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Ultrastructure of pigment in adrenocortical pigmented adenomas of Cushing's syndrome and in non-functioning pigmented nodules with respect to tissue steroid analyses*

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Summary. Ultrastructural and morphometrical analysis of brown pigment in pigmented (black) and non-pigmented adrenocortical adenomas of Cushing's syndrome and non-functioning pigmented adrenocortical nodules was performed in reference to tissue concentrations and in vitro production of steroids by the adenoma tissue. Pigment in pigmented adenomas was of membrane-bound lysosomal nature, while that of pigmented nodules contained membrane-unbound droplets of lipoid character. The morphometrical study showed little difference among individual adenomas. There was no difference between pigmented and non-pigmented adenomas in the amount of production and tissue concentrations of steroids. The steroid concentrations in a pigmented nodule were lower than those in an adenoma of Cushing's syndrome, but not significantly. Discussion is focused on the difference of pigment of lysosomal nature and of lipoid peroxidation.

Key words: Pigmented adenoma – Cushing's syndrome – Lipofuscin – Ultrastructure – Steroid analysis

Introduction

Adrenocortical pigmented adenomas or nodules are divided into two groups according to their functional state; adenomas with endocrine manifestations, usually of Cushing's syndrome (Symington 1969; Mackay 1969; Mizutani and Igarashi 1974; Bahu et al. 1974; Visser et al. 1974; Uras et al. 1978; Takagi et al. 1980; Shigematsu et al. 1982; O'Leary et al. 1982; Neville

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Table 1. Adrenocortical adenoma of Cushing's syndrome obtained at surgery

	No.	Cases	Age	Sex	Tumor		Examination	
					Size (cm)	Weight (g)	Morph.	Steroid
Pigmented	1	T.N.	14	M	4.8 × 3.3 × 2.5	8.0	−	+
	2	S.N.	17	F	2.6 × 2.2 × 2.2	8.5	+	+
	3	M.T.	29	F	1.2 × 1.1 × 1.0		+	−
	4	Y.T.	31	M	2.5 × 2.4 × 1.1	5.0	+	+
Non-pigmented	5	S.I.	22	F	5.0 × 3.0 × 3.0	20.6	+	−
	6	K.S.	25	F	5.0 × 1.9 × 1.4	8.0	+	−
	7	S.U.	28	F	3.4 × 3.2 × 2.3	17.5	+	+
	8	T.M.	28	F	3.0 × 3.2 × 2.3	12.0	+	+
	9	S.O.	30	F	3.0 × 2.4 × 2.1	9.0	−	+
	10	N.S.	33	F	3.0 × 2.7 × 2.2	15.5	+	+
	11	A.S.	35	F	2.4 × 2.1 × 2.0	10.0	+	+
	12	S.S.	36	F	4.4 × 3.6 × 2.8	30.0	+	+
	13	S.O.	42	F	3.2 × 2.8 × 2.3	12.1	−	+
	14	M.O.	44	F	3.0 × 2.4 × 2.1	15.5	+	+
	15	M.Y.	46	M	4.8 × 3.3 × 2.5	13.9	−	+
	16	I.T.	47	M	3.0 × 2.8 × 2.3	12.1	−	+
	17	T.S.	49	F	2.0 × 1.5 × 1.2	8.2	+	+

Morph. = Morphological examination, Steroid = Steroid analyses
+ = examined, − = not examined

Table 2. Adrenocortical pigmented nodules obtained at autopsy

No.	Cases	Age	Sex	Major diseases	Largest diameter
18	T.O.	54	F	Acute meningitis	4 mm
19	K.T.	71	F	Gastric carcinoma	5
20	U.T.	72	M	Cerebral infarction	7
21	R.W.	79	M	Carcinoma of colon	7

and O'Hare 1982) and adenomas or nodules without endocrine manifestations (Lucksch 1912; Tuczek 1914; Schmidt 1925; Baker 1938; Lüders 1953; Macadom 1971; Robinson and Rywlin 1972; Robinson et al. 1972; Garret and Ames 1973).

Histochemical examinations of pigment in pigmented adenomas revealed lipofuscin (Lucksch 1912; Tuczek 1914; Schmidt 1925; Baker 1938; Lüders 1953; Symington 1969; Macadom 1971; Robinson and Rywlin 1972; Robinson et al. 1972; Garret and Ames 1973; Mizutani and Igarashi 1974; Bahu et al. 1974; Visser et al. 1974; Uras et al. 1978; O'Leary et al. 1982; Neville and O'Hare 1982). No differences between the groups with and without endocrine manifestations were described.

