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# Ultrastructural Appearance and Cytoskeletal Architecture of the Clear, Chromophilic, and Chromophobe Types of Human Renal Cell Carcinoma *in Vitro*

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***The clear, chromophilic, and chromophobe types of human renal cell carcinoma have been defined as distinct morphological entities and can be clearly separated by differences of ultrastructural appearance, cytoskeletal architecture, enzyme synthesis, and cytogenetic aberrations. In this report, the cytomorphological aspects of these tumor types are compared* in vitro, *showing that essential ultrastructural and cytoskeletal characteristics of each tumor type are expressed even after prolonged* in vitro *cultivation. The pattern of intermediate filament proteins of each tumor type was preserved* in vitro, *permitting the separation of exclusively cytokeratin-positive chromophobe tumor cells from clear and chromophilic tumor cells with a co-expression of vimentin and cytokeratins.* In vitro, *the chromophobe tumor cells continued to exhibit abundant cytoplasmatic microvesicles and sparsely distributed "studded" vesicles, which are known to be characteristic features of this tumor type in* vivo. *This observation confirmed the structural similarity of the chromophobe cell to the 'intercalated cell' of the cortical collecting duct and provided further evidence for the histogenetic derivation of this tumor subtype from the collecting duct system. (Am J Pathol 1993, 142:851–859)***

The normal epithelium of the kidney consists of morphologically and functionally distinct cell types that are arranged along the nephron and collecting ducts.[1–4] The marked cytomorphological heterogeneity of normal renal epithelia is also reflected by the broad spectrum of cell types observed in different human renal cell tumors. The main cell type present in the majority of all human renal cell carcinomas is the clear cell showing a highly transparent and structureless ('empty') cytoplasm due to an abundance of glycogen and lipid.[5–7] Another type of human renal cell carcinoma has only recently been referred to as chromophobe renal cell carcinoma. This tumor type, which was first described by Bannasch et al[8,9] in experimental tumors and by Thoenes et al[10] for the first time in human tumors, likewise shows tumor cells with a translucent, but finely reticular (not 'empty') cytoplasm. The chromophilic tumor type[11] comprises tumors with cells, the cytoplasm of which is not clear and not chromophobe, but exhibits basophilia or a fine-granular eosinophilia.

The clear, chromophilic, and chromophobe types of human renal cell carcinoma were shown to make up the vast majority of all epithelial renal cell tumors in adults[11] and clearly can be separated from other rare tumor types such as the spindle-shaped/pleomorphic carcinoma,[11] the Bellini Duct carcinoma,[12,13] and the benign oncocytoma.[11,14,15] The cytomorphological separation between clear, chromophilic, and chromophobe carcinomas was further substantiated by differences in ultrastructural appearance, cytoskeletal architecture, antigenic profile, and enzyme synthesis,[10,11,15–18] as well as distinct cytogenetic aberrations.[19–23] Preliminary data also suggest a relationship between the different cytomorphological types of human renal cell tumors and patients' survival times.[24]

Because cell lines are irreplacable tools for investigations into the biological properties of renal cell

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carcinoma, the proposed subclassification of the original tumors should also be transferred to the corresponding cell lines. In previous reports on permanent cell lines, however, the distinction between the different types of human renal cell carcinoma has not been made (for review, see refs. 25 to 27). The aim of the present investigation, therefore, was to demonstrate that essential ultrastructural and cytoskeletal features of the clear, chromophilic, and chromophobe types of renal cell carcinoma are preserved even after prolonged *in vitro* cultivation. This observation underscores the validity of a consequent distinction between the different types of renal cell carcinoma as a basic prerequisite for future studies on differences in their biological behavior both *in vivo* and *in vitro*.

## Materials and Methods

### Tissue Culture

The original tumors were typical representatives of the clear, chromophilic, and chromophobe types of renal cell carcinoma, as became evident from light microscopy, transmission electron microscopy, and immunohistochemistry (data not shown) (*cf* refs. 10, 11, 15, 16, and 17).

Tumor samples were obtained immediately after nephrectomy from 1) a 52-year-old man with a renal cell carcinoma of the clear cell type (pT2, pNX, pMX); 2) a 64-year-old man with a renal cell carcinoma of the chromophilic type (pT3b, pNX, pMX); and 3) a 61-year-old man with a renal cell carcinoma of the chromophobe type (pT3, pNX, pMX).

