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CASE REPORT

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Well-differentiated endocrine tumours of the middle ear and of the hindgut have immunocytochemical and ultrastructural features in common

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Abstract The immunocytochemical analysis of two cases of well-differentiated endocrine tumours (carcinoids) of the middle ear revealed predominant cell populations producing pancreatic polypeptide (PP)-related peptides, glucagon-related peptides, and serotonin (the latter only in one case). In consecutive sections PP- and glucagon-related immunoreactivities mainly colocalized in the same tumour cells. Ultrastructurally tumour cells were characterized by medium-sized to large granules of moderate to high density, on which PP and glicentin were localized by the immunogold technique. No amphicrine cells were found. These features are consistent with those of similar tumours in the rectal mucosa that are mainly composed of L cells coexpressing both PP-related and glucagon-related peptides. Additional tumour antigens of hindgut type detected immunohistochemically were prostatic acid phosphatase and CAR-5 mucin. Expression of the CAR-5 antigen was also found in samples of normal middle ear mucosa, in which endocrine cells have not been identified. In case 1 peritumoral mucosal invaginations showed a proliferation of endocrine cells identical immunophenotypically to tumour cells, possibly representing a precursor lesion. It is concluded that well-differentiated endocrine tumours of the middle ear are a distinct pathological entity characterized by multiple hormone production, typically involving three classes of hormones (pancreatic polypeptide-related peptides, glucagon-related peptides, and serotonin) of the hindgut endocrine system.

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Introduction

The well-differentiated endocrine (carcinoid) tumour of the middle ear is a rare neoplasm. A survey of the literature up to 1992 by Manni et al. [18] yielded 20 cases, to which 2 additional cases have since been added [14, 19]. In spite of its rarity, however, this tumour is a very interesting pathologic condition because of its peculiar cytological characteristics: tumour cells have been found to store hormones that are usually produced in the gastro-entero-pancreatic endocrine system. Although endocrine cells have not been identified in the normal middle ear mucosa, this tumour hormone production indicates an intrinsic capability of epithelial cells of the middle ear to express intestinal hormones, at least following tumour transformation.

In the present study two cases of well-differentiated endocrine tumour of the middle ear were investigated by immunohistochemistry and electron microscopy in an attempt at better delineation of their cytological and hormonal characteristics.

Case reports

Case 1

A 35-year-old man presented with an 18-month history of tinnitus, ear fullness and hearing loss in the left ear. In addition, diarrhoea had been present for the last 6 months. A CT scan revealed a mass measuring 1×0.5 cm in the middle ear. On exploration performed in another institution, partial removal of the tumour for biopsy purposes had led to the diagnosis of carcinoid tumour. The patient was then referred to our hospital for treatment.

On admission, the results of routine laboratory tests on blood and urine were unremarkable, as was a chest roentgenogram. An open tympanoplasty was performed and revealed a hypotympanic mass that was adherent to the tympanic membrane and the promontory and surrounded the stapes. The tumour was removed easily

from the bony surface and from the stapes, and a myringoplasty was done.

The postoperative course was uneventful. A CT scan performed 6 months later revealed no residual tumour. One year after the operation the patient is in good health and no longer suffering from diarrhoea.

Case 2

A 66-year-old woman was referred in January 1991 because of headache and progressive hearing loss on the right side over the preceding 3 months. There was no history of otorrhoea, and no vestibular symptoms were reported. Otoscopy revealed a polypoid red-bluish mass arising from the anterior-superior part of the right external auditory canal corresponding to the tympanic annulus and atticus, and causing the anterior part of the tympanic membrane to bulge. Audiograms revealed a sensorineural deficit on the right. Impedance audiometry showed reduced compliance. CAT, MNR and angiography confirmed the presence of a poorly vascular non-bone-eroding neoplasm in the right middle ear.

A biopsy of the mass was performed and conventional histology indicated a well-differentiated endocrine tumour. The patient underwent a radical tympano-petro-mastoidectomy extending to the antero-superior part of the external auditory canal infiltrated by the neoplasm. She is alive with no evidence of recurrent disease 3 years after surgery.

Materials and methods

Histology and peptide immunohistochemistry

Samples of tumor tissues were fixed in formalin–calcium acetate or Bouin's fluid for 6–12 h, dehydrated through graded alcohols and embedded in paraffin. Serial, 5-μm-thick sections were stained with haematoxylin-eosin, PAS-Alcian Blue stain and the Grimelius silver technique. For immunohistochemistry the antibodies listed in Table 1 were used. The immunoreactivity was visualized with the avidin-biotin complex (ABC) procedure (Vectastain ABC kit, Vector Laboratories, Burlingame, Calif.), using diaminobenzidine tetrahydrochloride as chromogen substrate and nickel chloride as signal intensifier. In addition, an original haematoxylin-eosin section of the tumour removed in another institution in case 1 was available for revision. Samples of histologically normal middle ear mucosa collected from three patients who had undergone tympano-petro-mastoidectomy for non-chromaffin paragangliomas were also studied with the same methods.

