

Histochemical and Morphological Characteristics in Cultures of Normal Human Adrenal Cortex and Adrenocortical Tumors

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Received for publication, January 28, 1984

Histochemical and morphological studies in culture were carried out on 4 cases from the normal human adrenal cortex (NHA), 1 case from CUSHING'S adenoma (CS), 4 cases from CONN'S adenoma (primary aldosteronism, PA) and 1 case from adrenocortical carcinoma (ACC).

Cultured cells from CS and ACC transformed rapidly into fibroblast-like cells, while those from NHA and PA remained on the substratum as epithelial-like cells in the beginning but gradually changed into fibroblast-like cells.

Cultured cells from CS and ACC were negative for oil red O staining, while lipid droplets in the cytoplasm were prominent in the PA- and NHA-cultured cells.

Cells active for 3β -hydroxysteroid dehydrogenase (3β -HSD) were admixed among the ACC-cultured cells and present sporadically in the CS culture. In primary culture of PA and NHA, cells in early passage revealed activity for 3β -HSD but fibroblast-like cells hardly revealed activity.

In all the cases, ultrastructural features of the cultured cells varied from those of the original cells, but the cultured cells were contacted each other with interdigitation and desmosome-like structures.

INTRODUCTION

For the past 20 years or so, many studies have been published on the adrenocortical cell cultures from human as well as from animals. Most of them, however, dealt with the functions of the adrenocortical cells³⁾⁵⁾⁶⁾⁷⁾¹⁰⁾ and observations on the morphological features of the adrenocortical cells, especially of the tumor cells are quite a few²⁾⁴⁾⁸⁾¹³⁾¹⁵⁾¹⁷⁾¹⁸⁾¹⁹⁾.

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According to some of the previous reports, the culture cells from the adrenocortical tumor underwent fibroblastoid transformation or presented two types of cells, i.e. epithelial and fibroblastic types depending on the time for the cultivation, under a phase contrast microscope. It, however, has never proven whether these fibroblast-like cells really originate from the adrenocortical cells or pure fibroblasts intermingled in the adrenocortical cell cultures are regarded as these fibroblast-like cells by mistake. It is not generally agreed that the adrenocortical cells change into fibroblast-like cells.

The present study shows the transformation of normal and neoplastic adrenocortical cultured cells into fibroblast-like cells. Moreover, morphological characteristics of these cultured cells would be discussed based on the findings under a phase contract microscope in addition to the histochemical and electron microscopic observations.

MATERIALS AND METHODS

Materials used in the present study were as follows: 4 cases of normal human adrenal cortex except for the tumor in primary aldosteronism, 1 case of CUSHING's adenoma, 4 cases of CONN's adenoma and 1 case of adrenocortical carcinoma, based on the diagnoses made by clinical findings and laboratory data. All the cases were confirmed by routine histological examinations. The clinical data of these cases are shown in Table 1.

Pieces of the individual tissues were removed aseptically, transferred into PETRI dishes (6 cm in diameter, CORNING) containing phosphate buffered saline without CaCl_2

Table 1. Cases of CUSHING's adenoma, CONN's adenoma and abrenocortical carcinoma
CUSHING's adenoma

No.	Age	Sex	Site	Size(cm)	Plasma cortical ($\mu\text{g/ml}$)
1	34	F	Left	2.8×3.0×2.3	22.1—25.4

CONN's adenoma

No.	Age	Sex	Site	Size(cm)	Plasma aldosterone (ng/dl)
1	35	F	Right	2.3×2.0×1.5	52.9—58.8
2	49	F	Left	2.0×1.9×1.5	31.1—39.6
3	35	F	Left	2.0×1.6×1.2	44.2
4	32	F	Right	1.0×1.0×0.7	28.3—41.1

Adrenocortical carcinoma

No.	Age	Sex	Site	Size(cm)	Urine 17-KS (mg/day)
1	62	M	Left	17×11.5×10	91.1

and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ [PBS(-)], washed by several changes of PBS(-) and were chopped with scissors into small fragments measuring about 1 mm^3 in dimension. The fragments were placed into a flask containing 10 ml of dispase solution (2000 PU/ml, Gudo-Shuseki Co. Ltd.) and were suspended with a magnetic stirrer at 37° for 20 min. The supernatant was, then, collected through a platinized filter and was centrifuged at 800-1000 cpm for 7 min to obtain isolated cells, which were incubated at 37° in atmosphere of 5% CO_2 and 95% air. Modified EAGLE's Medium containing a 10% fetal calf serum (GIBCO) was utilized for incubation. The cells for subculture were removed with dispase solution at 37° for 10-30 min and were transferred into PETRI dishes. The cultured cells were examined using phase contrast microscopes and were stained with oil red O.

3β -HSD: After washing the dishes with PBS(-) several times, the reagent for 3β -HSD was poured into the dishes and left for 60 min to cause reaction¹¹⁾.

Electron microscopic examination: Cultured cells removed from PETRI dishes with dispase solution were centrifuged at 800-1000 cpm for 7 min. The cell pellets were fixed in 2.7% glutaraldehyde in phosphate buffer (pH 7.2, 0.1 M), rinsed with phosphate buffer, postfixed for 30 min in 1.25% osmium tetroxide in phosphate buffer, dehydrated in a graded ethanol series and acetone, and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate, and observed under a NIHON-DENSHI 100B electron microscope.