The tumor samples were minced under aseptic conditions with paired scissors. The resulting mechanically macerated tissue mass was repeatedly washed by centrifugation and finally seeded into 25 $cm^2$ Nunclon culture flasks (Gibco, Karlsruhe, Germany) with Dulbecco's modified Eagle's medium (Gibco), supplemented with fetal calf serum, penicillin, and streptomycin. The cultures were maintained at 37 C in an atmosphere with 5% $CO_2$. For subculturing, cells were disaggregated by exposure to 0.05% EDTA (Biochrom, Berlin, Germany).

The tumor cells became adherent within 7 to 10 days after seeding, forming small colonies during the next few days. Fibroblastic contamination did not prove to be a major problem because fibroblasts could be removed by selective trypsinization during the following passages. The tumor samples obtained from the clear and chromophilic types of renal cell carcinoma gave rise to permanent cell lines. The clear-cell carcinoma cell line is presently at the 148th passage after 4 years in permanent culture with a mean population doubling time of about 72 hours. The chromophilic carcinoma cell line is presently at the 192th passage after 4 years in permanent culture with a mean population doubling time of about 24 hours. The chromophobe renal cell carcinoma could only be established as a short-term culture for about 8 months with a splitting ratio of 1:1 up to passage number 18. After an initial period of rapid outgrowth, the proliferation of the chromophobe tumor cells slowed down and during the last passages the tumor cells showed degenerative changes with an increase in cell size and cytoplasmatic vacuolization.

Our studies were performed with cells from passages 10 to 15, describing the different cell types at an early *in vitro* stage before the selection of certain tumor subpopulations by prolonged *in vitro* cultivation. Nevertheless, the morphologic features of the clear and chromophilic carcinomas remained remarkably stable and were still present after 4 years of permanent culture.

### Light Microscopy

The tumor cells cultivated *in vitro* were seeded on microscope slides and fixed *in situ* by immersion in 4% formaldehyde. The slides were stained with hematoxylin-eosin, periodic acid-Schiff (PAS) hemalum and Hale's acid colloidal iron method modified by Mowry.[28]

### Immunocytochemistry

For immunocytochemistry, the cultivated tumor cells were seeded on microscope slides, fixed *in situ* by exposure to methanol (5 minutes) and acetone (10 second) at −20 C and then air-dried. Primary antibodies[15,16,29] were applied to the slides and allowed to incubate for 30 minutes at room temperature in a moist chamber. The mouse monoclonal antibodies used (and their specificity) were MAb AE14 (cytokeratin 5), kindly provided by Dr. T.-T. Sun, Department of Dermatology, New York University, Medical Center, New York; MAb CK-7 (cytokeratin 7), from Boehringer, Mannheim, Germany; MAb CAM 5.2 (cytokeratin 8), from Becton-Dickinson, Neckargemünd, Germany; MAb $K_s$18.174 (cytokeratin 18), MAb $K_s$19.2.105 (cytokeratin 19), and MAb IT-$K_s$ 20.3, 5 and guinea pig antibodies (cytokeratin 20), from Progen Biotechnics, Heidelberg, Germany; and MAb VIM-9 (vimentin), from Viramed, Martinsried, Germany. The visualization of the primary antibody was achieved by the immunoperoxidase method [*cf* ref. 16]; reagents were from DAKO (Hamburg, Germany).