Ultrastructural differences between pigmented adenoma cells of inactive cases and of Cushing's syndrome have been documented since Mackay 1969 (Robinson et al. 1972; Garret and Ames 1973; Mizutani and Igarashi 1974;

Table 3. Results of stainings for adrenocortical pigment

Staining	Cells in the reticularis	Adrenal adenoma		Pigmented nodule
		Pigmented	Non-pigmented	
Eosinophilia	+	+	+	+
Basophilia	−	−	−	−
Acid-fast (long Ziehl-Nelsen)	+	±	±	++
Sudan black	+	+	+	+
PAS	+	±	±	++
Schmorl	+	+	+	+
Chrome hematoxylin	+	±	±	++
Nile blue	+	+		
Alternative nile blue	−	−	−	−
Luxol fast blue	+	±	±	++
Fontanta-Masson	+	±	±	++
Ultraviolet Fluorescence	orange	orange	orange	orange

Table 4. Tissue concentrations of steroid

Steroids	Normal control	Non-pigmented adenoma (Case 15)	Pigmented nodule (Case 21)
Pregnenolone (ng/g tissue)	650–1710	1362	203.0
17-hydroxy-pregnenolone (ng/g tissue)	620–1850	1150	121.2
Progesterone (ng/g tissue)	920–1600	876	60.7
17-hydroxy-progesterone (ng/g tissue)	860–1650	513	79.6
DHEA (ng/g tissue)	720–1200	8.1	21.5
Aldosterone (ng/g tissue)	350– 700	9.2	0.4
Cortisol (μg/g tissue)	8.1–18.6	12.4	18.6

Bahu et al. 1974; Takagi et al. 1980; Shigematsu et al. 1982; Neville and O'Hare 1982). However, the association of pigment granules with the endocrine activity of adrenocortical cells has not been investigated previously. The present paper will report on pigment granules studied by means of light and electron microscopy and in vitro steroid analyses. The ultrastructural findings of pigment granules in pigmented nodules are analyzed with regard to tissue concentrations of steroid hormones.

Materials and methods

Light microscopy. Seventeen adrenocortical adenomas including 4 pigmented ones were surgically removed from patients with Cushing's syndrome (Table 1), and 4 non-functioning pigmented nodules were obtained at autopsy (Table 2). The material for histologic observations was fixed in 10% buffered formalin. Paraffin sections 2.5 micron thick were stained with hematoxylin and eosin. For histochemical observations of the pigment, the staining procedures listed in Table 3 were applied.

Electron microscopy: Three pigmented and 9 non-pigmented adenomas with Cushing's syndrome and 1 pigmented nodule were obtained. Normal control was provided by adrenalec-

Fig. 1. Cut surface of a pigmented adenoma, black in colour with sharp demarcation from the adjacent cortex

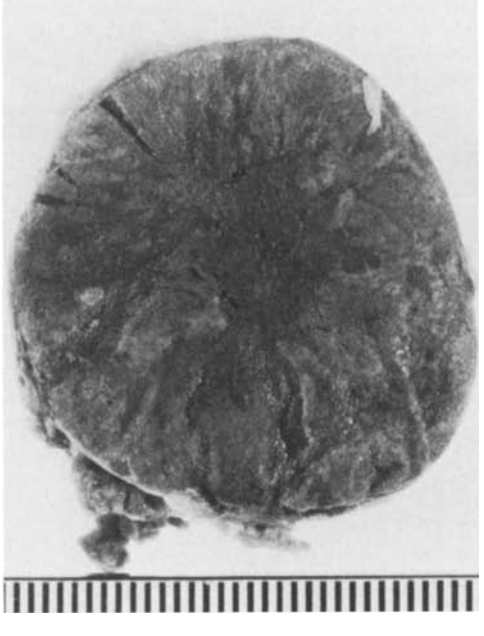


Fig. 2. Cut surface of a non-pigmented adenoma composed of combined yellow and brown lesions