Radioimmunoassay: After solvent extraction and preliminary purification with the method of AIKAWA *et al.*¹⁾, the content of aldosterone and cortisol in the culture media were purified by paper chromatography and measured in one case each from CS and PA by radioimmunoassay. Anti-aldosterone and cortisol-3-BSA antisera were obtained from Miles-Yeda Ltd. and stored at -40° . Before use, they were diluted each with 0.05M potassium phosphate buffer at pH7.4 containing 0.15 NaCl, 0.5% BSA and 0.1% NaN_3 , and with 0.05M Tris-HCl buffer at pH8.0 containing 0.1M NaCl, 0.1% NaN_3 and 0.1% gelatin. The standard and labeled corticosteroids were obtained from Sigma Co. and New England Nuclear Co.

RESULTS

In the primary cultures of NHA, epithelial-like cells formed colonies and were laden with lipid droplets in the cytoplasm. Most of the cells were polygonal but some of them were spindle-shaped (Fig. 1A). All the cells revealed activity for 3β -HSD (Fig. 1B). About 5 days later fibroblast-like cells appeared at the peripheries of the colonies, which were gradually invaded by the fibroblast-like cells as well (Fig. 1C). Petri dishes for subculturing were mainly covered with fibroblast-like cells, most of which hardly revealed activity for 3β -HSD. Electron microscopic examination revealed similar cells to those of the original adrenal cortex until the 20th day of culture (Fig. 2A), but a minority of the cultured cells gradually changed from the original cells²⁰⁾ into those which formed microvilli and interdigitation. Mitochondria were mostly spherical but occasionally

rod-like or ellipsoidal. The cultured cells contained abundant free ribosomes, microfilaments and stress fibers. In the second passage culture a large number of myelinated dense bodies were found in the cytoplasm (Fig. 2B).

Cells from CUSHING'S adenoma developed fibroblast-like cells about 4 days later without showing lipid droplets, but lipid containing cells were sporadically present. The fibroblast-like cells were evidently negative for oil red 0 staining (Fig. 3A) and hardly revealed activity for 3β -HSD (Fig. 3B). Cells in the confluent dishes tended to form multilayers with neighbouring cells and to pile up (Fig. 3C). Electron microscope disclosed two apparently distinctive types of cells from the original tumor (CUSHING'S adenoma); one type occupying the most of the cultured cells was scanty in lipid granule but contained lipofuscin-like dense bodies, and the other showed lipid granules and lipofuscin. Common characteristics to either of the two types were irregularly protruded filamentous microvilli. The junction complex were poorly developed, and the cells were joined with interdigitation and desmosome-like structure. The former type cells were abundant in organelles. Mitochondria were spherical and elongated with tubular cristae. Rough endoplasmic reticulum and the GOLGI apparatus were well-developed. Numerous free ribosomes were seen throughout the cytoplasm. Multiple microfilaments were recognized running in various directions and often formed stress fibers. The latter type cells, which were abundant in lipid granules and lipofuscin, contained organelles similar to those of the former type cells. Intermediate or transformed cells were observed (Fig. 4).

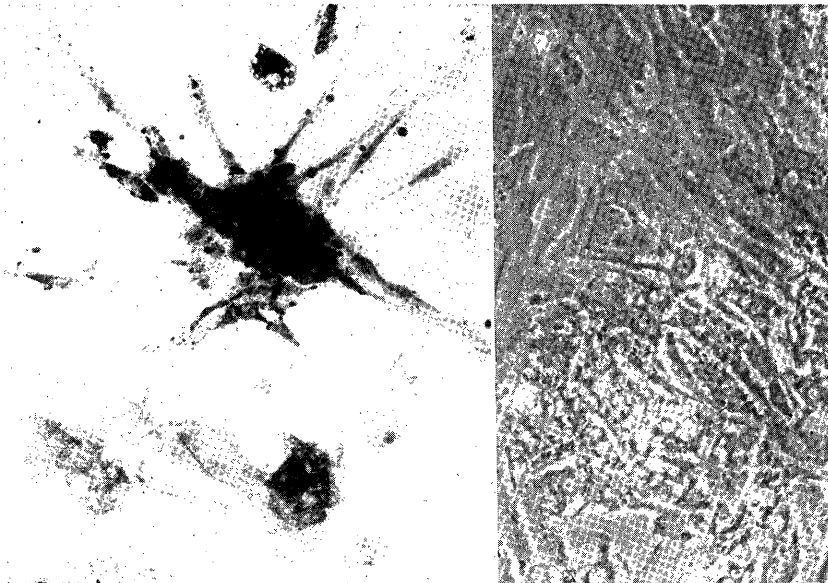


Fig. 1. Normal human adrenocortical cells 4 and 10 days after culture. A. Epithelial-like cells formed a colony and were laden with lipid droplets. Oil red 0 stain. $\times 200$. B. All the Cells revealed activity for 3β -HSD. $\times 200$. C. The cultured cells 10 days later. The fibroblast-like cells occupied the spaces between the colonies. Phase contrast. $\times 200$.