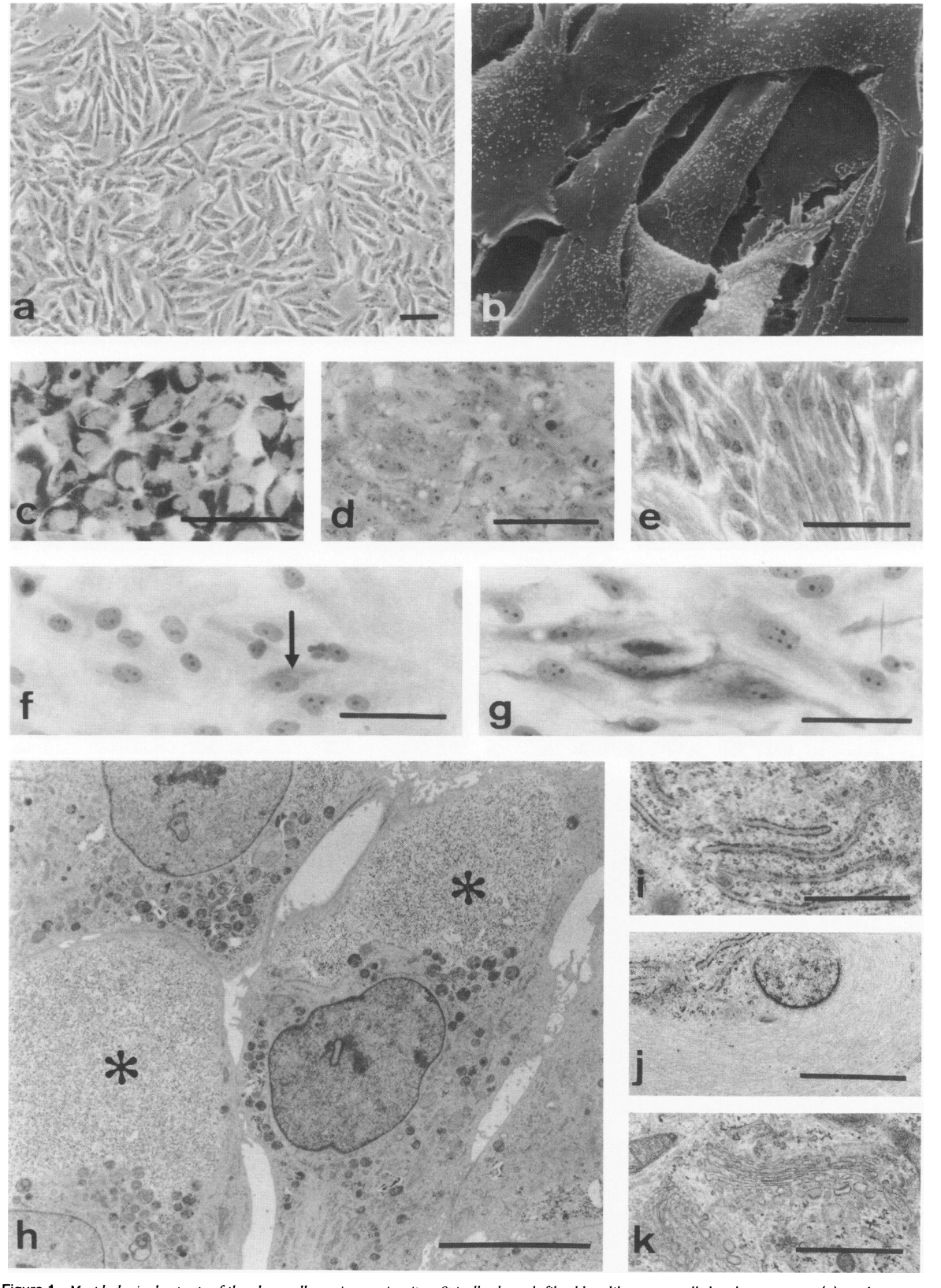
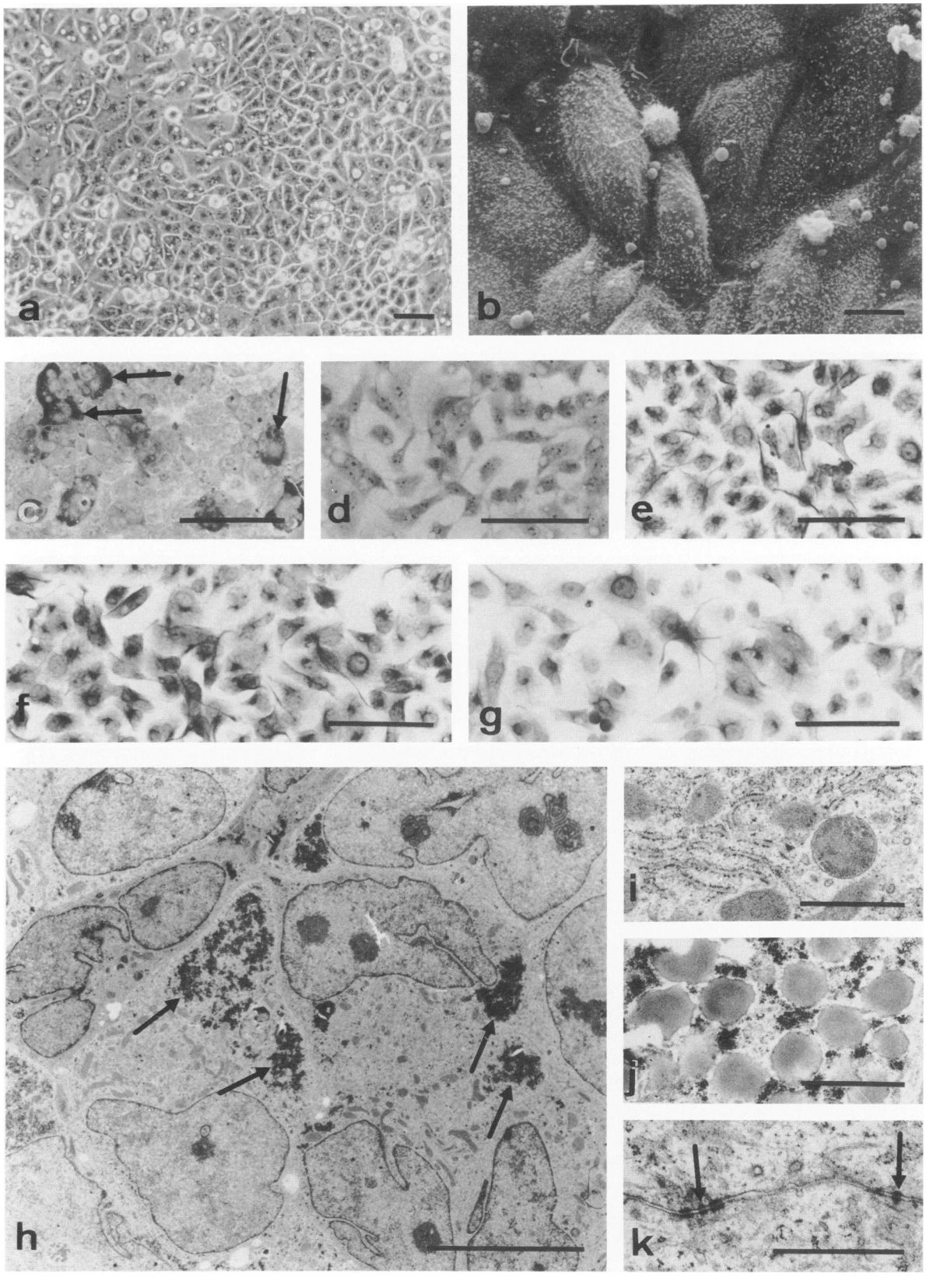



