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Evidence for proximal tubular cell origin of a sarcomatoid variant of human renal cell carcinoma

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Summary. A pure sarcomatoid variant of renal cell carcinoma obtained from a hydronephrotic kidney of an elderly white female was grown in tissue culture. Two parallel cell lines, one from the primary neoplasm and the other from a seeded metastasis within the same kidney have been cultured for more than 60 passages over a period of three years. Structural and functional studies of this neoplasm confirmed that it originated from proximal tubular cells.

Key words: Renal carcinoma – Proximal tubule – Cell culture – Viral susceptibility – Membrane transport

Introduction

Renal cell carcinomas comprise over 80% of all primary malignant renal neoplasms [6]. Nephroblastomas and sarcomas are less common. The older term of renal cell adenocarcinoma has been challenged in view of the mesenchymal origin of the kidney. According to Ewing [13], Robin and Waldeyer were the first to suggest the origin of renal neoplasms from tubular epithelium of the nephron. In contrast, Grawitz [17] postulated that renal cell carcinomas arose from adrenal nests, and therefore erroneously labeled them hypernephromas. Renal cell carcinomas have two distinctive cytomorphologic variants, epithelioid and sarcomatoid. Ultrastructural analysis of clear and granular epithelioid variants of renal cell carcinoma by Oberling et al. suggested a proximal tubular cell origin [32]. The site of origin has subsequently been confirmed by Tannenbaum [38]. The cell of origin of the sarcomatoid variant, on the contrary, has not yet been established.

The purpose of the present study was to characterize the histogenesis of a sarcomatoid variant obtained from a patient with a primary renal

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neoplasm of this type. In vitro techniques were used to investigate this neoplasm. Two parallel lines of cells were grown and maintained in culture for more than 60 passages. The studies described herein evidence that the proximal tubular epithelial cell is the cell of origin for this sarcomatoid variant.

Material and methods

Nephrectomy specimen. The right kidney was surgically removed from a 64-year-old patient with longstanding unilateral ureteral obstruction from postsurgical adhesions. The markedly hydronephrotic kidney was examined by gross and microscopic evaluation. A primary malignant neoplasm was identified, and numerous polypoid metastases had seeded onto the internal surfaces of the distended calyceal system. The patient died six months post-nephrectomy with widespread metastases from the primary renal neoplasm. Prior to death surgical sampling of several metastatic sites revealed neoplastic cells analogous to those of the original primary.

Cell harvest and culture. Small portions of the metastatic papillary projections on the inner lining of the dilated calyces (RCSC) and the solid primary neoplasm (RCC) were aseptically removed from the nephrectomy specimen and placed in chilled (5° C) Hanks balanced salt solution (HBSS) containing the antibiotics amphotericin B (10^{-6}M), penicillin (200 U/ml), and streptomycin (0.2 mg/ml). The two separate tissue fragments labeled RCSC and RCC, were washed three times in HBSS and mechanically minced using sterile scissors. The minced tissues were placed in trypsinization flasks with magnetic stirrers at 37° C and digested in 0.25% trypsin solution (700 U/ml) (K.C. Biological, Lenexa, KS, USA), in HBSS for 45 min [3, 4]. Cell suspensions were centrifuged (600 G) and washed twice. The final cell pellets were resuspended in minimal essential medium (MEM) enriched with 20% calf serum and containing the above antibiotics, and were placed in sterile 25 cm^2 plastic flasks (Falcon 3013, Becton Dickinson Labware, Oxnard, CA, USA). The flasks were incubated at 37° C in a 5% CO_2 incubator. Cell monolayers were formed in the flasks within 14 days. Subcultures were prepared by dispersing the monolayers with trypsin (0.25%) and growing the dispersed cells in the same growth medium but containing 10% calf serum in 35 mm plastic tissue culture dishes (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) as described above. In some experiments either calf serum or antibiotics were omitted. Cells were also grown in Primaria (TM) Falcon tissue culture dishes (Becton Dickinson Labware, Oxnard, CA, USA).