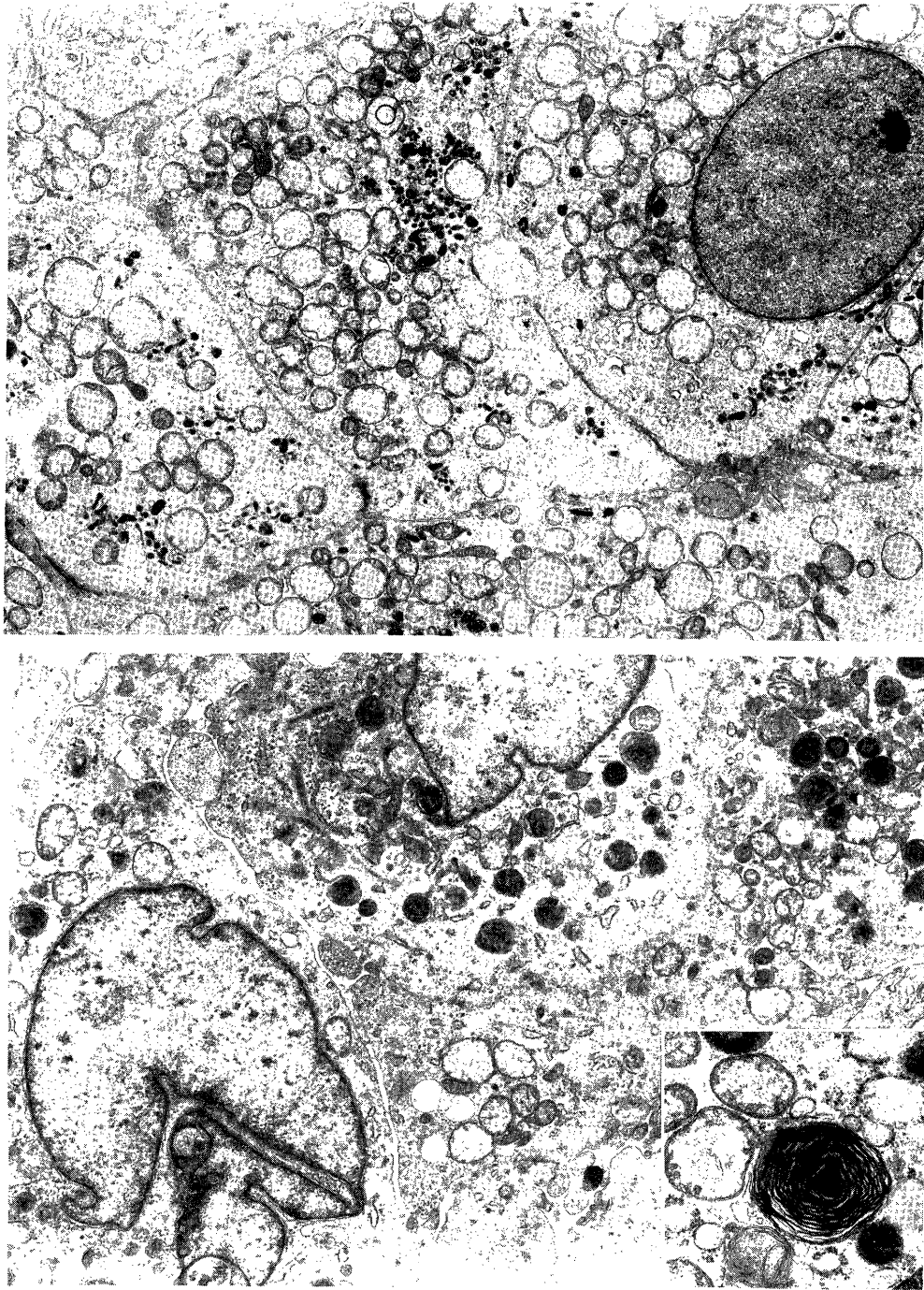


Fig. 2. Electron micrographs of normal human adrenocortical cells in culture. A. Culture 20 days later. $\times 8500$. B. Culture 40 days later. $\times 3600$. Inset shows myelinated dense body. $\times 10,000$.