Electron microscopy

Tumour samples were immediately fixed in a mixture of 2% or 4% paraformaldehyde and 2% or 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 3 h at room temperature, post-fixed in 1% osmium tetroxide, dehydrated and embedded in Araldite or Epon-Araldite. Thin sections were collected on 300 mesh copper grids, stained with uranyl acetate and lead citrate and examined in a Zeiss EM 109 electron microscope. The primary antisera used for immunolocalization of PP-related peptides and glucagon-related peptides at the ultrastructural level are specified in Table 1. As detailed elsewhere [4], the procedure was performed on sections deosmicated with sodium metaperiodate using the protein A–gold complex method intensified by further incubation with rabbit antiserum against protein A.

Results

Case 1

Histologically, the original tumour removed elsewhere revealed a typical, pure structure of a well-differentiat-

Table 1 Antibodies used in the present study (*M* monoclonal, *P* polyclonal)

Antigen	Type	Code	Dilution	Source
Chromogranin A (CgA)	M	Phe5	1:400	Ortho, Diagnostic System, Raritan, N.J., USA
Chromogranin B (CgB)	M	B11	1:4000	Dr. A. Siccardi, Milan, Italy
Serotonin (5HT)	M	YC5	1:5000	Serotec, Oxford, UK
	P		1:800	Sanbio, Uden, Holland
Alpha subunit of HCG (alpha HCG)	M	5E8	1:5000	Dr. S. Ghielmi, Brescia, Italy
	P		1:1600	Dr. M. Fukayama, Tokyo, Japan
N-terminus human gastrin 34 (G34)	P	AC90	1:500	Cambridge Research Biochemicals Cambridge, UK
	P	2604	1:1600	Dr. J. Rehfeld, Copenhagen, Denmark
C-terminus pancreatic polypeptide (Ct-PP)	P	221	1:2000	Dr. M.T.T. O'Hare, Belfast, N. Ireland
Bovine PP[a]	P		1:4000	Dr. R. Chance, Indianapolis, Ind., USA
Synthetic cyclic (1–14) somatostatin (SOM)	P	A566	1:400	Dakopatts, Copenhagen, Denmark
C-terminus porcine pancreatic glucagon	P	B31-1	1:1250	Milab, Malmoe, Sweden
N-terminus glucagon	P	05Y	1:800	Dr. R.H. Unger, Dallas, Tex., USA
N-terminus glucagon/glicentin	P	B37-1	1:1250	Milab, Malmoe, Sweden
Synthetic bovine neurotensin	P	B44-1	1:1600	Milab, Malmoe, Sweden
Prostatic acid phosphatase (PAP)	P	A627	1:400	Dakopatts, Copenhagen, Denmark
Calcitonin	P	596010	undiluted	Ortho, Diagnostic System, Raritan, N.J., USA
Polypeptide YY (PYY)	P	690	1:1000	Dr. L. Terenius, Uppsala, Sweden
HISL19	M		1:800	Dr. G. Eisenbarth, Boston, Mass., USA
7B2	P		1:200	Prof. J.M. Polak, London, UK
Insulin	M	029	1:200	Biogenex Laboratories, San Ramon, Calif., USA
	P	A546	1:1600	Dakopatts, Copenhagen, Denmark
C-terminus porcine glicentin[a]	P	R-4804	1:1000	Dr. N. Yanaihara, Shiznoka, Japan
Neuron specific enolase (NSE)	M	M16-N3	1:100	Sanbio, Uden, Holland
Car-5 human sulfated colorectal glycoprotein	M	BD5	1:20	Dr. P.M. Comoglio, Turin, Italy
Human foveolar gastric fucomucins	M	M1	1:500	Dr. D. Burtin, Villejuif, France

