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Immunohistochemical demonstration of cytokeratins in endocrine cells of the human pituitary gland and in pituitary adenomas*

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Summary. Ten non-neoplastic pituitary glands and 22 pituitary adenomas producing different hormones were studied by immunofluorescence microscopy as well as peroxidase-antiperoxidase and biotin-avidin techniques on frozen sections and formalin-fixed, paraffin-embedded material using antibodies to cytokeratin, vimentin, GFAP, neurofilament protein and different pituitary hormones. The endocrine cells in non-neoplastic pituitary glands as well as in most pituitary adenomas were cytokeratin-positive. The cytoplasmic cytokeratin distribution patterns of non-neoplastic and tumor cells were similar and typical of the type of hormone produced: GH-producing normal cells showed a paranuclear condensation of cytokeratin-reactive intermediate filaments; this accumulation was even further accentuated in GH-producing adenomas resulting in fibrous bodies (Kovacs and Horvath 1978) decorated by cytokeratin antibodies. Prolactin-producing cells showed a less intense cytoplasmic cytokeratin-specific staining with focal paranuclear accentuation in non-neoplastic as well as in neoplastic glands. ACTH-producing cells in normal pituitary glands as well as in adenomas exhibited a strong and more uniform cytoplasmic cytokeratin staining. The cytokeratin reactivity in glycoprotein hormone-producing cells of non-neoplastic tissue and adenomas was weak. Vimentin and GFAP reactivity was confined to agranular folliculo-stellate cells. The specific and different distribution patterns of cytokeratins in pituitary cells can, therefore, provide an (indirect) indication to the production of a specific hormone if immunocytochemistry fails to demonstrate hormone production.

Key words: Immunohistochemistry – Pituitary gland – Pituitary adenomas – Cytokeratins – Intermediate filaments

* Dedicated to Prof. Dr. J.H. Holzner on the occasion of his birthday

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Cytokeratins (CK) are constituents of the intermediate filament cytoskeleton of epithelial cells. In their chemical composition and antigenicity they are heterogeneous and consist of several polypeptides with different molecular weights and isoelectric points (see Moll et al. 1982; for further information and references). In secretory epithelial cells, such as hepatocytes and intestinal epithelial cells, the CK polypeptide composition is rather simple, whereas in stratified epithelium and epidermis it is more complex and varies depending upon the degree of maturation. Reports on presence and distribution of CK in cells of normal human pituitary glands are not found the literature. Only Nagle et al. (1983) included two pituitary adenomas in their studies on the occurrence of CK in human neoplasms.

The aim of the present investigation was to study presence and distribution of cytokeratin filaments in cells of normal human pituitary glands and pituitary adenomas using CK antibodies with broad range of immunological reactivity, such as antibodies to liver cytokeratin D (for further specifications of these antibodies see Franke et al. 1981; Denk et al. 1981). In addition, CK content was related to hormonal production of the endocrine cells (EC), as determined by immunohistochemistry. Moreover, human pituitary glands and pituitary adenomas were also tested with respect to their immunoreactivity with antibodies to other intermediate filament proteins, such as vimentin, glial fibrillary acidic protein (GFAP) and neurofilament protein (70 kD) (see Osborn and Weber 1983, for review and further information).

Material and methods

Material. The material studied included 10 non-neoplastic pituitary glands, either obtained by surgery (5) or at autopsy (5). Two of these specimens, one from a male and one from a female patient, were snap-frozen immediately after removal in isopentane at the temperature of liquid nitrogen and were stored at −80° C. Three non-neoplastic pituitary glands obtained at autopsy and non-neoplastic pituitary gland tissue from the periphery of surgically removed adenomas (5 patients) were fixed immediately after surgery in 10% phosphate-buffered formaldehyde solution and embedded in paraffin by conventional techniques. Twenty two adenomas were studied: 17 were obtained by surgery and 5 at autopsy. These specimens were paraffin-embedded after formalin fixation. Material from 5 surgically removed adenomas was also fixed in 3% glutaraldehyde for 4 h, postfixed in 2% osmium tetroxide and embedded in Epon 812 according to standard techniques. The adenomas were classified by immunohistochemistry according to their hormone production: 7 adenomas were reactive with prolactin-, 7 with human chorio-gonadotrophin (HCG)- and 3 with ACTH-antibodies; in all cases the patients showed the corresponding clinical symptomatology prior to surgery. In one adenoma tumour follicle stimulating hormone (β-FSH) was found, in another luteinizing hormone (β-LH) and in a further one the α-subunit of glycoprotein hormones were demonstrated. Two adenomas did not show hormone production as revealed by immunocytochemistry, nor did the patients display symptoms of increased hormonal activity.