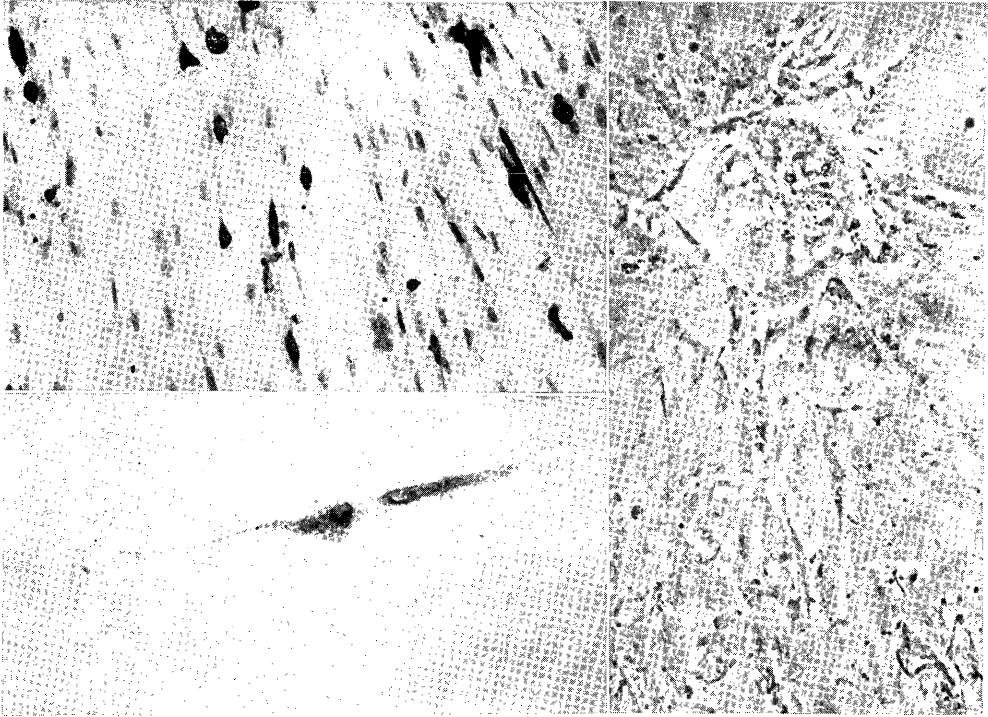


Fig. 3. CUSHING's adenoma in culture. A. Mainly fibroblast-like cells proliferated with occasional lipid-containing cells 4 days after culture. Oil red O stain, $\times 200$. B. 3β -HSD, $\times 200$. C. The fibroblast-like cells overgrew or piled up 31 days after culture. Phase contrast, $\times 230$.

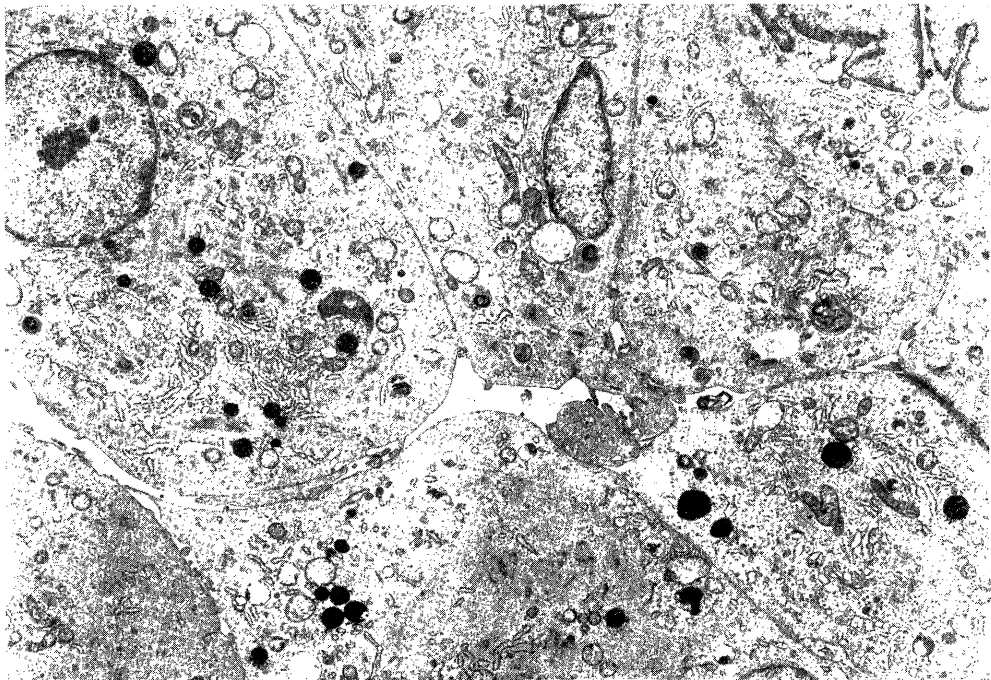


Fig. 4. Electron micrograph of CUSHING's adenoma in culture. The cultured cells were scant of lipid granules but showed lipofuscin-like dense bodies, $\times 2900$.

In the primary culture of CONN's adenoma, the cells in early passage formed colonies, and were generally somewhat elongated or rather polygonal, containing various-sized lipid droplets within, and active for 3β -HSD (Fig. 5A and B). As time passed, lipid-containing cells became spindle-shaped to protrude themselves from the peripheries of the colonies. Cells in the confluent PETRI dishes were mainly spindle-shaped or fibroblast-like, but a minority of them were active for 3β -HSD. The cultured cells gradually overlapped each other (Fig. 5C). Their electron microscopic findings were different from those of the original tumor. The cultured cells exhibited extensive development of numerous microvilli, presenting lipid granules at the periphery of the cytoplasm. Well-demarcated GOLGI apparatus and small vesicles were observed in the cytoplasm where rER, free ribosomes and multiple microfilaments were recognized (Fig. 6). Mitochondria were large rod-shaped with tubular cristae, and myelinated dense bodies (5, 22) were occasionally found.

