An ultrastructural stereologic study of aldosterone secreting adrenal adenomas and of adjacent zona glomerulosa

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Summary. The ultrastructure of four aldosterone secreting adenomas and of the adjacent zona glomerulosa has been described by the use of stereological techniques. Adenomatous cells (about 2800 μm^3 in volume) invariably displayed a striking abundance of lipid droplets, which occupied about 30% of the cytoplasm. Mitochondria prevalently contained tubulo-lamellar or lamellar cristae, but some cells exhibited organelles with vesicular cristae. Smooth endoplasmic reticulum (SER) was not very abundant. Small lipofuscin-pigment granules were frequently seen and in a few cells they were exceedingly numerous. Zona glomerulosa cells were smaller (about 950 μm^3 in volume) and possessed mitochondria with typical tubulo-lamellar cristae, a plentiful SER and few lipid droplets. They showed the ultrastructural features of elements actively engaged in steroid synthesis. The possible origin of aldosteronoma cells from the zona glomerulosa is discussed.

Key words: Aldosteronomas, Adrenal cortex, Human, Electron microscopy, Stereology

Introduction

Previous investigations on the ultrastructure of adrenal adenomas causing primary aldosteronism do not report uniform findings, since tumor cells resembling those of the zona glomerulosa, zona fasciculata and sometimes also of the zona reticularis have been described (for review, see Neville and O'Hare, 1982; Nussdorfer, 1986). Moreover, many of these studies examine tumors obtained from patients preoperatorily treated with spironolactone, so that adenomatous cells contained spironolactone bodies, which caused a profound disruption of their morphology.

It therefore seemed worthwhile to carefully re-examine the ultrastructure of aldosteronomas, obtained from patients not pre-treated with spironolactone, by using stereology, in order to provide baseline data for future investigations. Moreover, we have studied the morphology of the adjacent zona glomerulosa, which, as far as we are aware, has never been examined in earlier investigations.

Materials and methods

Four female patients (aged between 38 and 58 years) with primary hyperaldosteronism due to adrenal adenoma were studied. On admission, they showed a mild hypertension (170-190/100-115 mmHg). Plasma potassium was decreased (2.7-3.3 mEq/l). Plasma aldosterone concentration was elevated (15-83 ng/dl supine, and 17-90 ng/dl upright), as well as aldosterone excretion rate (19-100 $\mu g/24$ h). PRA was suppressed at 0.02-0.07 ng/ml/3h and could be only slightly stimulated by standing (0.22-0.27 ng/ml/3h). After operation (from two weeks to one month) blood pressure and serum potassium normalized (140/90 mmHg and 4.3-5.2 mEq/l). Plasma aldosterone concentration ranged from 3-12 ng/dl supine to 5-20 ng/dl upright. Urinary aldosterone excretion rate was below 5.11 $\mu g/24$ h. PRA was no longer suppressed: 0.47-1.04 ng/ml/3 h supine, 2.34-2.70 ng/ml/3h upright. All adenomas were histologically verified.

Fragments of adenomas and adjacent gland tissue were processed for electron microscopy. They were sliced and immediately fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer and then embedded in epon. 0.5 μm-thick sections were cut with LKB III ultramicrotomes and observed with the light microscope. Adjacent thin sections were counterstained with lead-hydroxide and observed in a Hitachi H-300 electron microscope at a direct magnification of 7,000 ×.

For each patient, three tissue blocks of adenoma and

another three blocks containing zona glomerulosa were examined. Two series of thick and thin sections for each block were selected. Three light micrographs were recorded for each series of thick sections at a magnification of 1,000 ×. For each series of thin sections, six electron micrographs at a final magnification of 21,000 × and three electron micrographs at a final magnification of 42,000 × were recorded for stereological analysis.