**Figure 1.** *Morphological aspects of the clear cell carcinoma in vitro. Spindle-shaped, fibroblast-like tumor cells by phase contrast* (a) *and scanning electron* (b) *microscopy. Intensively positive cytoplasmatic staining with PAS* (c) *and diffuse, weakly positive staining with Hale* (d). *Positive immunostaining for vimentin* (e) *in all tumor cells and for cytokeratins 18* (f, *arrow*) *and 19* (g) *in approximately 10% and 60% of the tumor cells, respectively. Large amounts of monoparticulate glycogen* (h, *asterisks*) *in the cell periphery and cytoplasmatic organelles such as rough endoplasmatic reticulum* (i), *intermediate filaments* (j), *and Golgi complexes* (k) *around the nucleus.* a, c *to* g, *scale bar = 100 μ;* b, h, *scale bar = 10 μ;* i *to* k, *scale bar = 1 μ.*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### *Transmission Electron Microscopy*

For transmission electron microscopy, tumor cells seeded on glass coverslips were fixed by exposure to 2.5% sodium-cacodylate–buffered glutaraldehyde solution (0.1 mol/L, pH 7.4) and postfixed in 1% sodium-cacodylate–buffered osmium tetroxide solution (0.1 mol/L, pH 7.4) before Epon embedding. Thin sections were contrasted with uranyl acetate and lead citrate. Electron photomicrographs were taken with an EM 410 Philips transmission electron microscope.