[a] Antisera used for immunoelectron microscopy

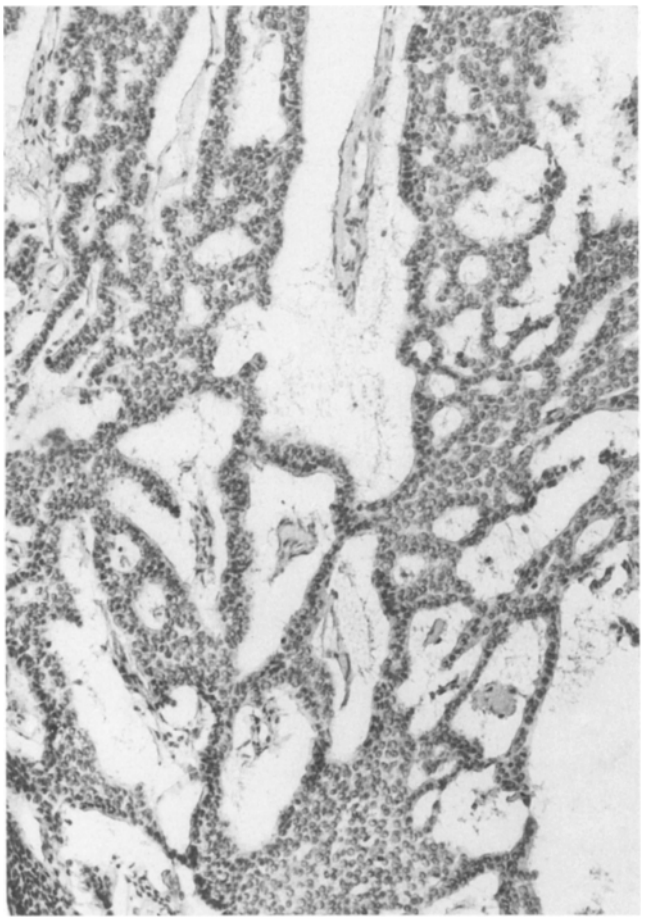

Fig. 1 Case 1. The tumour tissue shows a trabecular or ribbon like-arrangement, typical of foregut well-differentiated endocrine tumours. H&E, ×140

ed endocrine tumour, with uniform cuboidal to tall cells forming trabecular, anastomosing cords or more solid aggregates with little intervening stroma (Fig. 1). "Zellballen" were not observed. The tumour cells showed round nuclei with rare mitoses and finely granular eosinophilic cytoplasm. Neither haemorrhagic nor necrotic changes were present. Tumour tissue from the second operation, on which the immunohistochemical and ultrastructural studies were performed, differed from that removed at the first operation in the abundance and, often, predominance of dense fibrous stroma. In this stroma, solid clusters of tumour cells, otherwise consistent with the previous description, were scattered (Fig. 2). They varied in size from a few cells to quite large aggregates and were diffusely argyrophilic after the Grimelius staining. The mucosa covering the tumour mostly had squamous epithelium. In addition, it showed deep invaginations lined with cuboidal or columnar monostratified epithelium containing numerous argyrophil cells. This epithelium presented areas of pluristratified cell proliferation gradually merging with neoplastic cell clusters, a finding possibly reflecting tumour histogenesis.

Immunohistochemistry revealed that tumour cells were positive for CgA (Fig. 2A) and, less intensely, for neuron-specific enolase (NSE), HISL-19 and 7B2. Similar results were found in numerous cells basally located in the epithelium lining adjacent mucosal invaginations. Numerous cells immunoreactive for PP, PYY, serotonin, the N-terminal region of the glucagon molecule and C-terminal glicentin were observed in both tumour cell clusters and mucosal invaginations (Fig. 2B–D). Less frequent cells with similar distribution were found to react with the antiserum against C-terminus glucagon. Occasional isolated alpha-HCG immunoreactive cells were also seen. Antisera against insulin, somatostatin, calcitonin and gastrin consistently gave negative results. Although simultaneous localization of different hormones in the same cells could not be performed, analysis of consecutive sections indicated that PP, glucagon and serotonin were partially colocalized in tumour cells.

The basic ultrastructural findings in the tumour cells were abundant secretory granules 150–250 nm in diameter and the frequent occurrence of large perinuclear aggregates of intermediate filaments (Fig. 3). These findings were consistent with those reported in previous papers [13–15, 20, 24, 26]. The morphology of secretory granules was markedly heterogeneous, with different patterns variously associated in the same cells. The majority of granules showed a medium to high density, round to slightly elongated core, and a thickened, sometimes interrupted surrounding membrane separated from each other by a narrow but sharp clear space (Fig. 3, inset). Other granules presented less distinct, floccular content or, especially in poorly preserved cells, condensed cores with crystalloid profiles, both surrounded by large, vesicular spaces. Mucous granules were never observed.

Electron microscopic immunolocalization of PP was mostly seen on the dense, large granules of tumour cells (Fig. 4A). Unfortunately, and in spite of positive control reaction, no specific ultrastructural staining for glucagon was found in the tumour cells investigated.