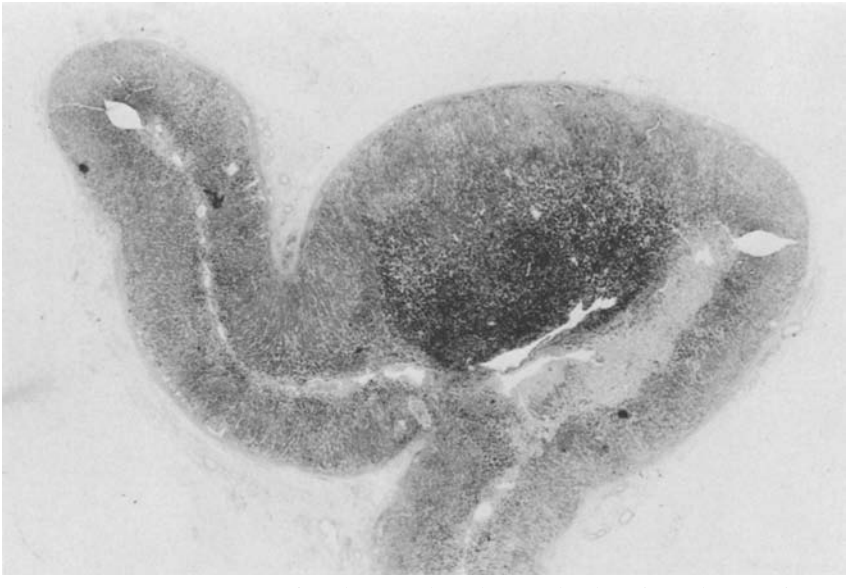


Fig. 3. Transparent picture of a slide of the adrenal gland with a pigmented nodule. The nodule involves the corticomedullary junction and is poorly demarcated from the adjacent cortex

tomized tissue obtained from 2 patients with advanced breast cancer, aged 47 and 53 years respectively. Tissue specimens cut in small pieces were immediately fixed in a mixture of 5% glutaraldehyde and 4% paraformaldehyde buffered at pH 7.4. After postfixation in osmium tetroxide, the specimens were dehydrated and embedded in Epon. Semithin sections of 1 μm were stained with toluidine blue for light microscopy. Thin sections of 60–80 nm were stained with uranyl acetate and lead citrate and examined with a JEM 100 B electron microscope.

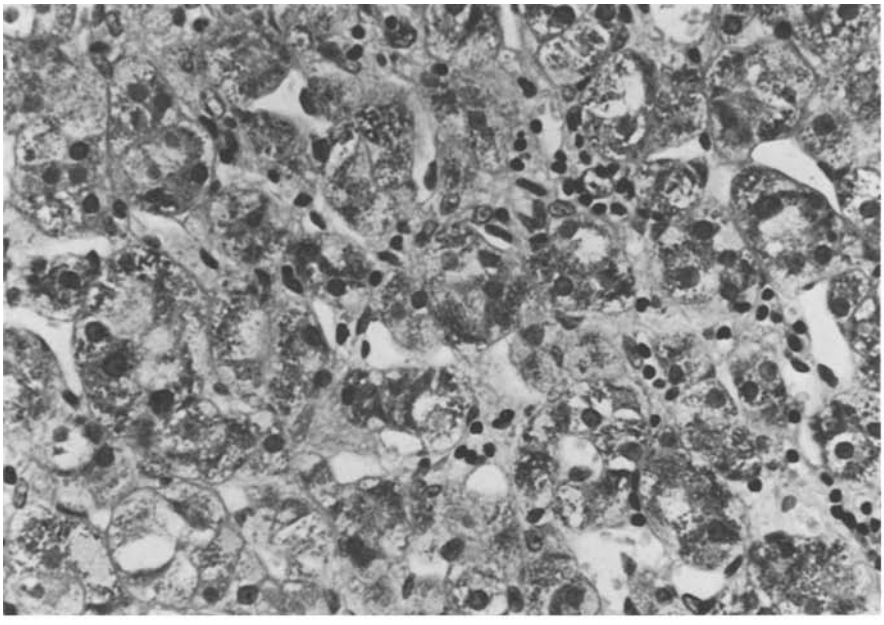
For morphometrical analysis of pigment granules in pigmented and non-pigmented adenomas with Cushing's syndrome and non-functioning pigmented nodules, respective 10 electron micrographs at a final magnification of ×8,000 were taken at random from five blocks in each case. Score of each case was obtained by analyzing 50 photographs using a Kontron-made image analyzer Videoplan.

There were two kinds of pigment granules; 1) membrane-bound (Fig. 10) and 2) membrane-unbound manifesting a conglomerate (Fig. 10). Membrane-unbound pigment granules were calculated as a conglomerate. The number of pigment granules calculated was more than 2,000.

For morphometrical analysis, the following two parameters were adopted:
1) volume density of pigment granules within cytoplasm, and
2) mean area of each pigment granule.

Statistical treatment of results:
Students' *t*-test was used for the statistical evaluation. The difference between two mean values was considered to be significant if the probability of error (p) was found to be less than 0.05.