Cultured cells from adrenocortical carcinoma were polygonal, round, spindle-shaped about 2 days later (Fig. 7A). On the 14th day the surface of the cultured cells looked wavy in phase contrast microscopic pictures and overlapped presenting an alveolar pattern. These cells were not containing lipid droplet (Fig. 7B) but active for 3β -HSD (Fig. 7C). Electron microscopic examination on the primary cultured cells revealed numbers

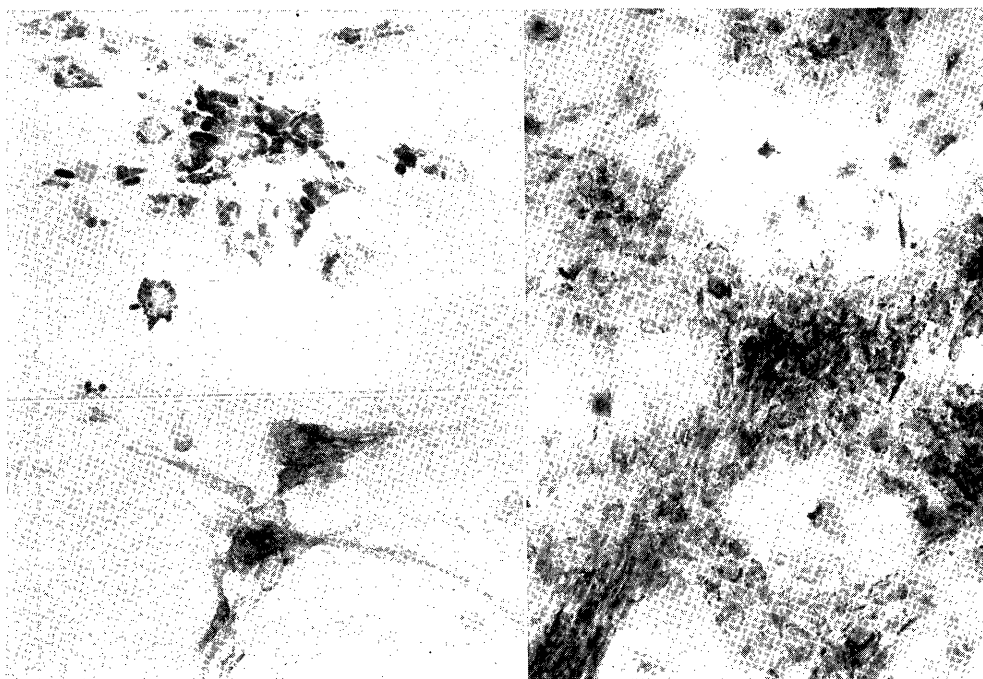


Fig. 5. CONN's adenoma in culture.

A. The cultured cells were generally rather polygonal than spindle-shaped 7 days after culture. Oil red O stain. $\times 200$. B. These cells revealed active for 3β -HSD. $\times 200$. C. The cultured cells tended to overlap 33 days after culture. $\times 100$.

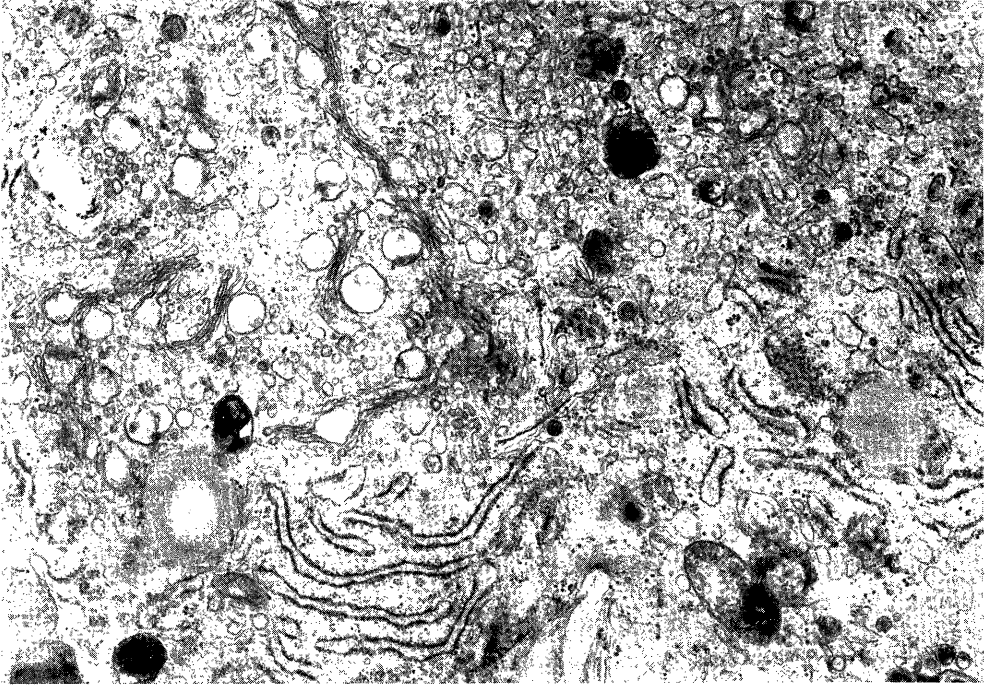


Fig. 6. Electron micrograph of CONN's adenoma in culture. GOLGI apparatus and small vesicles were well-developed. $\times 9000$.