### *Scanning Electron Microscopy*

For scanning electron microscopy, the tumor cells were seeded on glass coverslips, fixed *in situ* by exposure to 2.5% phosphate-buffered glutaraldehyde solution (0.1 mol/L, pH 7.2) and postfixed in 2% osmium tetroxide solution. After dehydration in an ascending acetone series, the tumor cell monolayer was dried by the critical point method and sputtered with gold. Electron photomicrographs were taken with a PSEM 501 scanning electron microscope.

## *Results*

### *Clear Renal Cell Carcinoma* in Vitro

Using phase contrast and scanning electron microscopy it was found that the tumor cells exhibited a spindle-shaped, fibroblast-like appearance, being loosely apposed in semiconfluent cultures and growing strictly anchorage dependent (Figure 1, a and b). PAS staining revealed considerable amounts of glycogen in almost every tumor cell (Figure 1c). In contrast to the negative staining reaction of the original tumor in vivo, the Hale staining for acid mucopolysaccharides produced a diffuse, weakly positive staining of the cytoplasm *in vitro* (Figure 1d).

Immunocytochemically, all the tumor cells exhibited an intensively positive staining reaction with antibodies against vimentin (Figure 1e). Only 10% of the tumor cells showed a positive reaction with antibodies against cytokeratins 8 (not shown) and 18 (Figure 1f), but about 60% of the tumor cells exhibited a staining reaction for cytokeratin 19 (Figure 1g). No staining reaction was observed with antibodies against cytokeratins 5, 7, and 20.

With transmission electron microscopy, most tumor cells showed large amounts of monoparticulate glycogen (Figure 1h), whereas lipid droplets were only occasionally observed. Cytoplasmatic organelles such as mitochondria, rough endoplasmatic reticulum, Golgi complexes, and bundles of intermediate filaments were concentrated around the nuclei (Figure 1, i to k). Microvillus-like cytoplasma protrusions and desmosome-like junctions were rarely observed.

### *Chromophilic Renal Cell Carcinoma* in Vitro

Using phase contrast and scanning electron microscopy, the tumor cells were found to exhibit an epithelium-like aspect, growing as a strict monolayer in tightly apposed tumor cell complexes (Figure 2, a and b) with numerous short microvillus-like cytoplasmatic projections (Figure 2b). PAS staining revealed a considerable heterogeneity in the amount of glycogen deposition. Thus, tumor cells with an intensively positive PAS-staining reaction were scattered between tumor cells without appreciable cytoplasma staining (Figure 2c). The Hale staining for acid mucopolysaccharides produced a diffuse weakly positive staining of the cytoplasm (Figure 2d).

Immunocytochemically, the tumor cells showed an intensively positive staining reaction with antibodies against vimentin (Figure 2e) and cytokeratins 8 (not shown) and 18 (Figure 2f). Antibodies against cytokeratin 19 produced a positive staining reaction in tiny groups of tumor cells (not shown). Antibodies against cytokeratin 20 revealed a positive staining in 80% of the tumor cells (Figure 2g). No staining reaction was observed with antibodies against cytokeratins 5 and 7.

By transmission electron microscopy, the tumor cells were closely apposed, sometimes exhibiting large deposits of monoparticulate glycogen (Figure 2h). Mitochondria and profiles of rough endoplasmatic reticulum (Figure 2i) were distributed rather evenly throughout the cytoplasm, sometimes intermingled with small aggregates of lipid droplets (Figure 2j). Desmosome-like junctions were only occasionally observed (Figure 2k).

**Figure 2.** *Morphological aspects of the chromophilic carcinoma in vitro. Closely apposed, epithelium-like tumor cell complexes by phase contrast* (a) *and scanning electron* (b) *microscopy. Intensively positive cytoplasmatic PAS staining in some tumor cells* (c, *arrows*) *and a diffuse, weakly positive staining for Hale* (d) *in most tumor cells. Positive immunostaining for vimentin* (e) *in all tumor cells and for cytokeratins 18* (f) *and 20* (g) *in 100% and 80% of the tumor cells, respectively (part of the immunoreaction for cytokeratins is concentrated in paranuclear aggregates). Deposits of monoparticulate glycogen* (h, *arrows*) *in some tumor cells. Evenly distributed cytoplasmatic organelles such as rough endoplasmatic reticulum and mitochondria* (i) *as well as lipid droplets* (j) *and occasional desmosome-like junctions* (k, *arrows*). a, c *to* g, *scale bar = 100 μ;* b, h, *scale bar = 10 μ;* i *to* k, *scale bar = 1 μ.*