Methodology

Conventional stainings. Four µm thick sections of formaldehyde-fixed, paraffin-embedded material were stained with haematoxylin-eosin, PAS- and Masson/trichrome-stain.

Table 1

Antiserum to (species	Dilution[a]	Source/References
(gp) mouse liver cytokeratin D (CK/D)	1:3,000 1:100[b]	Denk et al. (1981)
(ra) human vimentin	1:300 1:15[b]	Denk et al. (1983)
(ra) glial fibrillary acidic protein (GFAP)	undiluted	Dako, Kit (Denmark)
(m) human neurofilament protein (70 kD), monoclonal	1:10 1:5[b]	Sanbio (Holland)
(ra) human prolactin	1:3,000	NIAMDD (USA)
(m) human prolactin, monoclonal	1:15,000	Hybritech (USA)
(ra) human GH	1:640	Milab (Sweden)
(m) human GH, monoclonal	1:10,000	Hybritech (USA)
(ra) porcine ACTH	1:320	Milab (Sweden)
(ra) porcine ACTH	1:500	Immuno Nuclear Corporation (USA)
(ra) human β-FSH	1:1,000	NIAMDD (USA)
(ra) human β-LH	1:1,000	NIAMDD (USA)
(ra) human β-TSH	1:4,000	NIAMDD (USA)
(ra) alpha HCG	1:7,500	NIAMDD (USA)

Abbreviations. gp = guinea pig; ra = rabbit; m = mouse; GH = growth hormone; TSH = thyroid stimulating hormone; NIAMDD (National Institute of Arthritis, Metabolism and Digestive Diseases, USA) (other abbreviations in the text)

[a] Peroxidase-antiperoxidase reaction except [b]

[b] Indirect immunofluorescence microscopy

Immunohistochemistry

For immunofluorescence microscopy frozen, unfixed tissues were cut at 5 µm. The air-dried sections were fixed in cold acetone (−20° C) for 10 min, dried and incubated with the primary antibodies for 20 min at room temperature. The sections were rinsed in phosphate-buffered saline (PBS; pH 7.4) and subsequently incubated with the secondary antibodies, conjugated with FITC or TRITC, for 30 min. The sections were then again rinsed with PBS, dehydrated for 5 min in absolute ethanol, dried, embedded in Moviol and viewed with a Zeiss fluorescence microscope (Fotomikroskop III). For photographic documentation a Kodak Tri-X-Pan black-and-white film was used.

For immunoperoxidase techniques 5 µm thick sections were cut from paraffin-embedded formalin-fixed material and, after removal of paraffin with xylene and rehydration, were subjected to the peroxidase-antiperoxidase (PAP)- (Sternberger 1979) or the biotin-avidin-method (ABC; Vector, Burlingham USA). For amplification of the color reaction cobalt chloride (1%) and nickel ammonium sulfate (1%) were added to the (3,3-diaminobenzidine) incubation medium according to the procedures of Adams (1981). For the demonstration of cytokeratin and vimentin the sections were pretreated with protease type VII (Serva, Heidelberg, FRG) in a concentration of 0.6% for 10 min at 37° C (Denk et al. 1977). For the simultaneous demonstration of two antigens in tissue sections the first peroxidase reaction was developed with 3,3-diaminobenzidine with or without addition of heavy metals, and in the second reaction 4-chloro-1-naphthol or 3-amino-9-ethylcarbazole were used as substrates. Antibodies used are listed in Table 1. Pre-immune sera and non-immune sera from other species applied instead of the specific immune sera served as controls. The sections were viewed with a Leitz Orthoplan photomicroscope and photographed using a Kodak Tri-X-Pan black-and-white film.