Case 2

Pathological examination of the tumour showed cuboidal to columnar cells forming trabeculae, solid nests and gland-like spaces within the fibrous stroma. In several areas the tumour was located immediately beneath the epithelial lining of the tympanic cavity, but continuity between the tumour and the middle ear epithelium was not observed. Tumour cells were uniform, with a moderate amount of granular eosinophilic cytoplasm and round nuclei showing finely dispersed chromatin. Mitotic figures were not seen. Alcian Blue stain demonstrated scanty extracellular mucin within the gland-like spaces. Grimelius staining showed argyrophilic granules mainly located in the basal portion of tumour cells.

Immunohistochemically, the majority of tumour cells showed diffuse cytoplasmic staining with antibodies

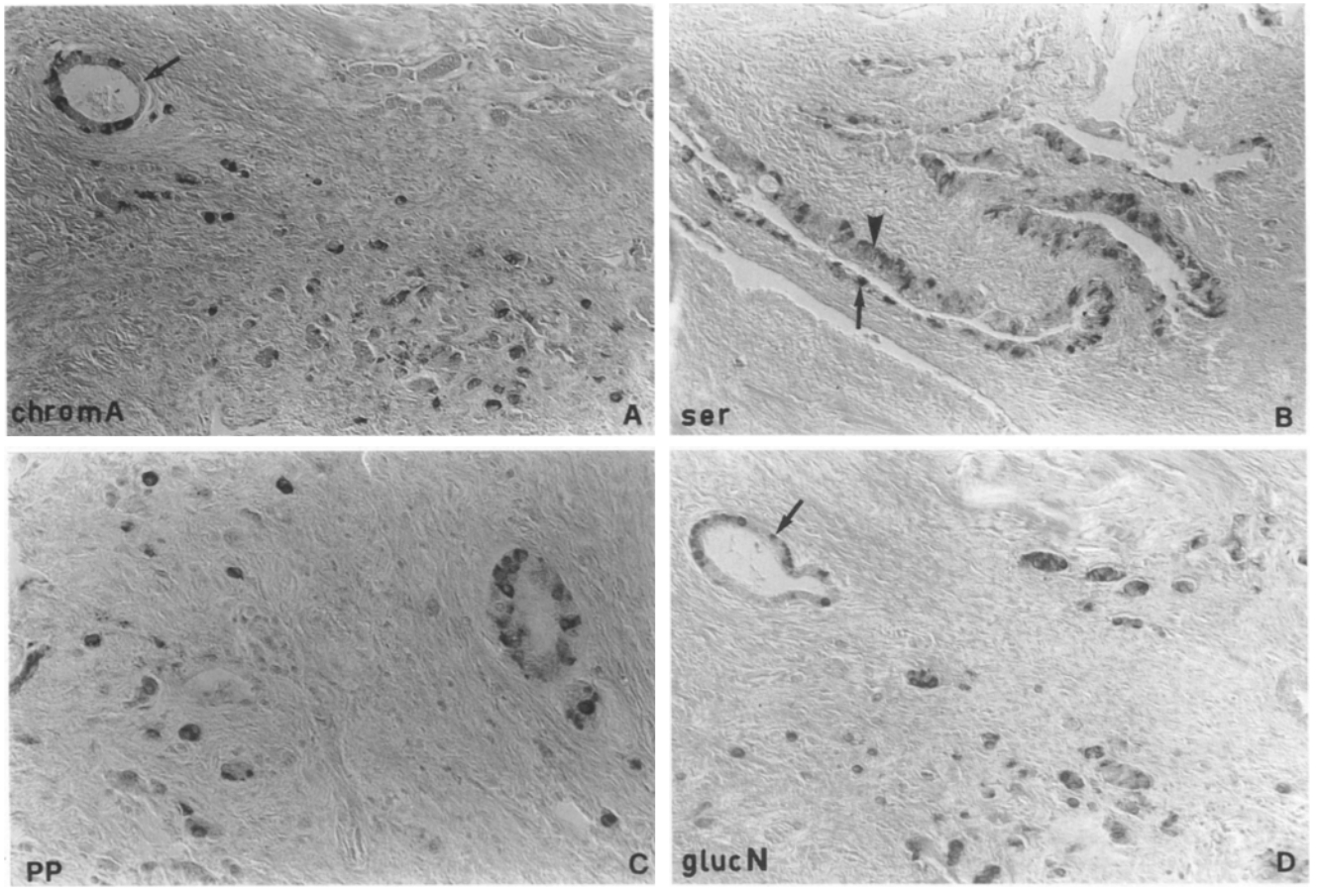

Fig. 2A–D Case 1. Immunostaining for **A** chromogranin A **B** serotonin, **C** pancreatic polypeptide, and **D** N-terminus glucagon is present in tumour tissue and associated mucosal invaginations. The latter are lined with either monostratified, cuboidal (*arrows*) or pluristratified, hyperplastic (*arrowhead*) epithelium. Avidin–biotin complex immunoperoxidase; **A**, **B**, **D** ×130; **C** ×180

against NSE, CgA, CgB, PAP (Fig. 5A), PP, PYY and N-terminus glucagon. A few cells were positive for somatostatin, alpha HCG and serotonin, while no positive staining was found for insulin, C-terminus glucagon, calcitonin and gastrin. Some lumina of the gland-like spaces showed CAR-5 immunostaining (Fig. 5B). In adjacent histological sections the pattern of distribution was often the same for PP and N-terminus glucagon and C-terminus glicentin, suggesting that the two immunoreactivites could be accounted for by the same cells (Fig. 6).