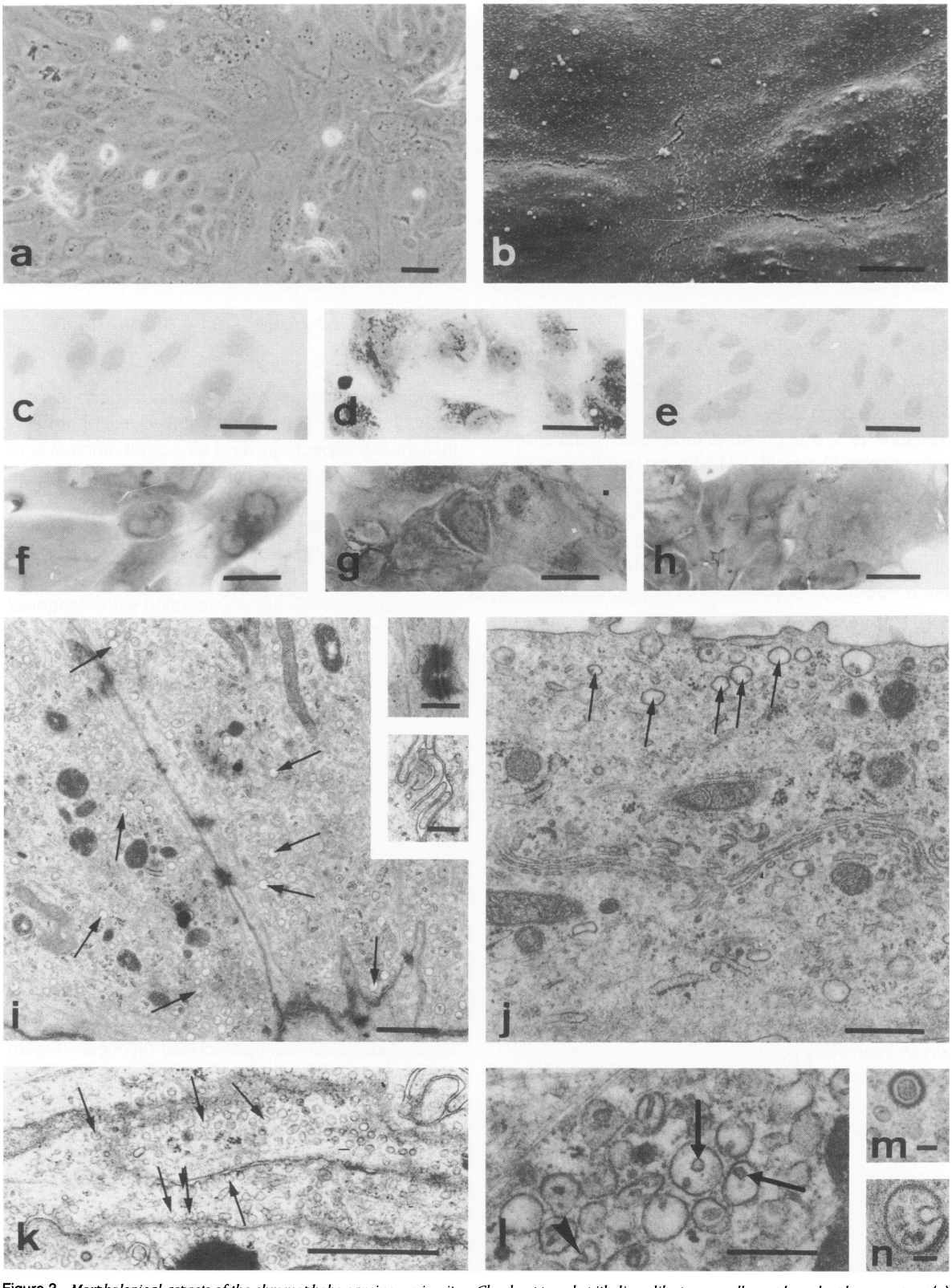


Figure 3. *Morphological aspects of the chromophobe carcinoma in vitro. Closely apposed epithelium-like tumor cell complexes by phase contrast* (a) *and scanning electron* (b) *microscopy. Negative staining results with PAS* (c) *and a finely granular staining of the cytoplasm with Hale* (d). *Negative immunostaining for vimentin* (e) *and a positive staining reaction for cytokeratins 7* (f), *18* (g), *and 19* (h) *in all tumor cells. Closely apposed tumor cells* (i) *with typical desmosomes* (i, *inset*) *and intricate cytoplasmatic interdigitations* (i, *inset*). *Abundant cytoplasmatic microvesicles* (i *to* l, *arrows*), *concentrated near the apical surface as revealed by tangential* (i, k) *and vertical* (j) *sectioning. Cytoplasmatic microvesicles showing 'inner vesicles'* (l, *arrow;* m), *a 'studded' surface* (m), *or vesicular invaginations* (l, *arrowhead;* n). a, *scale bar = 100 μ;* b, *scale bar = 10 μ;* c *to* h, *scale bar = 50 μ;* i, k, *scale bar = 1 μ;* j, l, *scale bar = 0.5 μ;* m, n: *scale bar = 0.1 μ; insets in* i, *scale bar = 0.25 μ.*

### *Chromophobe Renal Cell Carcinoma* in Vitro

With phase contrast and scanning electron microscopy, the tumor cells exhibited an epithelium-like aspect growing as strict monolayers in tightly apposed tumor cell complexes (Figure 3, a and b). PAS staining revealed no appreciable deposits of glycogen (Figure 3c). Hale's staining for acid mucopolysaccharides produced a diffuse, but sometimes also finely granular staining of the cytoplasm (Figure 3d).

Immunocytochemically, the tumor cells did not show a staining reaction with antibodies against vimentin (Figure 3e). Antibodies against cytokeratins 7 (Figure 3f), 8 (not shown), 18 (Figure 3g), and 19 (Figure 3h) produced a positive staining reaction in all tumor cells.

Transmission electron microscopy showed that the tumor cells were closely apposed, exhibiting either a smooth cell surface with typical desmosomes or intricate cytoplasmatic interdigitations (Figure 3i). Most tumor cells showed abundant cytoplasmatic microvesicles (diameter, 150 to 300 nm) that were concentrated near the apical surface as revealed by tangential (Figure 3, i and k) and vertical (Figure 3j) sections. The microvesicles near the cell surface were often continuous with the extracellular space, whereas microvesicles positioned deeper in the cytoplasm proved to be closed structures (Figure 3l). A proportion of the microvesicles showed 'inner vesicles,' vesicular invagination, or a 'studded' surface (Figure 3, l, m, and n). Autophagosomes containing numerous microvesicles were occasionally observed. Golgi complexes were rather prominent (Figure 3j). Mitochondria, profiles of rough endoplasmatic reticulum, polyribosomes, and bundles of intermediate filaments were evenly distributed throughout the cytoplasm. Deposits of monoparticulate glycogen were not observed, whereas lipid droplets were occasionally seen.