In vitro production of steroids. Tissue slices of 3 pigmented and 11 non-pigmented adenomas and of the normal adrenals removed from 6 patients with advanced breast cancer, aged 46–52 years, were incubated in Krebs-Ringer bicarbonate buffer (KRB) (pH 7.4) under a gas mixture of O_2 and CO_2 (95:5) at 37° C for 30 minutes. Synthetic $^{1\text{-}24}$ACTH (10^{-8}–10^{-7} M) was added to the incubation buffer to study its effect on steroid synthesis. After incubation, the buffer was extracted with dichloromethane (CH_2Cl_2). Cortisol (F), 11-deoxycorticosterone



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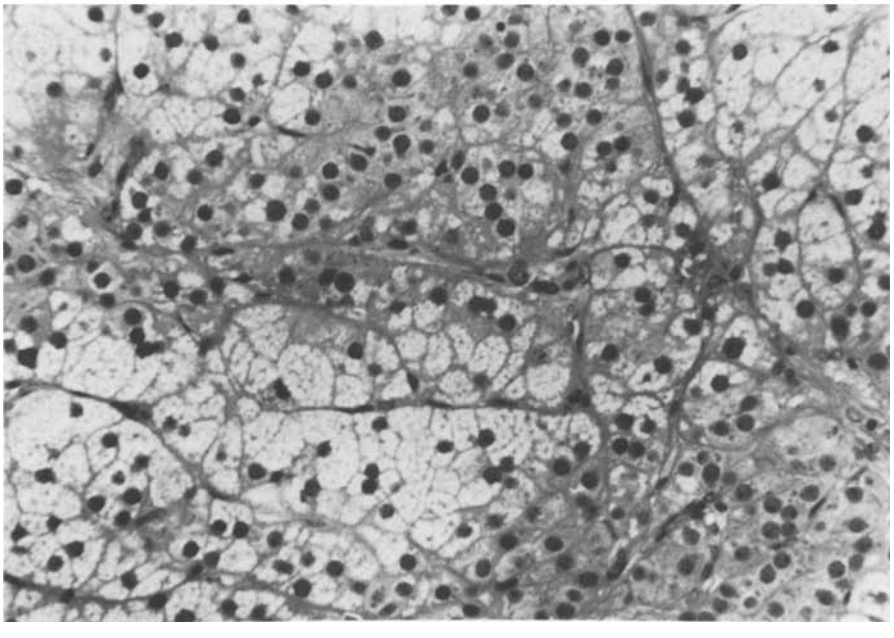

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Fig. 4. Cells of a pigmented adenoma of Cushing's syndrome. Note polyhedral cells with numerous intraplasmic fine pigment granules. PAS stain ×400

Fig. 5. Non-pigmented adenoma of Cushing's syndrome composed of both clear and compact cells. The latter contain less pigment granules than those of pigmented adenoma. Hematoxylin and eosin stain, ×250

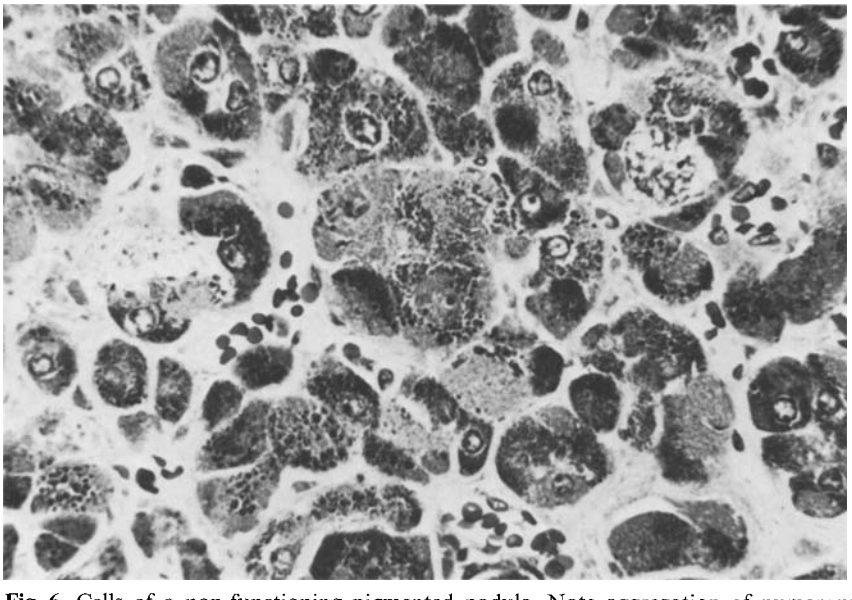


Fig. 6. Cells of a non-functioning pigmented nodule. Note aggregation of numerous coarse pigment granules. Luxol fast blue stain, × 100

(DOC) and dehydroepiandrosterone (DHEA) were purified by three types of Sephadex LH-20 column chromatography. The amount of steroids was measured by radioimmunoassay (RIA).