The volume densities (V_v) of nuclei and cytoplasm of parenchymal cells and stroma (mm^3/mm^3 of tissue) were estimated on the light micrographs by differential point counting, using a square lattice test system of type A (400 test points/dm^2) (Weibel, 1979). On the same micrographs the numerical density (N_a) of parenchymal nuclear profiles (number/mm^2 of tissue section) was computed. The number of nuclei of parenchymal cells per mm^3 of tissue (N_v) was estimated according to the formula of Weibel and Gomez (Weibel, 1979):

$$N_v = \frac{K}{\beta} \times \frac{N_a^{2/2}}{V_v^{1/2}}$$

The shape coefficient β, which depends on axial ratio of estimated nuclei, was calculated from the curve for ellipsoids of Weibel (1979) and found to be about 1.350 for tumor cells and 1.500 for zona glomerulosa cells. The size-distribution coefficient K was assumed to be 1 (Weibel, 1979). Since tumor and zona glomerulosa cells are mononucleated, N_v corresponds to the number of cells per mm^3 of tissue. Subsequently, the average volume of parenchymal cells was computed according to the formula: (1 - V_v of stroma)/N_v.

On the electron micrographs at a final magnification of 21,000 ×, the V_v of mitochondria, «membrane space» (i.e. the cellular space occupied by smooth endoplasmic reticulum membranes, including Golgi apparatus) (Loud, 1962), lipid droplets, lysosomes and lipofuscin-pigment granules ($\mu m^3/100$ μm^3 of cytoplasm) were estimated by differential point counting, using a test system of type A (100 test points/dm^2) (Weibel, 1979). On the electron micrographs at a final magnification of 42,000 ×, the surface densities (S_v) of mitochondrial cristae ($\mu m^2/\mu m^3$ of mitochondria), smooth (SER) and rough (RER) endoplasmic reticulum ($\mu m^2/\mu m^3$ of membrane space) were evaluated according to Loud (1962), employing a parellel-line test system (100 cm of test line/dm^2).

The mean values ± SD of the various stereological parameters were calculated for each patient. χ^2 test revealed that the data were not different from normal distribution and ANOVA showed that differences among the four patients were not significant Therefore the data were pooled. Statistical comparison between V_v and S_v of adenomatous and zona glomerulosa cells was done by Student's t-test. All the statistical procedures were performed using an IMB Personal Computer.

Results

Adenomatous cells (about 2800 μm^3 in volume) were arranged in alveolar clusters, surrounded by a well-developed stroma with numerous capillaries, and possessed a round or irregularly-shaped nucleus (about 150 μm^3 in volume) (Fig. 1). Tumor cells always contained abundant lipid droplets (Figs. 2-4), which occupied almost a third of the cytoplasm. Mitochondria were round, ovoid or elongated and mostly displayed tubulo-laminar or lamellar cristae (Figs. 2, 4). However, few cells showed mitochondria with tubulo-vesicular cristae (Fig. 3). Membrane space was rather well developed and contained a not very abundant tubular SER and many RER cisternae frequently arranged in stacks (Fig. 3). The Golgi apparatus appeared small and fragmented. Lysosomes were present (Figs. 2, 3), as well as lipofuscin-pigment granules. However, these last inclusions were unevenly distributed, being absent in some cells and exceedingly abundant in others (Fig. 4). Stereological parameters are shown in Table 1.

Zona glomerulosa cells were arranged in small islets. In the central portion of the zone, they were rather elongated (about 950 μm^3 in volume) and contained a spindle-shaped nucleus (about 120 μm^3 in volume). Ovoid mitochondria invariably displayed tubulo-lamellar cristae (Fig. 5). Membrane space was abundant and filled with SER tubules. Several RER profiles can be observed, as well as a large juxtanuclear Golgi apparatus (Fig. 5). Lipid droplets were sparse. Lysosomes were present, while lipofuscin-pigment granules seemed to be lacking. Stereological parameters are displayed in Table 1.