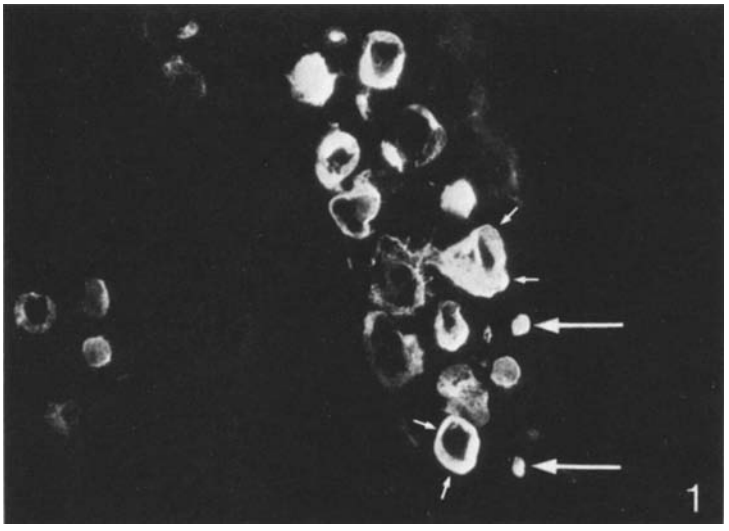

Fig. 1. Normal human pituitary gland. Different cytoplasmic distribution patterns of cytokeratin-reactivity in endocrine cells: in some cells a patchy (only the fluorescent cytoplasmic spots are visible, *long arrows*) and in other cells a diffuse (*short arrows*) cytoplasmic staining is present (see also Fig. 3). Antibodies to cytokeratin, indirect immunofluorescence microscopy, × 640

Results

1. Normal human pituitary gland. Antibodies to CK/D reacted with EC in frozen sections of normal human pituitary glands. The pattern of cytoplasmic keratin-specific staining was, however, variable and several types were observed: (i) large cells with a broad rim of cytoplasm and evenly distributed intense filamentous CK staining, (ii) cells with a narrow rim of specifically stained cytoplasm and (iii) cells with focal paranuclear staining (Fig. 1 and 3). The intensity of immunostaining was somewhat variable. Although formalin-fixed paraffin sections were less reactive, patterns comparable to those obtained by immunofluorescence were observed. However,

Fig. 2. Edge of a (GH-producing) pituitary adenoma (*top*); ACTH-reactive cells in non-neoplastic tissue (*bottom*). Antibodies to ACTH, PAP/differential interference contrast optics, × 420

Fig. 3. Same case and identical area as in Fig. 2. ACTH-reactive cells in non-neoplastic tissue reveal strong diffuse reactivity with cytokeratin antibodies (*short arrows*). Cells with focal patchy paranuclear cytokeratin staining are interspersed (long arrows, see also Fig. 1). (GH-producing) adenoma cells show only patchy paranuclear immunostaining (*top*). Antibodies to cytokeratin, PAP/differential interference contrast optics, × 420

Fig. 4. Intense and diffuse cytoplasmic staining in cells of an ACTH-producing adenoma. Antibodies to cytokeratin, PAP/differential interference contrast optics, × 360

Fig. 5. "Signet-ring"-like cytokeratin-specific immunoreactivity in a prolactinoma. Antibodies to cytokeratin, PAP, × 320