Tissue steroid concentrations. Repressentative parts of a non-pigmented adenoma in case 15 and of a pigmented nodule in case 21 wer e homogenized in 10 times volume of KRB and extracted with 15 times volume of CH_2Cl_2. The extract was purified by 3 types of Sephadex LH-20 column chromatography and analyzed for steroids by RIA. For the normal control, non-nodular adrenocortical tissue from cases 20 and 21 was examined by the same method (Table 4).

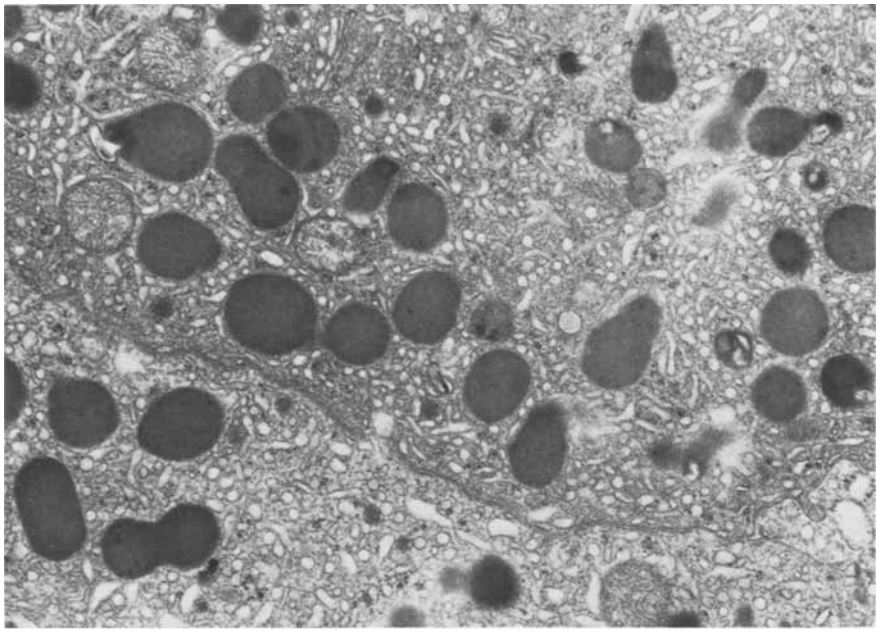
Results

Macroscopic findings. Pigmented adenomas were predominantly black or black-green in color on the cut surface (Fig. 1).

Non-pigmented adenomas were composed mainly of combined yellow and dark-brown lesions (Fig. 2). Black pigmented nodules were located at the corticomedullary junction, not encapsulated, and ill-defined from the adjacent cortex (Fig. 3).

Histologic findings. Pigmented adenomas with Cushing's syndrome were predominantly composed of compact cells which were arranged in clusters and separated by delicate fibrovascular stroma. Within the eosinophilic cytoplasm of compact cells were finely dispersed pigment granules (Fig. 4).

Non-pigmented adenomas were composed of clear and compact cells. Compact cells were usually predominant and had a small amount of fine pigment granules (Fig. 5). Clear cells less in number were full of lipid but devoid of pigment granules.



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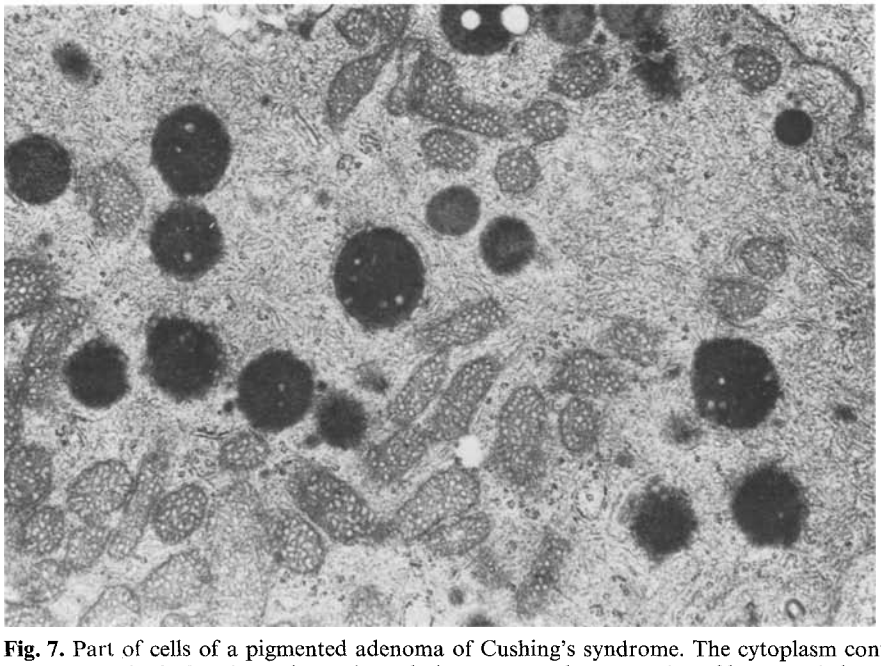