Morphologic studies. Microscopic preparations were made from the nephrectomy specimen using 4% buffered formaldehyde fixative, paraffin embedding, sectioning and staining with hematoxylin and eosin by standard techniques.

For ligth microscopy, the cultured cell monolayers in petri dishes were fixed with 4% phosphate buffered formaldehyde and stained with Giemsa. Stained monolayers were photographed in a Zeiss photomicroscope [36].

Ultrastructural evaluation of the monolayers was performed using both transmission (TEM) and scanning (SEM) electron microscopy. The monolayers in petri dishes were fixed overnight in phosphate buffered (pH 7.3) 2% glutaraldehyde and postfixed with osmium tetroxide. Samples for TEM were embedded in acrylic resin L.R. White (London Resin Co., England), sectioned in an LKB ultramicrotome III, stained with uranyl acetate and lead citrate and photographed in a Zeiss EM 10 transmission electron microscope [11]. Samples for SEM were dehydrated in graded ethanol, critical point dried in a carbon dioxide chamber, and coated with gold. The mounted specimens were photographed in a JEOL-35 scanning electron microscope [11].

Chromosome analysis. RCC cells (passage 34) and RCSC cells (passage 37) were treated with colchicine (added to the culture medium in a concentration of 0.02 mg/ml) for 5 h at 37° C. The cultured monolayers were dispersed with trypsin and washed. Centrifuged pellets were

treated with a hypotonic solution of 75 mM KCl at room temperature for 25 minutes. The cells were fixed overnight in a 3:1 mixture of methanol-acetic acid and stained with Wright's stain [4].

Heterotransplantation into athymic nude mice. Monolayers of cultured cells (RCC passages 43, 45 and 50, and RCSC passage 56) were dispersed with trypsin, centrifuged, washed twice with HBSS and resuspended in the same medium. Cell viability was assessed using the trypan blue exclusion method [3]. Cells were counted and loaded into a 1 ml syringe and injected through a 19 gauge caliber needle. Cells in amounts of $2.5–9.9 \times 10^6$ cells in 0.50 ml HBSS were injected subcutaneously into athymic nude mice. Viability of cells forced through the injection needles was again determined by trypan blue exclusion method. Additional aliquots of cells were injected through the needle into petri dishes and incubated in order to determine growth potential. The mice were examined daily for the development of neoplasms at the sites of injection over a period of four weeks.

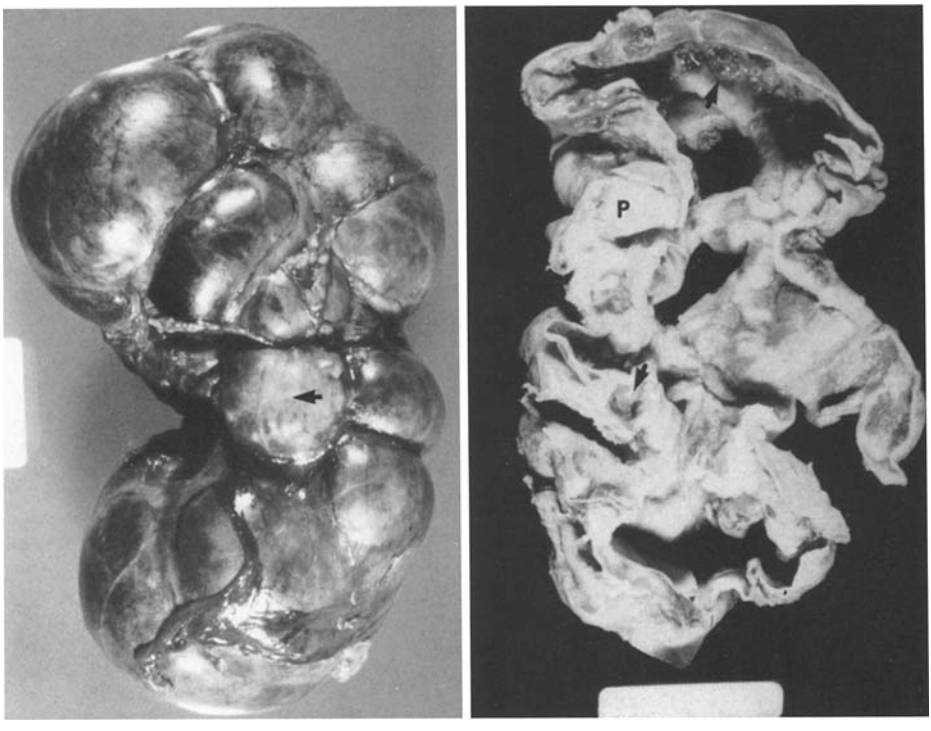
Viral susceptibility tests. RCC cells (passage 42) and RCSC cells (passage 47) were evaluated for the presence of viral receptors, as judged by the appearance of viral cytopathic effects (CPE) using several DNA (herpes simplex virus types 1 and 2 and adenovirus type 5) and RNA (echovirus type II and coxsackievirus B type 4) viruses. Tubes of cultured cells inoculated with the above viruses were examined daily for cytopathic effects over a period of seven days [3].