Ultrastructurally, virtually every cell examined contained numerous round, membrane bound secretory granules measuring 130–290 nm. The majority of granules were homogeneously electron dense with some occasional pale granules admixed. Other conspicuous features included paranuclear whorls of intermediate filaments and occasional junctional complexes, but amphicrine cells also featuring mucous granules were not found. Lumen formation with microvilli was partly seen in some tumour cells. Immunogold localization of PP and C-terminus glicentin (Fig. 4B) was mostly found on dense and large secretory granules of tumour cells.

Histologically normal middle ear mucosa

The epithelium lining the tympanic cavity was partly squamous and partly cuboidal to columnar and was provided with cilia. The apical border of the ciliated epithelium was stained by Alcian Blue. Pan-endocrine markers, including Grimelius' silver method, and immunostaining for NSE, CgA, and CgB did not reveal the presence of any endocrine cells within the squamous or the ciliated epithelium. Staining for CAR-5 was found in the apical border of ciliated cells (Fig. 7), while positivity for M_1 antigen was observed in the apical cytoplasm of some columnar mucin-producing cells.

Discussion

The histological differential diagnosis of well differentiated endocrine tumours (or carcinoids) of the middle ear from non-endocrine tumours such as adenoma and adenocarcinoma or paraganglioma can be difficult (for a detailed discussion of differential criteria see [20, 23, 26]). It is thus possible that the true incidence of well-differentiated endocrine tumours of the middle ear is underes-

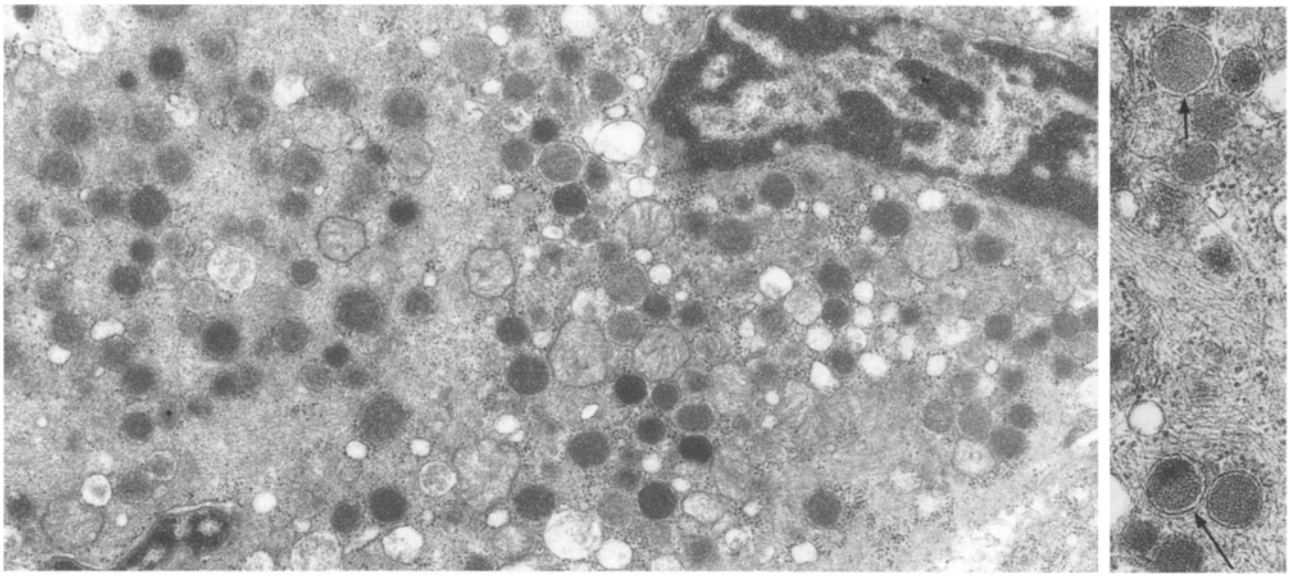

Fig. 3 Case 1. Ultrastructure of tumour cells, revealing heterogeneous granule population with abundance of round, dense and large forms and bundles of microfilaments (×21150). *Inset* Details of the granule structure with thick, interrupted perigranular membrane separated from the core by a distinct clear space (*arrows*). ×42650