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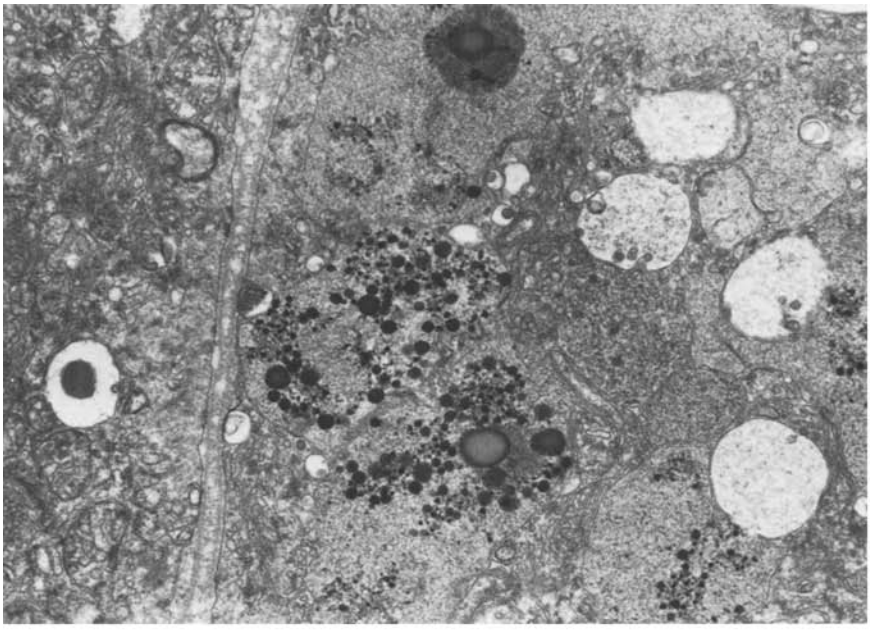
Fig. 7. Part of cells of a pigmented adenoma of Cushing's syndrome. The cytoplasm contains numerous spherical and membrane-bound pigment granules suggestive of lysosomal character. They are homogeneous in electron density. × 25,000

Fig. 8. Part of cells of a non-pigmented adenoma. Pigment granules in compact cells are spherical and limited by a membrane. They are composed of dense particles with little pale matrix. × 25,000



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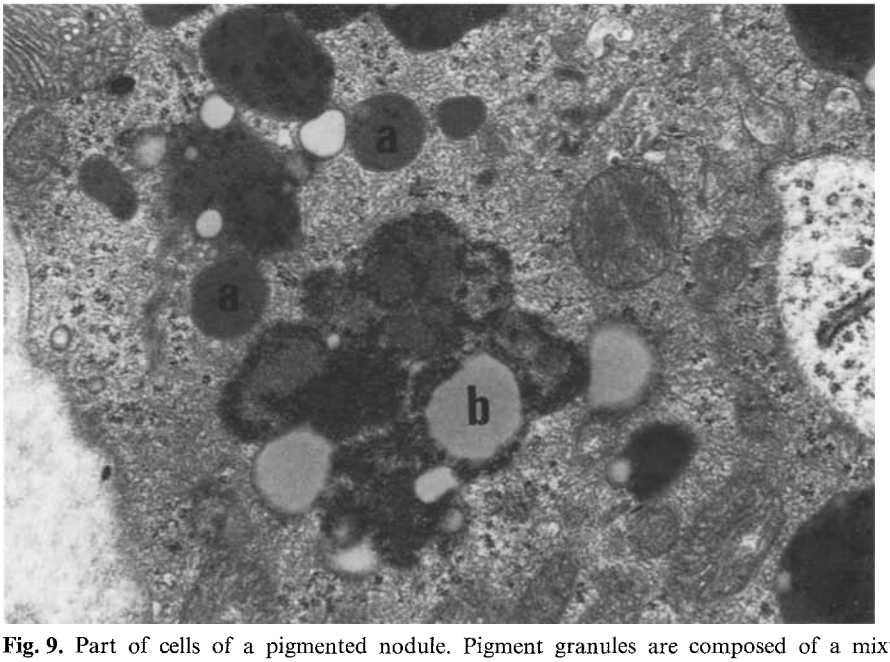