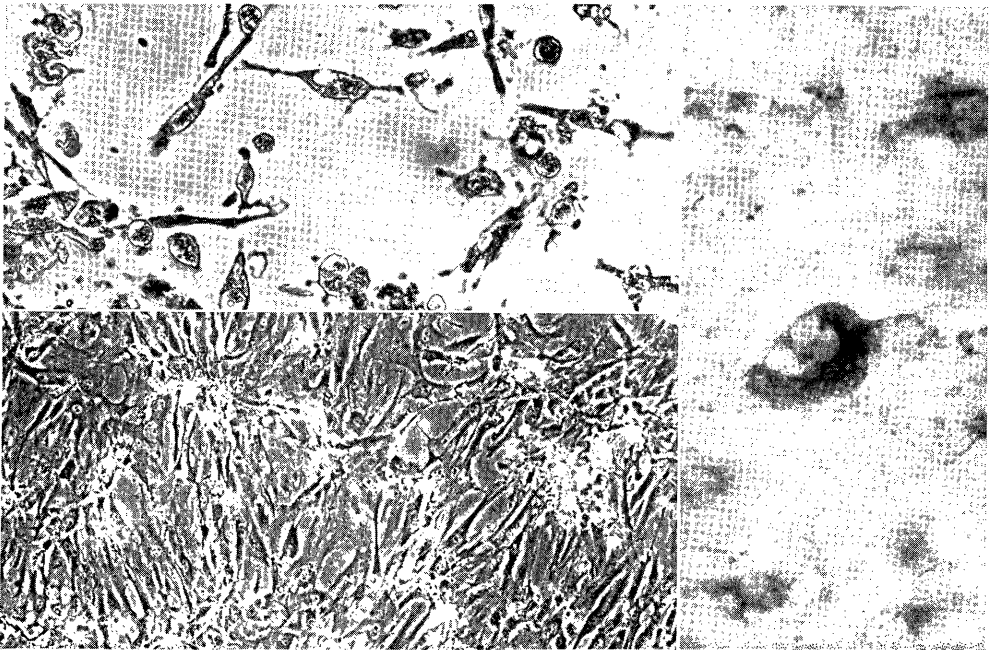


Fig. 7. Adrenocortical carcinoma in culture.
 A. Phase contrast micrograph 2 days after culture. $\times 200$.
 B. Phase contrast micrograph 14 days later. $\times 100$.
 C. stained with 3β -HSD. $\times 400$.

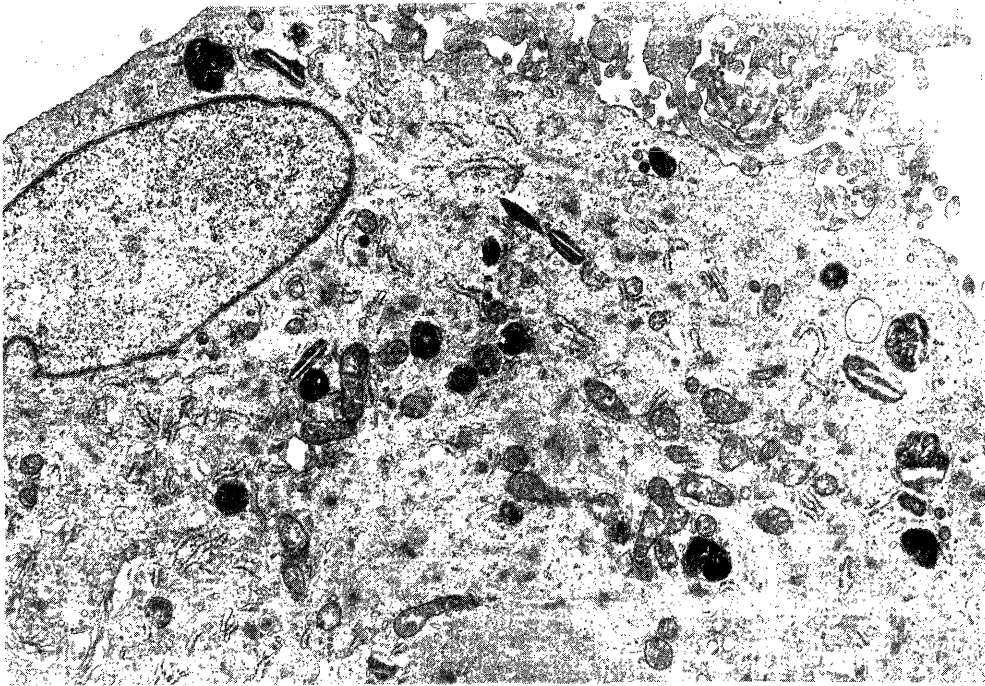


Fig. 8. Electron micrograph of adrenocortical carcinoma in culture. The cultured crystalline-shaped bodies. $\times 3100$.

Table 2. Cortisteroid contents in culture medium

	Time of culture (days)	Aldosterone (ng/dl)	Cortisol (μ g/dl)
CUSHING's adenoma	8	6	18.3
	11	4	3.4
CONN's adenoma (No.1)	38	7	49.4
	67	undetectable	undetectable
Control		undetectable	undetectable

control: Modified EAGLE's Medium containing a 10% fetal calf serum

microfilaments and microtubules. The majority of the microfilaments were irregularly arranged in the matrix, partially bundled up, and whirled the vacuoles. Multiple microvilli and filopodia protruded irregularly from the cell surface, and some of them contacted each other forming interdigitation or desmosome-like structure. The nuclei were frequently indented and the cytoplasm was containing numerous rER, polysomes and free ribosomes. The rER were not parallel but irregular in arrangement. Mitochondria were round and large, rod-shaped with tubular cristae. Occasionally GOLGI apparatus and small vesicles were recognized relatively near the nucleus. There were crystalline-shaped bodies, vacuoles and lysosomes in the matrix (Fig. 8). In the 3rd

passage, myelinated dense bodies⁵⁾²²⁾ were present and many rER in parallel arrangement were well-developed throughout the cytoplasm. The hormone content in the culture media of CS and PA are shown in Table 2. These contents were significantly high both in CS and PA-cultures, when the fibroblast-like cells appeared throughout the dishes. As time goes by, the content decreased in value.