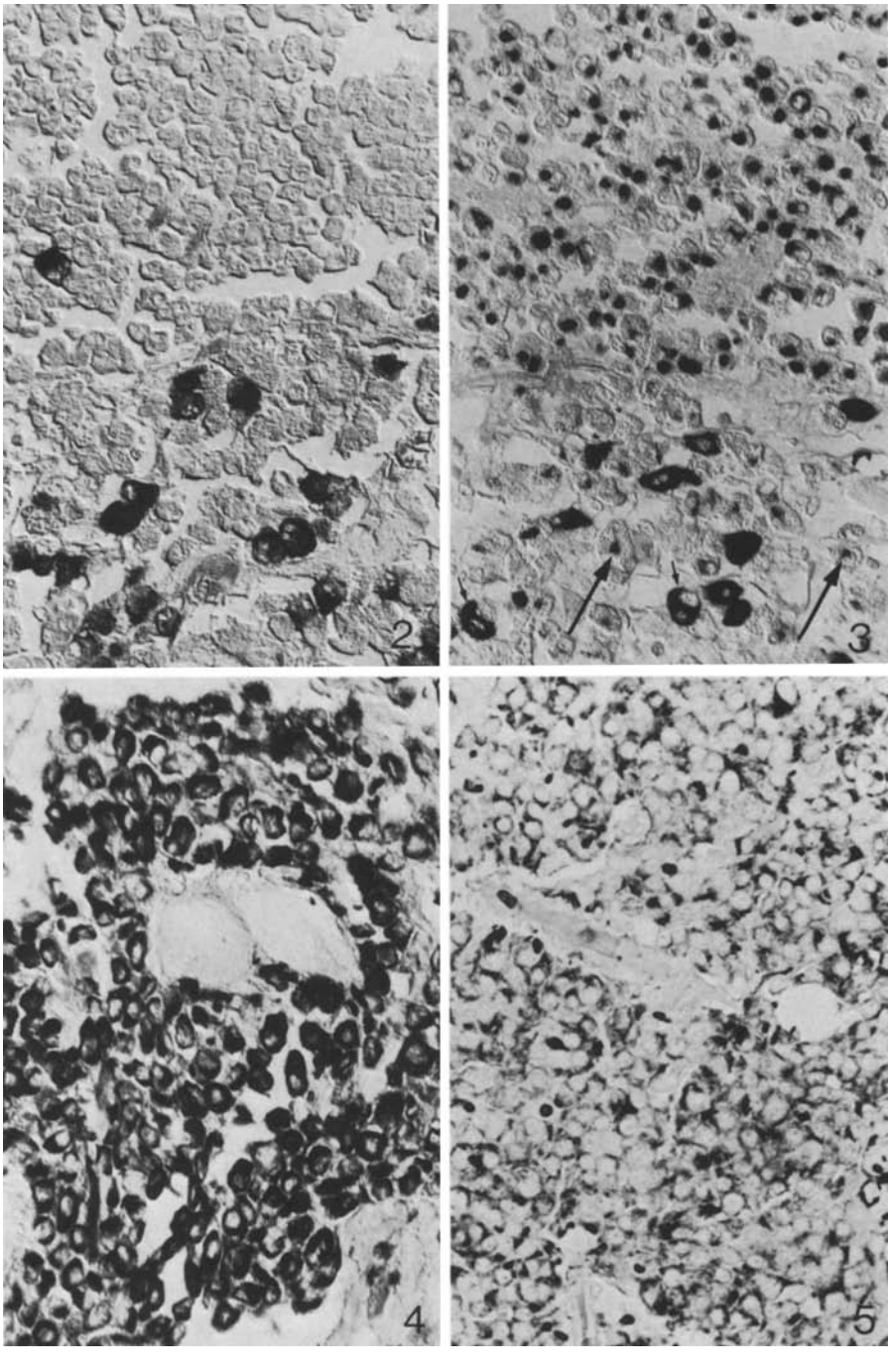
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Table 2. Pattern of cytokeratin immunoreactivity in normal pituitary cells

Cells	Pattern of cytoplasmic staining
GH-producing	Patchy (strong)
Prolactin-producing	Diffuse-patchy (weak)
ACTH-producing	Diffuse (strong)
β-FSH/TSH/LH-producing	Diffuse (weak) or negative
Folliculo stellate cells	Negative

some EC in fixed tissue failed to bind CK antibodies to a significant extent, possibly due to CK antigenicity below the sensitivity threshold of the immune reaction. Simultaneous demonstration of CK and pituitary hormones revealed that cells showing strong cytoplasmic CK-specific staining contained ACTH whereas in GH-producing cells a less distinct focal paranuclear CK reactivity, and in prolactin-producing cells a less intense cytoplasmic CK reactivity, sometimes in paranuclear arrangement, was revealed (Fig. 2 and 3). In Masson/trichrome stained sections the whole cytoplasm of ACTH-producing cells was stained red, whereas in GH-producing ones paranuclear red clusters were revealed. β-FSH-, TSH- and LH-producing cells were either negative or only weakly positive with respect to CK, sometimes in a perinuclear fashion (Table 2). Folliculostellate cells (FSC) reacted with GFAP antibodies but were negative, in double immunohistochemical staining, with respect to all pituitary hormones studied. Some FSC reacted with vimentin antibodies (a detailed study on the immunoreactivity of FSC is presented elsewhere). Neurofilament protein-related immunoreactivity was only demonstrable in a few neurons in frozen sections. Neurofilament-positive nerve cells were absent in the anterior lobe of the pituitary gland as well as in adenomas.

2. Adenomas. The CK reactivity of adenoma cells essentially resembled that of cells in non-neoplastic pituitary glands, particularly with regard to its relationship to hormone content. Adenoma cells producing GH showed paranuclear patches, intensively stained with CK antibodies (Fig. 3), apparently equivalent to the paranuclear red globules in Masson/trichrome stained sections. In electronmicroscopy, these paranuclear bodies resembled felt-like accumulations of intermediate-sized filaments with diameters between 8–10 nm, sometimes including endocrine granules and other cell organelles (Fig. 6). The diameters of CK-positive patches in cells of GH-producing adenomas were larger and more intensively stained than in non-neoplastic GH-producing cells. In adenomas producing prolactin the EC showed a more diffuse and less intense immunoreaction with CK antibodies and in only a few cells it was focally accentuated. The intensity of staining in these cells was less than in those producing GH (Fig. 5). Two of the 7 prolactinomas were CK – negative although positive immunoreactivity was found in non-neoplastic cells surrounding the adenoma. According to their ultrastructure, these tumours belonged to the "sparsely granulated" type