Fig. 1. Alveolar cluster of voluminous aldosteronoma cells, surrounded by a well-vascularised stroma. × 1,350

Fig. 2. Tumor cell displaying mitochondria (M) with laminar cristae and several lipid droplets (ld). N, nucleus; A, brown adipose cell. × 12,500

Fig. 3. Tumor cell showing mitochondria (M) with tubulo-vesicular cristae and a conspicuous RER stack. N, nucleus; ld, lipid droplets. × 11,500

Fig. 4. Aldosteronoma cell containing abundant lipid droplets (ld) and many lipofuscin-pigment granules (lf). N, nucleus; M, mitochondria. × 11,500

Fig. 5. Zona glomerulosa cells showing round mitochondria (M) with tubulo-vesicular cristae, a very well-developed SER, few RER cisternae (arrows), sparse small lipid droplets (ld), some lysosomes (L) and a prominent juxtanuclear Golgi apparatus (G). ICS, intercellular space. × 14,000
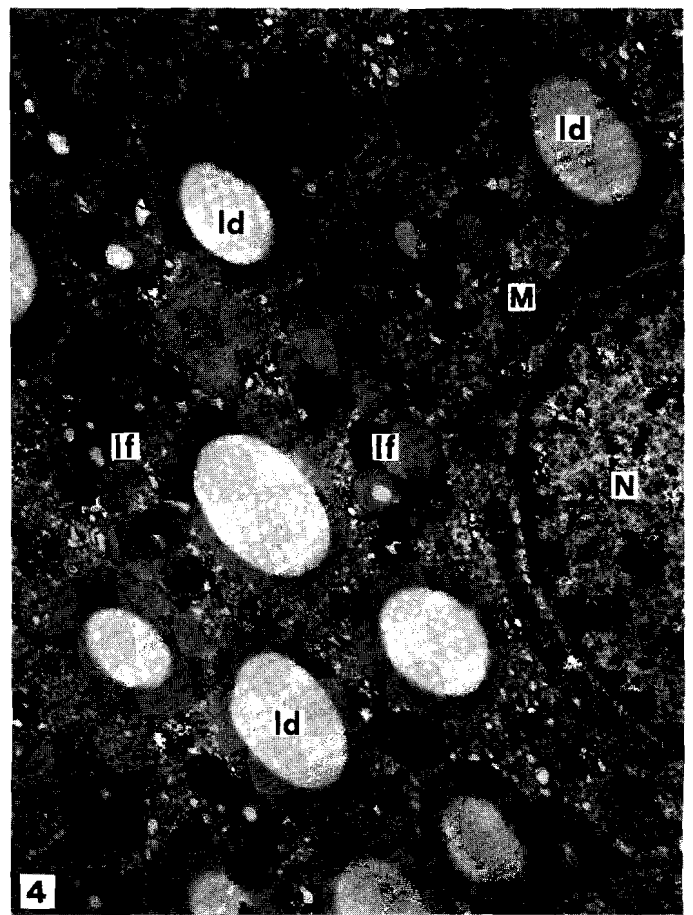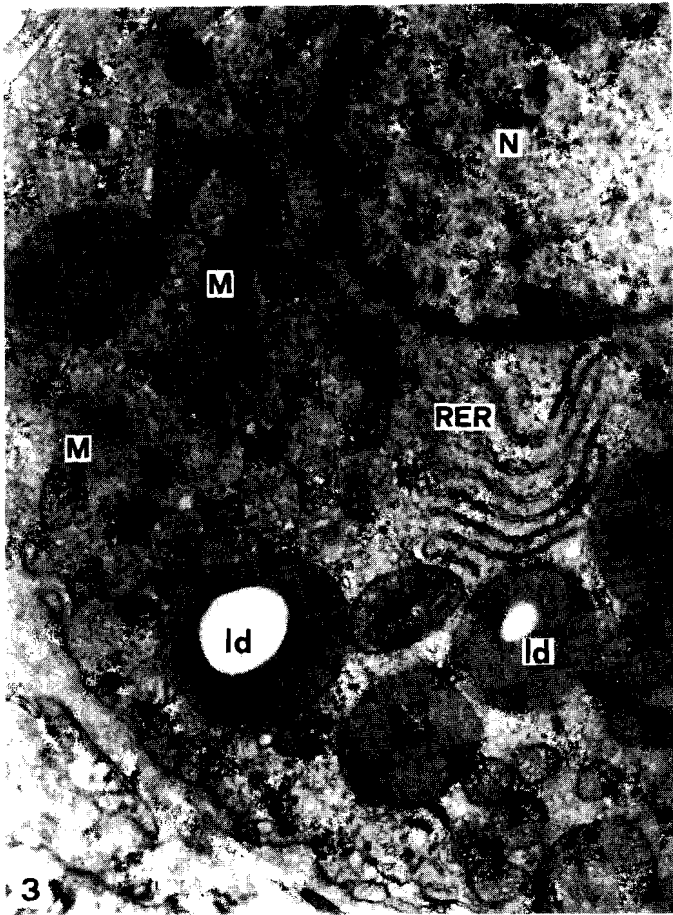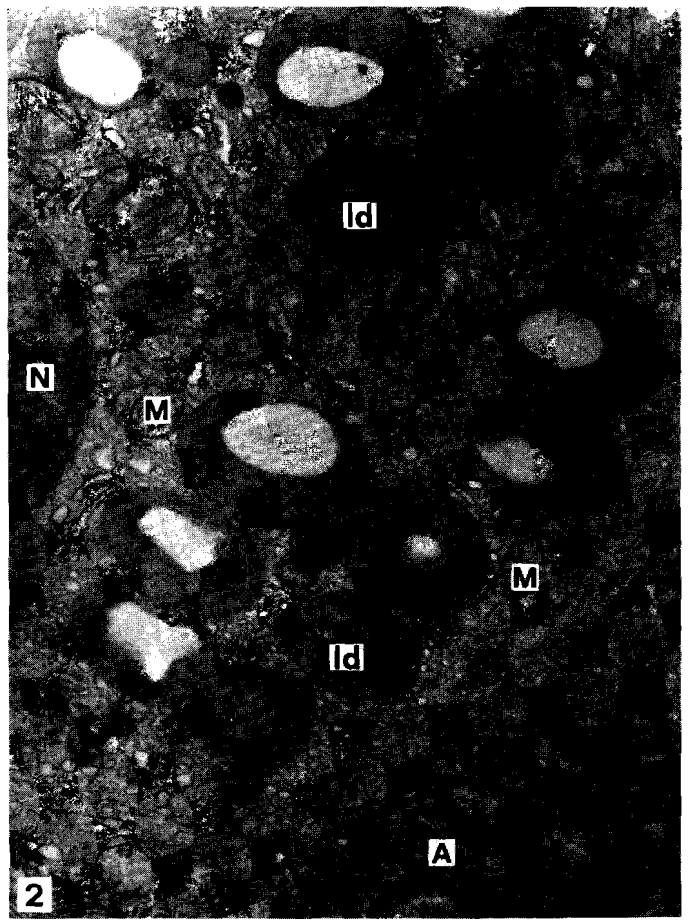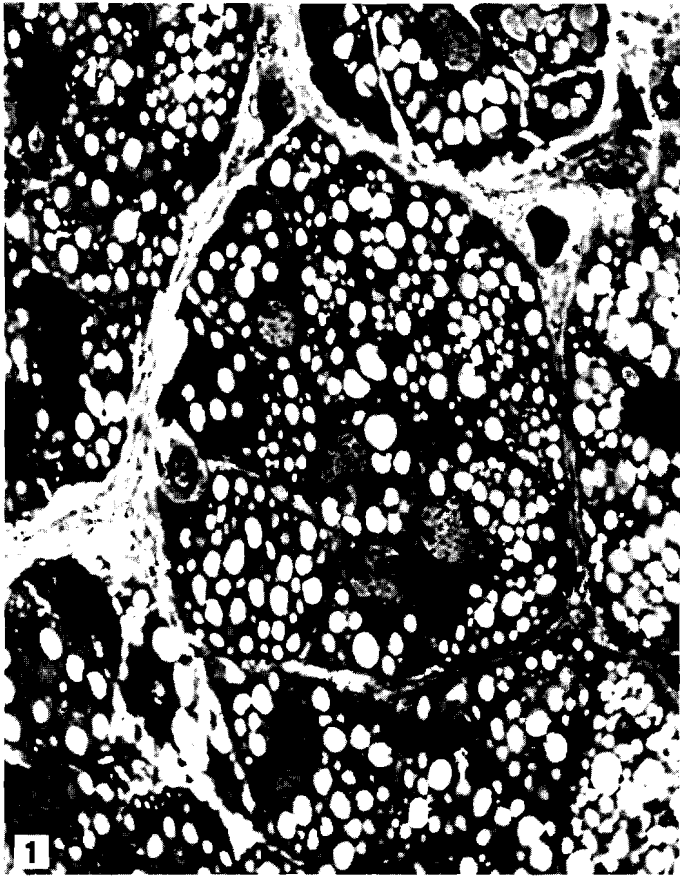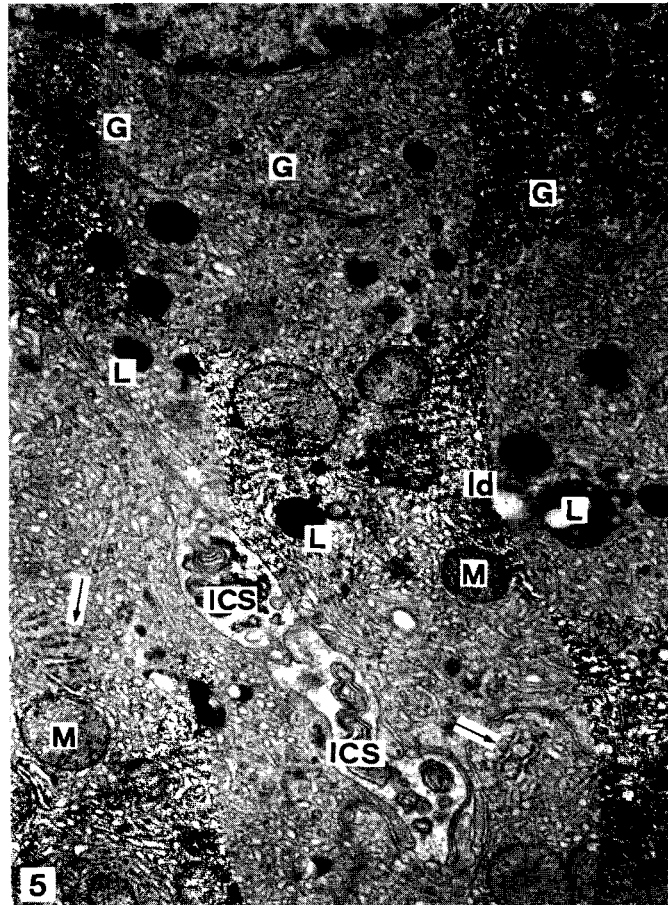
Table 1. Morphometric parameters of aldosteronomas and adjacent zona glomerulosa.