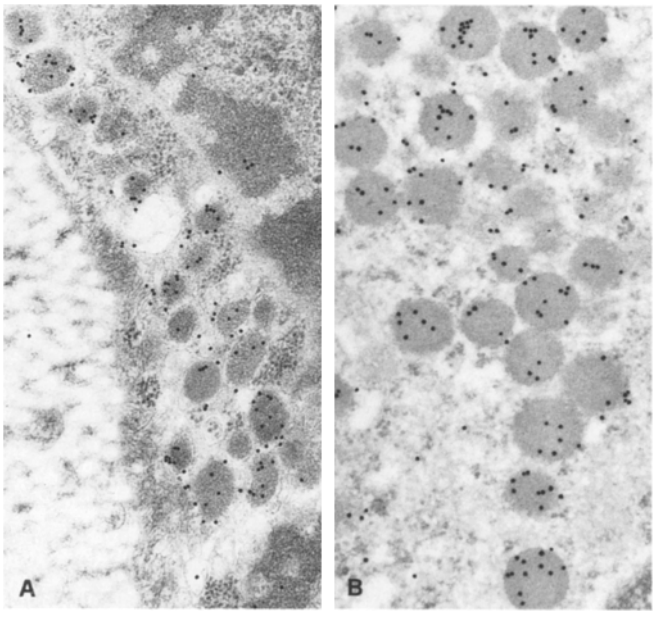

Fig. 4A, B Immunoelectron microscopic localization of PP (**A**, case 1) and of glicentin (**B**, case 2) in large dense granules of tumour cells. Protein A–gold technique, **A** ×32200, **B** ×45000

timated. In our patients the tumours fulfilled all four basic criteria proposed by Murphy et al. [20] for the differential diagnosis of adenoma: predominantly trabecular and acinar architecture, cytological uniformity, Grimelius argyrophilia, and ultrastructural demonstration of neurosecretory granules. In addition, absence of *zellballen* and of a prominent vascular component helped to exclude a paraganglioma [23]. Neither mixed histological endocrine and exocrine structures nor amphicrine cells containing mucous and endocrine granules were observed, supporting the pure endocrine nature of the tumours. The occurrence of microvilli bordering intercellular lumina does not indicate exocrine differentiation; it has been observed in typical, pure endocrine tumors [3, 7, 8].

Immunohistochemical study of the present cases revealed a positive reaction of tumour cells for CgA, and CgB and, to a lesser degree, for NSE, HISL-19 and 7B2. These substances are neuroendocrine proteins common to most endocrine cells regardless of the type of hormone produced [5]. Their demonstration, therefore, lends further support to the assumed endocrine nature of the middle ear tumours in our patients.

Immunohistochemistry also clearly documented the multihormonal production of middle ear well-differentiated endocrine tumours. Indeed, cells immunoreactive for PP-PYY, glucagon-glicentin and (at least in one case) serotonin were frequently found, whereas cells containing somatostatin and the alpha subunit of HCG were more occasional. In contrast, negative results were obtained for insulin and gastrin. These data are consistent with those of previous immunohistochemical investigations of such tumours [9, 14, 19]. When suitably investigated, PP immunoreactivity was found in all 11 previous cases, serotonin immunoreactivity in 9 of 12 cases and glucagon immunoreactivity in 6 of 11 cases. Moreover, serotonin was demonstrated by formalin-induced fluorescence in two additional cases [15, 20]. Taken together, our own and previous findings indicate that production of the constellation of serotonin, PP- and glucagon-related peptides is a regular feature of middle ear well-differentiated endocrine tumours. These findings may be used as differential diagnostic criteria to rule out a metastatic tumour, in addition to those proposed by Murphy et al. [20] (absence of clinical evidence of a primary tumor and absence of local destruction of bone).