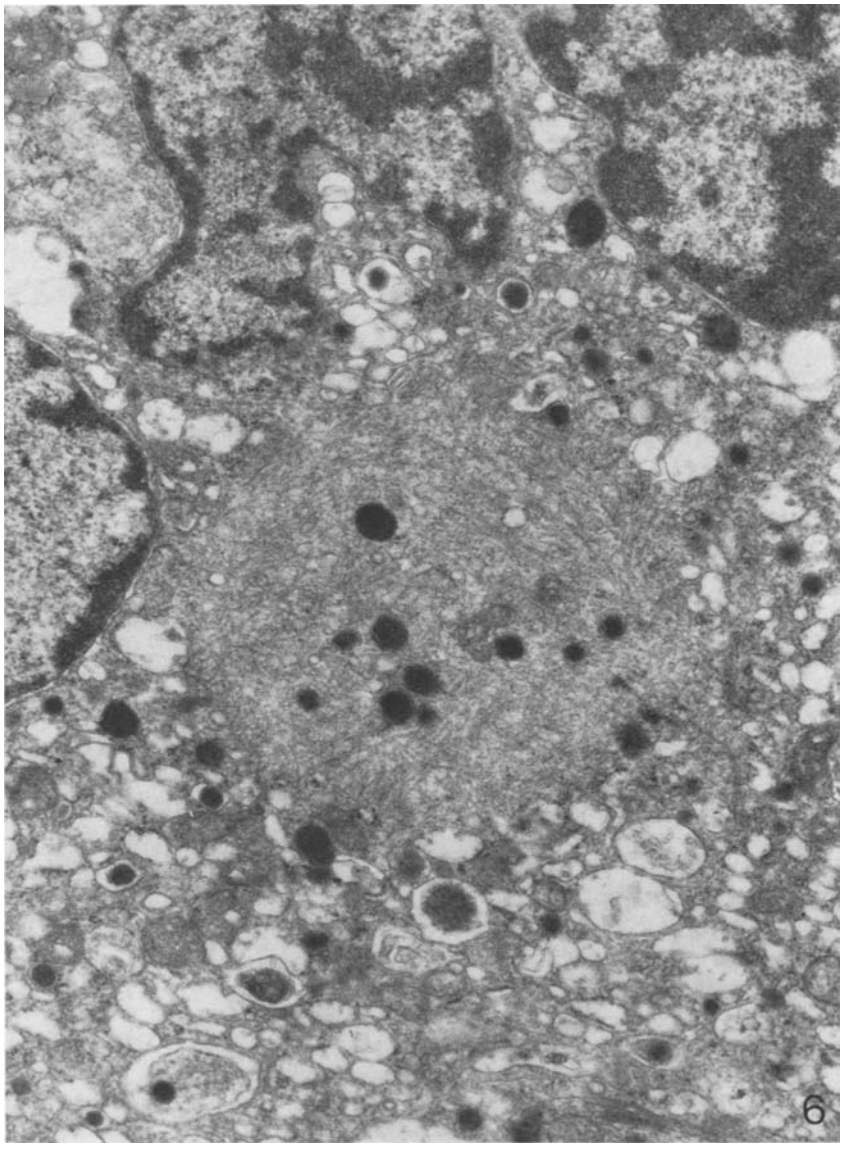

Fig. 6. Paranuclear concentration of intermediate-sized filaments ("fibrous body") in a cell of a GH-producing adenoma. Note inclusion of endocrine granules and other cell organelles within the filament meshwork. × 18,600

(Kovacs et al. 1981) and their cells contained only few intermediate filaments located in the perinuclear area; in some cells small paranuclear filament accumulation were seen. The ACTH-producing cells of adenomas strongly bound CK antibodies resulting in an intense cytoplasmic staining (Fig. 4). In two hormonally inactive pituitary adenomas less intense and evenly distributed CK-related immunoreactivity was seen without focal accentuation

Table 3. Cytokeratin immunoreactivity in pituitary adenomas ($n=22$)

Cells	Pos/n	Pattern of cytoplasmic staining
GH-producing	7/7	Patchy (strong)
Prolactin-producing	5/7	Diffuse-patchy (weak)
ACTH-producing	3/3	Diffuse (strong)
β-FSH-producing	0/1	Negative
β-LH-producing	0/1	Negative
α-subunit only-producing	0/1	Negative
Inactive	2/2	Diffuse (weak)

as spherical bodies. The β-FSH, β-LH and alpha-subunit-producing adenomas were CK negative (Table 3).