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Fig. 9. Part of cells of a pigmented nodule. Pigment granules are composed of a mixture of dense osmiophilic droplets and multiple vesicular bodies. They are not membrane-bound. × 25,000

Fig. 10. Part of cells in the zona reticularis of the normal adrenal. Two types of pigment granules, *a* membrane-bound and *b* membrane-unbound are observed. × 25,000

In most cells of pigmented nodules, cytoplasmic coarse granules of brown pigment were evenly distributed (Fig. 6).

In the normal adrenal gland, pigment granules were observed in the zona reticularis.

Pigment histochemistry. Table 3 summarizes the results of 12 kinds of staining and fluorescence for the histochemical determination of pigment. In every case, pigment granules were positive to the majority except for basophilia and alternative nile blue, and autofluorescence was orange in colour. These characteristics were common to those described for lipofuscin.

Ultrastructural findings. Cells of pigmented adenomas had numerous pigment granules, which were frequently homogeneous, high in electron density and mostly spherical and membrane-bound in shape (Fig. 7).

In non-pigmented adenomas, clear cells possessed few pigment granules, which were of small spherical, homogeneous and membrane-bound structure. Compact cells contained numerous pigment granules composed of fine dense particles and membrane-bound electron-lucent particles (Fig. 8).

Pigment in pigmented nodules mostly exhibited irregular grouping of osmiophilic droplets of various size dispersed in the pale matrix (Fig. 9). The droplets were not membrane-bound and were suggestive of lipoid droplets.

In the zona reticularis, two types of pigment granules were observed. One was relatively homogeneous in electron density and was a membrane-bound structure probably of lysosomal nature. Another was large in size, few in number and of membrane-unbound structure similar to non-metabolized lipid droplets (Fig. 10).

Morphometrical analysis: The results of morphometrical analysis of pigment granules are graphed in Fig. 11. A significant result was obtained in the volume density; 9.1–10.4% in pigmented adenomas and 1.2–5.1% in non-pigmented adenomas ($p < 0.01$). The volume density of pigment granules in pigmented adenomas was a little larger than that in non-pigmented adenomas, and also a little different from that in the normal zona reticularis.

The mean area gave no significant difference between pigmented and non-pigmented adenomas. However, the mean area in pigmented adenomas was smaller than that in the zona reticularis.

The volume density and mean area were seemingly larger in pigmented nodules than those in adenomas and the zona reticularis. However, the number of only one pigmented nodule examined was insufficient to make a definite conclusion.

In vitro steroid production: No significant difference in cortisol production was confirmed between pigmented and non-pigmented adenoma tissue. In 6 out of 11 cases, the basal production was high compared with that in the control and did not respond to ACTH. The basal production of DHEA was within the normal range and was not stimulated by ACTH (Fig. 12).

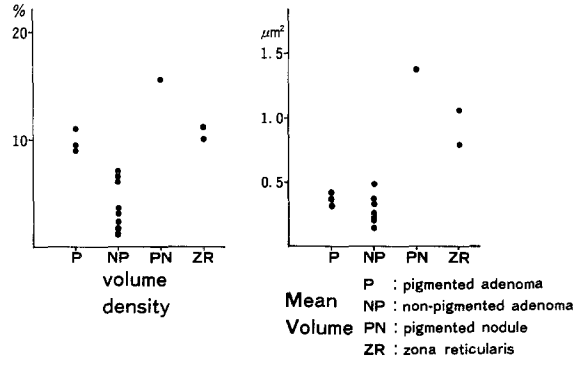

Fig. 11. Morphometrical analysis measured by a Videoplan. (Kontron). Dots represent the mean value of individual cases