## *Discussion*

The results of our investigation demonstrate that the characteristic cytoskeletal and ultrastructural aspects of the clear, chromophilic, and chromophobe tumor cell types are preserved to a large extent even after prolonged in vitro cultivation. This observation holds true especially for the chromophobe renal cell carcinoma, which has only recently been recognized as a distinct type of renal cancer.[10,11,15–17,30,31] Chromophobe renal cell carcinomas *in vivo* show a transparent, but fine-reticular cytoplasm with a positive Hale's staining reaction and numerous cytoplasmatic microvesicles as revealed by transmission electron microscopy. In contrast to the clear and chromophilic cell types, the chromophobe tumor cells exclusively express cytokeratins (nos. 7, 8, 18, and 19), but not vimentin. In our investigation, these cytomorphological characteristics of the chromophobe tumor type were almost completely preserved even after prolonged cultivation *in vitro* for 6 months. No appreciable amounts of glycogen could be detected *in vitro* either by PAS staining or by transmission electron microscopy, and the tumor cells exhibited exclusively cytokeratin polypeptides but not vimentin. Most importantly, however, the chromophobe tumor cells persistently showed abundant cytoplasmatic microvesicles in their apical poles, and 'studded' vesicles were occasionally observed. These *in vitro* observations further confirmed the close resemblance between chromophobe tumor cells and the intercalated cells of the collecting duct system. In the meantime, convincing arguments have been accumulated[17] suggesting that the chromophobe tumor cell closely resembles—and possibly also originates from—the intercalated cell, which has been observed in the collecting duct system of rat[3,32–34] and human[1,17] kidneys and which contains numerous cytoplasmatic microvesicles and occasional 'studded' vesicles. Furthermore, it has been shown that the chromophobe tumor cells exhibit a strong carbonic anhydrase activity,[17] which is known to be a characteristic feature of the intercalated cells.[35,36] (Unfortunately, the *in vitro* carbonic anhydrase activity could not be determined in the chromophobe tumor cells of our investigation). In this context, however, it was interesting to note that Hale's colloidal iron staining method, which exclusively shows a positive staining reaction with chromophobe tumor cells *in vivo*[10,11,16,17,31] lost its specificity *in vitro*. This observation suggests that the specific staining of chromophobe tumor cells *in vivo* with Hale's colloidal iron need not necessarily be associated with the formation of microvesicles as previously supposed.[17]