DISCUSSION

KAHRI¹²⁾ first described the growth of epithelial cells and cells of mesenchymal origin from explants of fetal and newborn rat adrenal cortex in tissue culture, where first outgrown epithelial cells from the adrenal explants were small with a narrow rim and sharply delimited from pure fibroblasts. YASUMURA *et al.*²¹⁾ reported two types of clonal strains of adrenal tumor cultures (transplantable mouse adrenal tumor) when they established Y-1 cell. One type was morphologically fibroblastic without performing adrenal specific functions and the other was epithelial cells. O'HARE *et al.*¹⁷⁾ eventually found the overgrowth of fibroblast-like cells in monolayer adrenal culture. LIE *et al.*¹⁴⁾ explanted fragments of adrenocortical tumor in order to cause CUSHING's syndrome associated with moderate hirsutism. The explant technique did not give rise to growth of any cells indicative of epithelial cell, but instead, yielded the growth of fibroblastic cells in culture. AUSLAENDER *et al.*⁴⁾ cultivated a human adrenocortical adenoma in vitro and described that the structure of the cultured cells changed in the course of time and underwent fibroblastoid transformation. It has been reported that isolated cells in adrenocortical cell culture begin to change into fibroblast-like cells. The transformation of the cultured cells from NHA and adrenocortical tumor into fibroblast-like cells was observed by a phase contrast microscope in the present study, too. In CS and ACC cultures fibroblast-like cells appeared throughout the dishes early in the first passage, but those in NHA and PA cultured developed from the periphery of the colonies after 10 days passage. Furthermore, lipid droplets were not present in the fibroblast-like cells of either CS and ACC but were found in those of NHA and PA. KAHRI¹²⁾ reported that epithelial cells in the culture of the rat adrenal cortex were moderately or strongly active for 3β -HSD for which pure fibroblasts were negative. O'HARE *et al.*¹⁷⁾ described that proliferating fibroblast-like cells stained intensely with 3β -HSD. Therefore, 3β -HSD staining could be employed to distinguish adrenocortical cells from fibroblasts. In this experiment, a few fibroblast-like cells of NHA, CS and PA were slightly active for 3β -HSD. Moreover, the ACC-cultured cells stained intensely with 3β -HSD. The contents of aldosterone and cortisol in the culture media of CS and PA were measured by radioimmunoassay, when the fibroblast-like cells appeared throughout the dishes. As a result, these contents were significantly high both in CS and PA-cultures and it is likely that these fibroblast-like cells might produce aldosterone and cortisol. PA-cultured cells, however, did not secrete aldosterone or cortisol on the 67th day of culture.

An electron microscope revealed characteristics varying from those of the original

adrenocortical cells in all cases, while the cultured cells of NHA in the first passage resembled the original cells in features. The cytoskeletal system²³⁾ was well-developed, particularly the ACC-cultured cells contained numerous microfilaments and microtubules. The microfilaments were seen running irregularly throughout the cytoplasm and were partly bundled up to form stress fibers. The GOLGI apparatus and rER were well-developed. Numerous free ribosomes and/or polysomes were seen throughout the cytoplasm. The shape of mitochondria in vivo²⁴⁾ varies in the zone of the adrenocortical cortex, which, in other words, can be discriminated by the shape of mitochondria one zone from another. Mitochondria of the cultured cells, however, were generally elongated with tubular cristae. KAWAOI *et al.*¹³⁾ reported that on section of Y-1 cell which maintains the ability to secrete corticosteroids in vitro, mitochondria were short, or rod-shaped or ellipsoidal. Consequently, the adrenocortical cells in vitro is likely to present the common morphological features of mitochondria to all. In all the cases microvilli were prominent on the cell surface, from which filopodia protruded. These cultured cells, however, contacted each other with interdigitation and desmosome-like structures, indicative of the epithelial origin.

It is generally agreed that the steroid-secreting cells in the ovary and testis derive from fibroblast-like cells (9, 16). Compared with this theory, the present result in which adrenocortical cells underwent fibroblastoid transformation in vitro is very interesting. It is desirable to perform further studies on the relation between fibroblastoid transformation and steroidogenesis of the neoplastic adrenocortical cells in culture.

ACKNOWLEDGMENTS

Grateful acknowledgement is made to Prof. H. TSUCHIYAMA and Assoc. Prof. K. KAWAI for their constant interest and guidance in this investigation. Special thanks go to Prof. H. SUGIHARA (Dept. of Pathology, Saga Medical College), Prof. K. MIFONE (Dept. of Microbiology, Oita Medical College), Prof. A. IGARASHI (Dept. of Virology, Nagasaki University Institute for Tropical Medicine) for their technical advice on culture and Prof. T. AIKAWA (Department of Physiology, Nagasaki University School of Medicine) for their technical advice on radioimmunoassay.