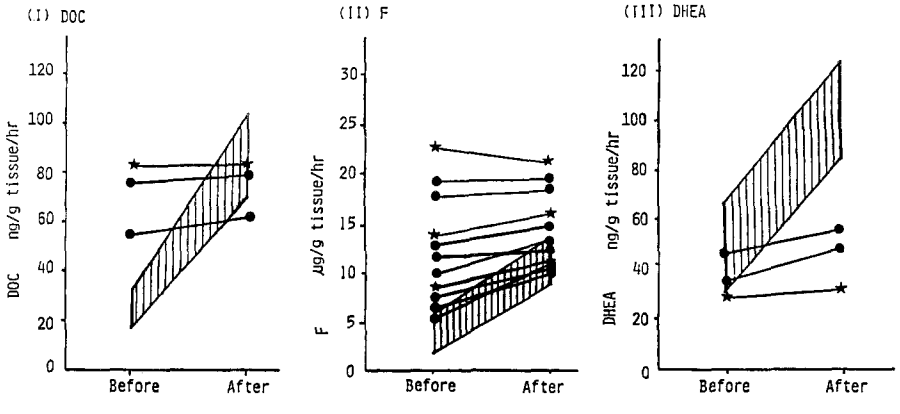

Fig. 12. In vitro steroid production by adenoma tissue. □□: normal range of in vitro production before and after ACTH stimulation; *–*: Pigmented; ●——●: Non-pigmented

Tissue steroid concentrations. Table 4 summarizes the tissue concentrations of 7 steroids: cortisol, DHEA, pregnenolone, progesterone, aldosterone, 17α-hydroxypregnenolone, and 17α-hydroxyprogesterone. The amount in the steroids examined except for DHEA and cortisol was markedly lower in a pigmented nodule than that in an adenoma of Cushing's syndrome and in the normal cortex.

Discussion

In the present study of 17 pigmented and non-pigmented adenomas with Cushing's syndrome, 4 pigmented nodules and 2 samples of normal adrenocortical tissue, a series of 11 stainings and an autofluorescence for cytoplasmic pigment revealed lipofuscin. This agreed with previous reports (Symington 1969; Macadom 1971; Robinson et al. 1972; Garret and Ames 1973; Bahu et al. 1974; Visser et al. 1974; Kovacs et al. 1976; Uras et al. 1978). The ultrastructure of pigment granules in adenomas of Cushing's syndrome,

however, showed some differences from those in non-functioning pigmented nodules.

Shigematsu et al. (1982) described pigment granules in multiple pigmented nodules with Cushing's syndrome as large irregularly fused granules with various electron densities. We, however, could not confirm such types of pigment granules in pigmented adenomas of Cushing's syndrome, but found them in non-functioning pigmented nodules. In our ultrastructural study, pigment granules in pigmented adenomas were small in size, homogeneous in electron density and membrane-bound. The findings were comparable to those in non-pigmented adenomas with Cushing's syndrome and suggested that they were of lysosomal nature. It has been suggested that the membrane-bound pigment granules in normal adrenocortical cells are lysosome and lipofuscin granules (Long and Jones 1976). Lysosomes are membrane-bound and could be generally classified into two categories (Rhodin 1974). One is the primary lysosome of homogeneous electron density bordered by an limiting membrane. The other is the so called lipofuscin; a multitude of varying-sized granular structures also bordered by an limiting membrane.

In our study, pigment granules in non-functioning pigmented nodules were histochemically identifiable as lipofuscin. But, electron microscopically those in non-functioning pigmented nodules had the lipoid character of a membrane-unbound ultrastructure. This type of pigment granules was infrequently observed in the zona reticularis. The volume density and mean area were larger than those of lysosomal pigments in the adenomas with Cushing's syndrome.

Hornsby and Crivello (1983) regarded the accumulation of pigment lipoids as the late stage of lipoid peroxidation caused by increasing steroid gradient and consequent steroid-cytochrome P-450 interactions in the course of aging of adrenocortical cells.

In the present study, the steroid concentrations in a pigmented nodule were of lower level than those in the normal cortex. These data suggest that pigment-rich cells in pigmented nodule as well as in the zona reticularis are damaged by lipoid peroxidation. On the other hand, in vitro steroid production of cortisol by pigmented adenomas of Cushing's syndrome was high and was not significantly different from that of non-pigmented adenomas of the same disease.

Sasano et al. (1980) observed black adenomas of Cushing's syndrome and detected pigment granules in clear cells as well as compact cells. Pigmented adenomas are not unique to Cushing's syndrome and can cause other forms of hypercorticalism, e.g. hyperaldosteronism or virilisation (Neville and O'Hare 1982). These facts corroborated the hypothesis that lysosomal pigment granules are elaborated by the longstanding cellular activity and are different from the fused lipoid pigments that are the terminal product of lipoid peroxidation.

In conclusion, pigment granules in pigmented adenomas of Cushing's syndrome may be of lysosomal nature and not different from those in non-pigmented adenomas. The functional role of lysosomal pigment in steroidogenesis is still uncertain. Further investigations are required.

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