The comparison of the cytoskeletal architecture both *in vivo* and *in vitro* further confirmed that renal cell carcinomas can be divided into two distinct groups: 1) clear and chromophilic renal cell carcinomas that express both vimentin and cytokeratins, and 2) chromophobe renal cell carcinomas that express cytokeratins but not vimentin.[15–17,30,31] The expression of both vimentin and cytokeratins in the nonchromophobe renal cell carcinomas is intriguing in that the co-expression of these cytoskeletal proteins has not been detected in their alleged cell type of origin, ie, proximal tubule epithelium.[37–43] It is known, however, that the embryonic morphogenesis

of the proximal renal tubules proceeds from exclusively vimentin-positive mesenchymal cells to exclusively vimentin/cytokeratin-positive pretubular vesicles and prospective proximal tubules.[29] The vimentin gene might, therefore, simply become reactivated in the proximal tubular cells during the process of malignant transformation.

The separation of the clear and chromophilic type of human renal cell carcinoma as distinct morphological entities was primarily based on cytomorphological criteria, eg, the abundance of cytoplasmatic lipid and glycogen deposits in the clear cell variant.[11] In addition, the predominance of the tubulopapillary growth pattern in chromophilic tumors[11] as well as distinct cytogenetic aberrations[19–23] further justified the separation of these two tumor types. Nevertheless, the clear and chromophilic tumor cell types seem to be more closely related to one another than to the chromophobe cell type. This assumption is supported by the observation that the rare 'mixed' forms of renal cell carcinoma (less than 3% of all renal cell tumors [*cf* ref. 11]) are exclusively composed of the clear and chromophilic cell types.[11] The close relation of the clear and chromophilic cell types also becomes evident from the extent of lipid and glycogen deposition. The deposition of large amounts of lipid and glycogen is one of the most important cytomorphological criteria for the definition of the clear cell type. Nevertheless, there are randomly scattered tumor cells with only minor deposits of lipid and glycogen in many clear cell carcinomas. Vice versa, there are randomly scattered tumor cells with considerable amounts of lipid and glycogen in many chromophilic renal cell carcinomas. This phenomenon also became evident during *in vitro* cultivation. Thus, the deposition of cytoplasmatic lipid, which had been a prominent *in vivo* feature of the clear renal cell carcinoma of our study, was inconspicuous *in vitro*. In contrast, the chromophilic renal cell carcinoma, which had not exhibited appreciable amounts of cytoplasmatic glycogen *in vivo*, produced randomly scattered tumor cells with abundant glycogen deposits *in vitro* resembling the clear cell type. Further investigations, therefore, will have to show whether the metabolic alterations leading to the excessive deposition of lipid and glycogen are exclusively genetically determined or possibly also modulated by as yet unknown microenvironmental factors.

In conclusion, the results of our investigation demonstrate that essential ultrastructural and cytoskeletal features of the different types of renal cell carcinoma are preserved to a large extent during *in vitro* cultivation. Therefore, the establishment of strictly defined types of renal cell carcinoma as permanent cell lines will be the basic prerequisite for future investigations into the biological characteristics of each tumor type.

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