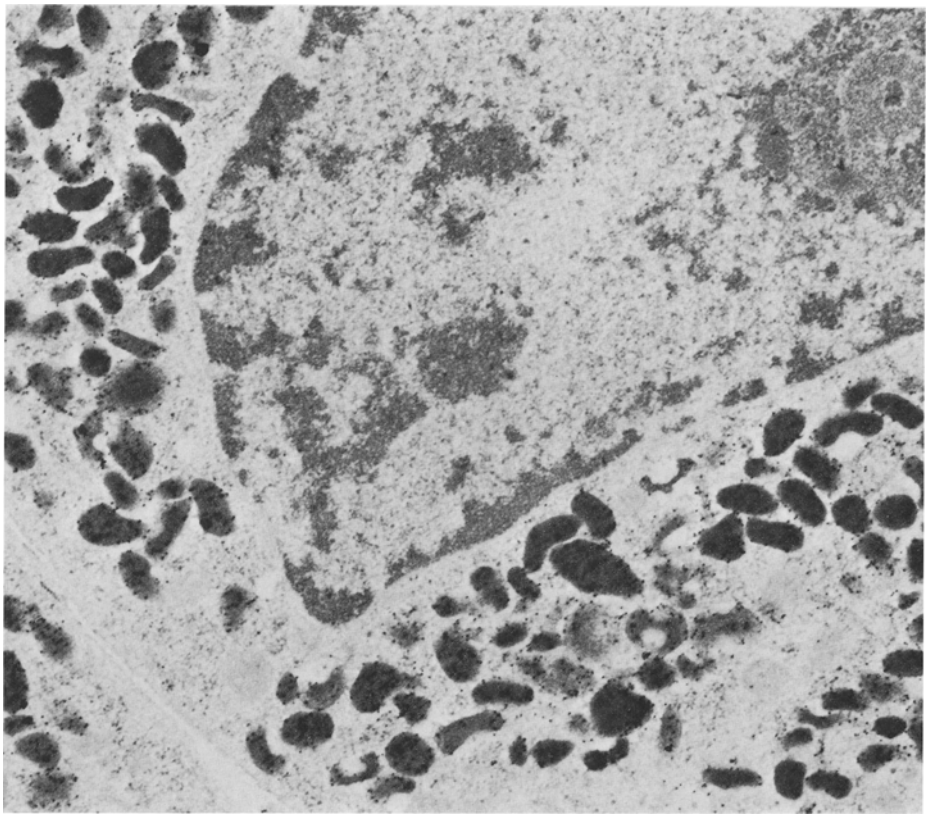

Fig. 7. Secretory granules closely resembling EC granules are also heavily labelled with gold particles, although membranous structures are not clear because of non-osmium fixation. From case 2. ×23,000

345 nm with a mean value of 212 ± 52 nm in case 1. Both smaller and larger granules were almost equally labelled with gold particles (Fig. 4).

Secretory granules were specifically labelled with gold particles even in the osmium-fixed specimen. Rough endoplasmic reticulum and Golgi complex were both free from gold particles (Fig. 5, 6). Some secretory granules closely associated with the Golgi complex, however, were clearly labelled (Fig. 6). EC granules found in case 2 were also heavily labelled with gold particles (Fig. 7). The labelling index mentioned above was much higher in the non-osmium fixed specimens than that in the osmium-fixed. Differences in the embedding material, however, did not affect the labelling index. The result is shown in Table 1.

A calcitonin immunoreactivity of amyloid was not observed in the present tumour sections. The nonspecific back ground staining and control staining was neglegible.

Serotonin immunoreactivity could not be detected in the secretory granules in the case 2 tumour cells by this method.

Table 1. Labelling index of the secretory granules

Resin	Fixation with Osmium	Labelling index[a]
Epon 812	+	1.21 ± 0.65
	−	5.83 ± 1.07
Stylenemethacryl	−	5.78 ± 1.28

[a] Mean values of gold particles per 10^2 μm^2 area of secretory granules ($n = 100$; Mean ± SD)

Discussion

Immunoelectron microscopy reveals the nature of content in the intracytoplasmic secretory granules satisfactorily. In this study the PAG technique (Romano and Romano 1977; Roth 1982) was used to demonstrate the ultrastructural localization of calcitonin in a thyroid medullary carcinoma. This technique showed high sensitivity and specificity as reported by the demonstration of other intracellular antigens (Roth 1982). The advantage of PAG method compared with some other immunocytochemical techniques is the use of the post-embedding methodology and the high electron-density of the gold particles. Even after counter-staining with uranyl acetate and lead citrate the gold particles specifically bound to the antigens are not masked.

The necessity to use only glutaraldehyde or paraformaldehyde plus glutaraldehyde-fixed tissues for the detection of many cellular antigens results in less good tissue preservation especially of membrane systems, when compared with the conventional electron microscopic techniques. Calcitonin, however, as shown in this study, could be clearly demonstrated in the osmium-fixed medullary carcinoma. Therefore, the membranous structures were fairly well preserved. The Golgi area, and also the rough endoplasmic reticulum were both apparent. The osmium-fixed specimen, however, was less heavily labelled than the non-osmium-fixed, but this disadvantage was not so serious as to confuse the interpretation of the specific labelling of the secretory granules.