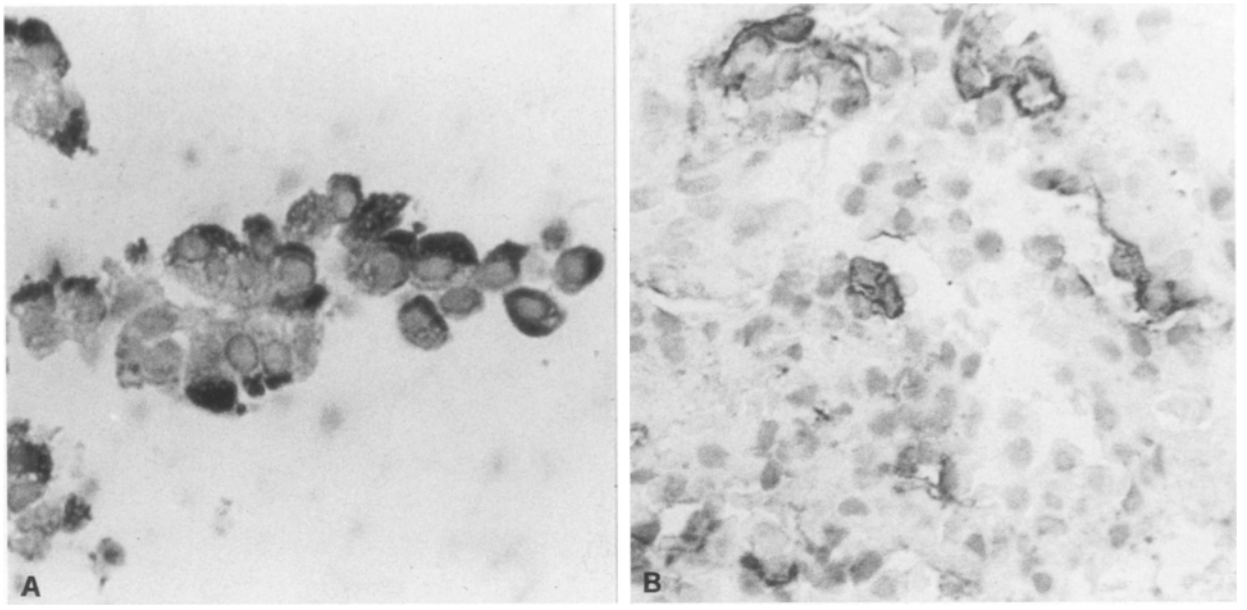

Fig. 5A, B Case 2. Immunoreactivity **A** for prostatic acid phosphatase in the cytoplasm of tumour cells and **B** for the colo-rectal mucin antigen CAR-5 in some lumina of gland-like spaces of the tumour. **A** ×630; **B** ×340

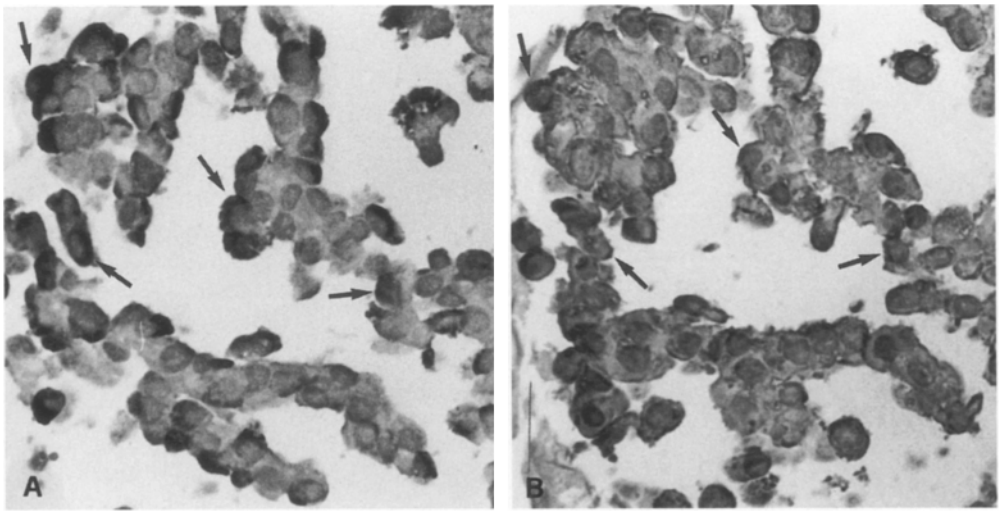

Fig. 6A, B Case 2. Immunostaining for **A** C-terminus glicentin and **B** N-terminus PP in serial sections, showing colocalization of the two immunoreactivites (*arrows*). ×400

Interestingly, this distinctive immunoreactive profile closely reflects that of hindgut (rectal) well-differentiated endocrine tumours. The cumultive analysis of five different series of these tumours comprising a total of 60 cases studied by immunohistochemistry [12] showed immunoreactivity for glucagon-related peptides in 33 cases (55%), for PP-related peptides in 37 (62%), for either one or both in 46 cases (77%), and for serotonin in 19 cases (32%). In addition, the coexpression of PP-PYY- and glucagon-glicentin-related immunoreactivities found in tumour cells from our patients corresponds to a pattern typical of intestinal L cells, the main constituents of rectal well-differentiated endocrine tumours [12]. The ultrastructural features and immunogold reactivities of tumour cells were also consistent with those of tumour L cells [11]. Additional evidence for a hindgut-like phenotype is provided by the widespread immunoreactivity of tumour cells for the prostatic acid phophatase, which is regarded as an excellent marker of the corresponding rectal tumours, possibly related to the common origin of rectum and prostate from the cloaca [25]. Moreover, from the clinical point of view rectal and middle ear well-differentiated endocrine tumours share a virtually universal silent endocrinological presentation and an excellent prognosis after radical extirpation [10, 19].