REFERENCES

- 1) AIKAWA, T., HIROSE, T., MATSUMOTO, I. and SUZUKI, T.: Secretion of aldosterone in response to histamine in hypophysectomized-nephrectomized dogs. *J. Endocr.* 81:325-330, 1979.
- 2) ANDREIS, P. G., MANTERO, F. and ARMATO, U.: The primary tissue culture of human adrenocortical CONN'S adenomata. *Path. Res. Pract.* 173: 66-81, 1981.
- 3) ARMATO, U., NUSSDORFER, G. G., NERI, G., DRAGHI, E., ANDREI, P. G., MAZZOCHI, G. and MANTERO, F.: Effects of ACTH and 3' 5'-Cyclic purine nucleotides on the morphology and metabolism of normal adult human adreno-

- cortical cells in primary tissue culture. *Cell Tiss. Res.* 190: 189-205, 1978.
- 4) AUSLAENDER, L., PHLEG, I. and BARZILAI, D.: A human adrenocortical adenoma in tissue culture. *Israel. J. Med. Sci.* 11: 1106-1113, 1976.
 - 5) BRANCHAUD, C. I., GOODYER, C. G., HALT, C. St. G., ARATO, J. S., SILMAN, R. E., and GIROUD, C. T. P.: Steroidogenic activity of hACTH and related peptides on the human neocortex and fetal adrenal cortex in organ culture. *Steroid* 31: 557-572, 1978.
 - 6) DOUGIAS, M. G.: Effects of high potassium diet angiotensin II receptor and angiotensin-induced aldosterone production in rat adrenal glomerulosa cells. *Endocrinology* 106: 983-990, 1980.
 - 7) DURAND, P., CATHIARD, A. M., LOCAELLI, A. and SAEZ, J. M.: Modification of the steroidogenic pathway during spontaneous and adrenocorticotrophin induced maturation of ovine fetal adrenal. *Endocrinology* 110: 500-505, 1982.
 - 8) FANG, V.: Establishment and characterization of a strain human adrenal tumor cells that secrete estrogen. *Proc. Natl. Acad. Sci. USA.* 74: 1067-1071, 1977.
 - 9) FUJITA, H. and HIURA, M.: Origin of steroid-secreting cells in the ovary. *The Cell* 12: 656-663, 1980. (in Japanese)
 - 10) HORNSDY, P. J. and GILL, G. N.: Hormonal control of adrenocortical cell proliferation. *J. Clin. Invest.* 60: 342-352, 1977.
 - 11) HORNSBY, P. J., SIMONIAN, H. and GILL, G. N.: Aging of adrenocortical cells in culture. *Internatl. Rev. Cytol. Suppl.* 10: 131-161, 1979.
 - 12) KAHRI, A.: Histochemical and electron microscopic studies on the cells of the rat adrenal cortex in tissue culture. *Acta Endocr. (Kbh), Suppl.* 108: 1-96, 1966.
 - 13) KAWAOI, A., UCHIDA, T. and OKANO, T.: Transmission and scanning electron microscopic observation of mouse adrenocortical adenoma cell (Y-1) in non-stimulated and stimulated states. *Acta Path. Jap.* 27: 841-856, 1977.
 - 14) LIE, S. O., HAVNEN, J. and DJØSELAND, O.: CUSHING's syndrome in infancy; a case report with studies of the hormone producing cells in vitro. *Acta Endocr.* 71: 141-152, 1972.
 - 15) MATTSON, P. and KOWAL, J.: Effects of cytochalasin B on unstimulated and adrenocorticotrophin-stimulated adrenocortical tumor cell in vitro. *Endocrinology* 111: 1632-1647, 1982.
 - 16) MURAKAMI, M.: Development of testicular steroid-secreting cells and its clinical elucidation. *The cell* 12: 648-655, 1980. (in Japanese)
 - 17) O'HARE, M. J., MONAGHAN, P. and NEVILL, A.: The pathology of adrenocortical neoplasia; a correlated structural and functional approach to the diagnosis of malignant disease. *Human Pathology* 10: 137-154, 1979.
 - 18) SHARMA, R. K. and HASHIMOTO, K.: Ultrastructural studies and metabolic regulation of isolated adrenocortical carcinoma cells of rat. *Cancer Res.* 32: 666-674, 1972.
 - 19) SZABO, D., GYEVAI, A., GLAZ, E., STARK, E., PETERI, M. and AIANT, O.: Change in the fine structure and function of hormone-secreting adrenocortical tumor investigated in tissue culture. *Virchows Arch. A.* 367: 273-286, 1975.
 - 20) TSUCHIYAMA, H., SUGIHARA, H. and KAWAI, K.: Electron microscopic observation of the human adrenal cortex. *J. Clin. Electron Microscopy* 4: 877-881, 1971.

(in Japanese)

- 21) YASUMURA, Y., BUONSNINI, V. and SATO, G.: Clonal analysis of differentiated function in animal cell cultures. *Cancer Res.* 26: 529-535, 1966.
- 22) UNSICKER, K., CHAMLEY, J. H. and MCLEAN, J.: Extraneuronal effects of 6-hydroxydopamine. *Cell Tiss. Res. Pract.* 174: 83-97, 1976.
- 23) WEBEC, K. and OSBORN, M.: Cytoskeleton; definition, structure and gene regulation. *Path. Res. Pract.* 175: 128-145, 1982.
- 24) ZWIERZINA, W. D.: Ultrastruktur eines hormonaktiven und eines endokrin stummen Nebennierenrindenzarzinoms. *Zb. allg. Pathol. u. Pathol. Anat.* 126: 269-276, 1982.