	Volume of cells (μm³)	Volume of nuclei (μm³)	V_v of mitochondrial compartment	S_v of mitochondrial cristae	V_v of membrane space
Aldosteronomas	2809.4 ±442.3	152.3 ± 8.5	20.5 ±2.5 [29.1]	13.5 ± 1.9	49.3 ± 6.0 [69.9]
Zona glomerulosa	942.1 ± 121.8*	119.7 ± 14.4*	26.5 ± 3.1* [29.0]	14.4 ± 1.6	64.0 ± 7.6* [70.3]

	S_v of SER	S_v of RER	V_v of lipid-droplet compartment	V_v of lysosome compartment	V_v of lipofuscin pigment compartment
Aldosteronomas	10.6 ± 1.3	1.15 ± 0.11	29.5 ± 3.9	0.62 ± 0.12	0.09 ± 0.2
Zona glomerulosa	14.8 ± 1.6*	1.20 ± 0.13	8.9 ± 1.0*	0.60 ± 0.11	0

Values are group means ± SD. V_v calculated per 100 μm³ of cytoplasm minus lipid-droplet compartment are shown in square brackets. *, $P < 0.01$

Discussion

A review of the literature dealing with aldosterone secreting adenomas stresses their evident ultrastructural pleiomorphism. Some tumors appear to be composed of zona glomerulosa-like cells (Cervos-Navarro et al., 1965; Tsuchiyama, 1967; Reidbord and Fisher, 1969; Hashida and Yunis, 1972; Tannenbaum, 1973; Beskid et al., 1978; Kano et al., 1979; Favre et al., 1980; Kuramoto and Kumazawa, 1985), while others seem to consist exclusively of zona fasciculata-like cells (Propst, 1965; Sommer and Terzakis, 1970). Aldosteronomas consisting of a mixture of zona glomerulosa- and zona fasciculata-like elements were also frequently described (Kovacks et al., 1974; Tsuchiyama et al., 1980; Mazzocchi et al., 1982a; Shigematsu, 1982; Matsuo and Tsuchiyama, 1986). The distinction between the two cell types was almost exclusively based on the morphology of mitochondrial cristae. In fact, it is current knowledge that mitochondria display tubular or tubulo-laminar cristae in the zona glomerulosa and tubulo-vesicular or vesicular cristae in the zona fasciculata. However, this contention is valid only for the rat adrenal cortex, inasmuch as human zona fasciculata mitochondria usually show short tubular cristae (Long and Jones, 1967; and for review, see Nussdorfer, 1986).

In the present study, we observed in all aldosteronomas either numerous cells with laminar-cristae mitochondria or sparse elements with vesicular-cristae organelles. S_v of vesicular cristae is slightly higher than that of laminar ones (15.0 versus 13.1; $P<0.05$), but stereology did not evidence any significant difference in the other parameters (including V_v of the mitochondrial compartment). On these grounds, it seems legitimate to assume that the two cell types belong to the same population and that mitochondrial-cristae morphology is not a reliable criterion for singling out the zonal origin of adrenal adenomatous cells. In this connection, it must be recalled that many lines of evidence indicate that mitochondria are very plastic organelles, which are able to change their inner membrane architecture under various experimental conditions: for instance, after prolonged treatment with ACTH tubular cristae of rat zona glomerulosa mitochondria transform into a homogeneous population of vesicles (Mazzocchi et al., 1986a; Riondel et al., 1987).

Parenchymal cells of the middle portion of the zona glomerulosa of the adrenal bearing the aldosteronoma display all the typical ultrastructural features described previously and look like well-functioning elements actively engaged in aldosterone synthesis (for review, see Nussdorfer, 1986). This is a very intriguing finding, since the patients showed a low PRA and a notably lowered kalaemia, and there is general agreement that angiotensin II and potassium ions are both involved in the maintenance of zona glomerulosa growth (for review, see Nussdorfer, 1986). However, an increasing mass of data is available indicating that zona glomerulosa growth undergoes a very complicated multifactorial control. In addition to the classic adrenoglomerulotrophic factors (i.e. angiotensin II, K+ and ACTH), some other hormones and regulatory peptides appear to be involved. These include prolactin (Mazzocchi et al., 1986c), α-MSH (Robba et al., 1986b), enkephalins (Robba et al., 1986a) and VIP (Mazzocchi et al., 1987c), which exert a stimulatory effect, and somatostatin (Rebuffat et al., 1984; Mazzocchi et al., 1985), dopamine (Mazzocchi et al., 1987b) and ANF (Mazzocchi et al., 1987a), which exhibit an anti-adrenoglomerulotrophic action. Moreover, the existence of an active intra-adrenal renin-angiotensin system has been demonstrated in both rats and humans (Medelsohn, 1982; Naruse et al., 1983; Doi et al., 1984). Further studies are required to gain insight into the functional status of the zona glomerulosa of patients with primary hyperaldosteronism.

Our findings do not permit us to draw a definitive conclusion about the zonal origin of aldosteronoma cells. At first glance, tumor cells appear rather different from those of the adjacent zona glomerulosa. They are about three-times more voluminous and contain a very large lipid-droplet compartment. Stereology shows additional significant differences concerning V_v of the mitochondrial compartment and membrane space. However, it must be considered that V_v is a relative parameter, whose value may be influenced by that of other subcellular compartments; thus a striking accumulation of lipid droplets may alter V_v of mitochondria and membrane space. If V_v of these two last compartments are recalculated per $100\mu m^3$ of cytoplasm minus lipid droplets, any difference between adenomatous and zona glomerulosa cells disappears (Table 1). Therefore, our stereologic data do not rule out the possibility that aldosteronoma cells originate from elements of the zona glomerulosa, which, after proliferation, underwent a hypertrophy involving the same rate of growth of the main organelles engaged in steroid synthesis (i.e. mitochondria and membrane space containing SER) (for review, see Nussdorfer, 1986), coupled with a striking accumulation of lipid droplets.