In the adenomas, in contrast to normal pituitary glands, GFAP- or vimentin-positive FSC were absent. Since the antibodies to neurofilament proteins did not reveal reproducible results in our hands on formalin-fixed, paraffin-embedded tissue the negative results with these antibodies in adenomas can not be regarded as definitive and the presence of axons can, therefore, not be excluded.

Discussion

The presence of cytokeratins, which are markers of epithelial differentiation in EC of the pituitary gland is in agreement with their epithelial nature and their development from the Rathkés pouch (for review and further references see Moll et al. 1982; Osborn and Weber 1983). The discrepancies between our findings and the results obtained by Nagle et al. (1983), who studied two pituitary adenomas and were unable to find keratins, can be explained by methodological differences. Our antibodies show a broad range of immunological reactivity with CK present in diverse epithelial cell types (Denk et al. 1981) whereas Nagle et al. (1982) used, in indirect immunofluorescence microscopy, antibodies to keratins isolated from human callus, and obtained negative results not only with respect to pituitary adenomas but also to some other endocrine tumors, like carcinoids, known to be keratin-positive (Höfler and Denk 1984). In principle, immunofluorescence microscopy on frozen sections is more sensitive than the peroxidase-antiperoxidase-technique on formalin-fixed, paraffin-embedded material. The lack of CK reactivity in five of the 22 formalin-fixed pituitary adenomas studied by us does therefore not exclude the presence of intermediate filaments of the CK type.

There was a close similarity in CK content and arrangement between normal and neoplastic pituitary cells with identical hormone production. It is noteworthy that cells producing different hormones differ in their CK-specific immunostaining patterns. The focal paranuclear staining, in agreement with focal accumulations of intermediate-sized filaments, is most pronounced in GH-producing tumor cells. This is in agreement with results presented by Horvath and Kovacs (1978) who, by electronmicroscopy,

cell shape, cellular mobility and stability, organelle interaction and secretory found "fibrous bodies" consisting of focal accumulations of filaments with intermediate dimensions between 8 and 17 nm ("type II microfilaments") exclusively in GH-producing adenomas. These filamentous inclusions ("fibrous bodies") were first described by Racadot et al. (1964). According to our immunohistochemical studies the accumulated "microfilaments" seem to be intermediate filaments of the CK type. The "type II" microfilaments found by Horvath and Kovacs (1978) in FSC are most probably identical with intermediate filaments of the vimentin and/or glial type.

Paranuclear accumulations of normal or abnormal intermediate-sized filaments have been found in diverse experimental and pathological conditions, including treatment of tissue culture cells with tubulin antagonists, in hepatoma cells in culture, during neuronal degeneration (e.g. Alzheimer's disease), but also in familial muscular disorders and during alcohol-intoxication leading to Mallory bodies (see Denk and Krepler 1982 for further references). Perinuclear accumulations of CK have been described in mitotic tissue culture cells and also after disorganization of the intermediate filament cytoskeleton following microinjection of CK or tubulin antibodies (Klymkowsky et al. 1983; Blose et al. 1984) or by microtubule and microfilament inhibitors (Knapp et al. 1983). Similar focal accumulations of intermediate-sized filaments were demonstrated by electronmicroscopy in bronchial and duodenal carcinoids as well as in neuroendocrine skin carcinomas (Berger et al. 1984; Höfler and Denk 1984; Höfler et al. 1984). The functional significance of these focal accumulations of intermediate-sized filaments, particularly in neoplastic conditions, as well as the underlying mechanisms are as yet unknown.

It is an interesting facet of our results that cells producing and secreting hormones differ in their intermediate filament cytoskeleton architecture. Whether there are any functional relationships awaits elucidation. The biological significance of an intact intermediate-sized filament cytoskeleton is still unclear, but it may play a role, for example, in the maintenance of processes (Lazarides 1980). In hormone-secreting cells an intact intermediate filament cytoskeleton may be involved in transport and exocytosis of endocrine granules. This speculation is supported by the intimate relationship between intermediate filaments and the Golgi apparatus (Horvath and Kovacs 1978), as well as by the close spatial association between microtubules, microfilaments and the perinuclear fibrous bodies, as described by Berger et al. (1984).

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