In this study, larger and smaller granules were almost equally labeled, differing from the appearances reported by Dämmrich et al. in 1984 in which only smaller granules were specifically labelled with gold particles by the immune gold method (Faulk and Taylor 1971). The reason for this discrepancy is not clear. It is possible that the PAG method might be more sensitive than the immuno-gold technique, or that the larger granules of the case studied by Dämmrich et al. (1984) might contain peptides other than calcitonin. There have been sporadic reports of medullary carcinoma with somatostatin, ACTH (Kameya et al. 1977; Capella et al. 1978) or GRP immunoreactivities (Kameya et al. 1983; Matsubayashi et al. 1984).

Serotonin immunoreactivity was demonstrated in a very few cells of case 2 by the PAP method, but reactivity failed to be detected at the ultra-

structural level. From the EC morphology of the secretory granules, serotonin should be demonstrated in these granules. Fixation and/or embedding substance might damage the antigenicity of serotonin. Recent methods (Roth et al. 1981; Kobayashi et al. 1984) may overcome this problem. Nevertheless, calcitonin immunoreactivity can be demonstrated even in the osmium-fixed specimen by the PAG technique, and it is confirmed that calcitonin containing granules in medullary carcinoma are not uniform but variable in size, shape and electron density.

This technique, therefore, will provide further information on the biological characteristics or nature of calcitonin producing tumours or thyroid C-cells, when fixed with osmium tetroxide.

Acknowledgement. The authors thank Mr. Satoshi Tamura for electron microscopy, Mr. Makoto Yoshida for photographs, and Miss Yumiko Shiga for assistance in the preparation of this article.

References

Bendayan M, Zollinger M (1983) Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. J Histochem Cytochem 31:101–109

Capella C, Bordi C, Monga G, Buffa R, Fontana P, Bofanti S, Bussolini G, Solcia E (1978) Multiple endocrine cell types in thyroid medullary carcinoma. Evidence for calcitonin, somatostatin, ACTH, 5HT and small granule cells. Virchow Arch A Pathol Anat Histol 377:111–128

Dämmrich J, Ormanns W, Schäffer R (1984) Electron microscopic demonstration of calcitonin in human medullary carcinoma of thyroid by the immunogold staining method. Histochem 81:369–372

DeLellis RA, Nunnemacher G, Wolfe HJ (1977) C-cell hyperplasia. An ultrastructural analysis. Lab Invest 36:237–248

Faulk WP, Taylor GM (1971) An immunogold method for electron microscopy. Immunochemistry 8:1081–1083

Graham RC, Karnovsky MJ (1966) The early stage of absorption of injected horse radish peroxidase in proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. J Histochem Cytochem 14:291–302

Huang SN, Goltzman D (1978) Electron and immunoelectron microscopic study of thyroidal medullary carcinoma. Cancer 41:2226–2235

Iwanaga T, Koyama H, Uchiyama S, Takahashi Y, Nakano S, Itoh T, Horai T, Wada A, Tateishi R (1978) Production of several substances by medullary carcinoma of the thyroid Cancer 41:1106–1112

Kameya T, Shimosato Y, Adachi I, Abe K, Kasai N, Kimura K, Baba K (1977) Immunohistochemical and ultrastructural analysis of medullary carcinoma of the thyroid in relation to hormone production. Am J Pathol 89:555–574

Kameya T, Bessho T, Tsumuraya M, Yamaguchi K, Abe K, Shimosato Y, Yanaihara N (1983) Production of gastrin releasing peptide by medullary carcinoma of the thyroid. Virchow Arch A Pathol Anat Histol 401:99–108

Kobayashi S, Suzuki M, Uchida T, Yanaihara N (1984) Enkephalin neurons in the guinea pig duodenum: A light and electron microscopic immunocytochemical study using an antiserum to methonine-enkephalin-Arg^6-Gly^7-Leu^8. Biomed Res 5:489–505

Matsubayashi S, Yanaihara C, Ohkubo M, Fukata S, Hayashi Y, Tamai H, Nakagawa T, Miyauchi A, Kuma K, Abe K, Suzuki T, Yanaihara N (1984) Gastrin-releasing peptide immunoreactivity in medullary thyroid carcinoma. Cancer 53:2472–2477

Romano EL, Romano M (1977) Staphyrococcal protein-A bound to colloidal gold: An useful reagent to label antigen-antibody sites in electron microscopy. Immunochem 14:711–715

Roth J (1982) The protein A-gold (PAG) technique. A qualitative and quantitative approach for antigen localization on thin sections. In: Bullock GR, Petrusz P (ed) Techniques in Immunocytochemistry vol. 1 Academic Press, London pp 107–133

Roth J, Bendayan M, Carlemalm E, Villiger W, Garavito M (1981) Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. J Histochem Cytochem 29:663–671

Slot JW, Geuze HJ (1981) Sizing of protein A-colloidal gold probes for immunoelectron microscopy. J Cell Biol 90:533–536

Sternberger LA, Hardy PH Jr, Cuculis JJ, Meyer HG (1970) The unlabeled antibody enzyme method of immunohistochemistry: Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J Histochem Cytochem 18:315–333

Suzuki T, Hirota M, Ito S (1984) Protein A-colloidal gold method: Its application to human pancreatic tissues and re-identification of specific endocrine granules at the ultrastructural level. Acta Med Biol 32:91–97

Yamada Y, Ito S, Kayamori R, Shibata A (1979) The effects of vitamin D on the somatostatin and calcitonin concentration in the rat thyroid. Horm Metab Res 11:184–185

Zabel M (1983) Ultrastructural localization of calcitonin in control and stimulated thyroid C-cells of the rat using protein A-gold immuncoytochemical technique. Histochemistry 77:269–273

Accepted June 20, 1985