Nevertheless stereology indicates a notable difference in the S_v of SER, which is about 40%-higher in zona glomerulosa cells (Table 1). The relative paucity of SER coupled with the great abundance of lipid droplets (and viceversa) accords well with previous morphological data on adrenocortical cells of various species (for review, see Nussdorfer, 1986). Adrenocortical cells obtain cholesterol both by endogenous synthesis from acetate and by uptake of lipoproteins released by the liver (for review, see Gwynne and Strauss, 1982). According to Frühling et al. (1973) and Mazzocchi et al. (1987d), only exogenous cholesterol is stored in the adrenocortical lipid droplets, while endogenously synthesized cholesterol is promptly utilized in steroidogenesis or may temporarily accumulate in the SER membranes (Black, 1972). There are no doubts that cholesterol

synthesis occurs in the SER (Boyd et al., 1983), and hence *the abundance* of this organelle in zona glomerulosa cells, coupled with the paucity of lipid dropets, may be indicative of a high rate of endogenous cholesterol production. This view is supported by investigations which demonstrated that, in rat zona fasciculata cells, the prolonged blockade of the exogenous cholesterol supply (induced by the administration of drugs which inhibit the synthesis and release of hepatic lipoproteins) provokes a striking proliferation of SER, along with an almost complete lipid-droplet depletion and maintenance of a normal rate of hormone output (Mazzocchi et al., 1982b, 1986b). The presence of a conspicuous lipid-droplet compartment in aldosteronoma cells can be interpreted as the morphological counterpart of an active uptake of exogenous cholesterol, whose rate exceeds that of its utilization in steroidogenesis. Cholesterol uptake by serum lipoproteins is a receptor-mediated process (for review, see Goldstein and Brown, 1985). Therefore, it seems reasonable to propose that the transformation of adrenocortical cells into adenomatous elements is associated with the synthesis of an elevated number of specific receptors for lipoproteins. This contention appears to be indirectly supported by evidence indicating that steroidogenic tumors possess a high rate of lipoprotein-cholesterol consumption (for review see Gwynne and Strauss, 1982), but investigations on lipoprotein receptors in adrenocortical adenomas are hitherto lacking. A large body of data suggests that the activity and the *de novo* synthesis of the key enzyme of cholesterol synthesis (i.e. HMG-CoA reductase) is suppressed by an increase in the intracellular level of the free-cholesterol pool (Balasubramaniam et al., 1977; Osborne et al., 1985). This may be a tentative explanation for the relative paucity of SER in aldosteronoma cells, since the molecules of HMG-CoA reductase are an integral part of the membrane of this organelle (Brown and Goldstein, 1980).

Electron microscopy revealed that many tumor cells contain sparse granules of lipofuscin pigment, which in a few elements accumulate in a striking abundance. This is not surprising, since analogous findings were previously reported by some of us (Mazzocchi et al., 1982a) and black adenomas producing primary aldosteronism have been described (Caplan and Virata, 1974; Sienkowski et al., 1984). Lipofuscin is a «wear-and-tear» pigment, whose accumulation inside the cells may be considered as an expression of ageing since it is the residue not further degradable of the lysosomal digestive activity (Fawcett, 1986).

According to the theory of migration, a continuous flow of cells occurs from the outer to the inner compartments of adrenocortical gland, the youngest cells being in the zona glomerulosa and the oldest ones in the zona reticularis (for review, see Wright and Alison, 1984). Thus it is obvious that zona glomerulosa never contains lipofuscin pigment, which is a typical feature of inner-zona fasciculata and zona reticularis cells (for review, see Nussdorfer, 1986). On these grounds, we suggest that the presence of lipofuscin pigment in

adrenocortical tumor cells cannot be taken as an indication of their zonal origin, but must be considered as the expression of their ageing inside the close tumoral compartment.

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