In this regard our observation of the expression of CAR-5, a mucin-like antigen expressed mainly in colorectal epithelium [21], by normal ciliated epithelial cells of the middle ear mucosa also indicates a common path-

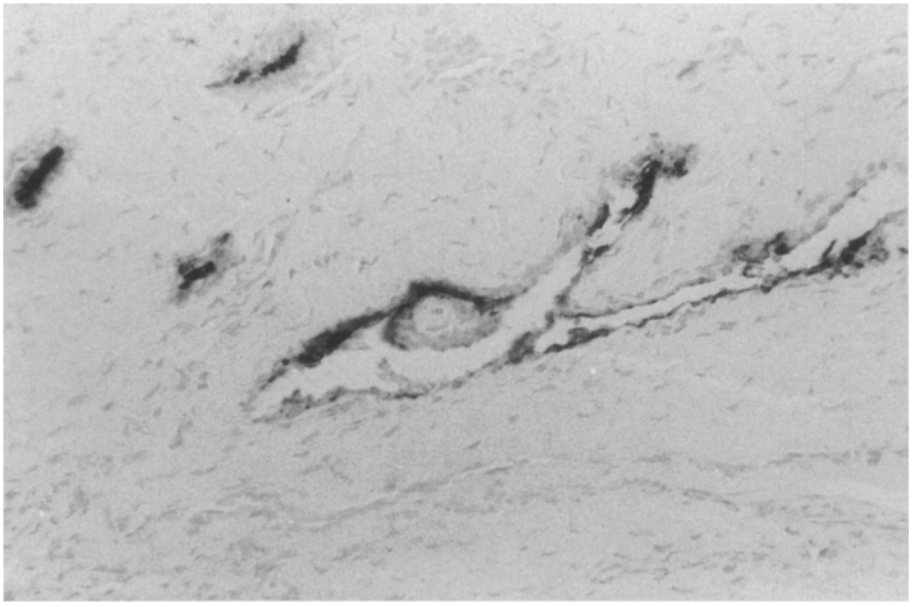

Fig. 7 Case 2. Immunostaining for CAR-5 in the apical border of ciliated cells of the normal mucosa of the middle ear. ×250

way of differentiation for hindgut- and foregut-derived epithelia also involving the mucin pool. Interestingly, CAR-5 is also expressed by nasal ciliated epithelium, which is strictly similar to middle ear epithelium and may give rise to intestinal type neoplasms, including colonic adenocarcinomas and composite carcinoid adenocarcinomas [1, 2]. Taken together, these findings may help to explain why in the process of tumorigenesis middle ear epithelial cells can assume hindgut phenotypes.

The origin of middle ear well-differentiated endocrine tumours remains a matter of speculation, as no endocrine cells have been documented so far in the ear [23]. The suggested hypotheses include derivation from totipotent stem cells capable of differentiating into both endocrine and non-endocrine cells or involvement of metaplastic changes [23]. In this regard our results do not provide new information. However, the finding of diffuse endocrine cell proliferation in the epithelium of the mucosa close to tumour cell clusters suggests that such proliferation is the precursor lesion from which middle ear tumours develop. A similar condition has been found in similar tumours of the stomach [6] and ileum [17] as well as in ampullary somatostatinomas [22].

In conclusion, our results indicate that the well-differentiated endocrine tumour of the middle ear is a specific type of neoplasm with distinctive cytological characteristics. These include simultaneous production of PP-related peptides, glucagon-related peptides and serotonin. These tumours share morphological features with similar tumours of the rectal mucosa and, like the latter, appear to be mainly composed of L cells coexpressing PP-related and glucagon-related immunoreactivities.

Although the majority of these middle ear tumours are endocrinologically silent, the postoperative disappearance of diarrhoea in one of our patients, already described in another case [16], suggests a possible relation of this disorder with active substance(s), possibly serotonin, produced by the tumour. In future cases circulating levels of relevant hormones should be determined before surgical removal of the tumour.

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Note added in proof Since this paper was accepted for publication we became aware of a detailed review discussing many issues addressed by the present paper (El-Naggar AK, Pflatz M, Ordonez NG, Batsakis JG (1994) Tumors of the middle ear and endolymphatic sac. Pathol Annual 29 (Part 2): 199–231). These authors were able to detect endocrine cells in a reportedly normal middle ear mucosa.