Cellular electrical measurements. Approximately five days after plating, monolayers of cells in petri dishes were placed on the stage of an inverted Unitron microscope (Uniton Inc., Plainview, NY, USA). The temperature of the medium was maintained at 33° C by means of circulating warm water from a temperature regulated source through polyethylene tube loops in contact with the external wall of the petri dish. Temperature was monitored with a fine tip telethermometer (Yellow Springs Instrument Company, Yellow Springs, OH, USA) placed within the medium. Electrical measurements were done as described elsewhere [39]. Intracellular electrode measurements were obtained by the placement within the cell cytoplasm of the fine tip of a glass micropipette (approximately 0.2μ diameter) filled with 3 M KCl. The advancement of the microelectrode from the incubation medium into the cell was accomplished by means of a screw-driven Emerson micromanipulator. The microelectrodes were connected through an Ag/AgCl interphase to a high input impedence electrometer (WPI 707, W.P. Instruments Inc., New Haven, CT, USA). The incubation media was grounded through a 2% agar Ringer bridge, a 3 M KCl chamber and a Ag/AgCl interphase, to the reference ground of the electrometer. The output of the electrometer was interphased with a strip chart recorder, a tape recorder, and a storage oscilloscope (Tektronix Inc., Beaverton, OR, USA). Micro-electrodes were pulled from borosilicated capillary glass in a vertical puller (Kopf, Tujunga, CA, USA). The micro-electrodes were back-filled with 3 M KCl by the use of a fine polyethylene tube. Micro-electrodes in the range of 70–150 MΩ were used. All measurements were performed in cells maintained in MEM culture medium containing 10% calf serum, pH 7.4 and osmolarity 290 mOsm. Antibiotics were excluded from the culture media at least 3 h before measurements were made.

Results

Pathology of nephrectomy specimen

The nephrectomy specimen weighed 900 g and measured $35 \times 17 \times 8$ cm. The kidney was markedly distorted by a distended pelvis and calyceal system (Fig. 1 A). A solid neoplasm measuring 5 cm diameter was present in its midsection. This primary neoplasm was poorly demarcated and infiltrated adjacent tissues in the hilus including the renal vein and ureter. On the



A B

Fig. 1A, B. Nephrectomy specimen. **A** Unsectioned kidney with distended calyceal system. Central, solid primary neoplasm (→). **B** Sectioned kidney with seeded metastases along surfaces of dilated calyceal system (→). *P*, primary neoplasm

intimal surfaces of the distended calyces numerous polypoid projections of brown seeded metastases were apparent (Fig. 1 B). The histomorphology of the primary and metastatic neoplasms were similar. The cells were of spindle shapes with oval nuclei containing granular chromatin and eosinophilic cytoplasm (Fig. 2). Nuclear pleomorphism was abundant, and numerous mitoses were present.

Morphology of cultured cells

The cultured cells of both the primary neoplasm (RCC) and the seeded metastases (RCSC) appeared similar in morphology in all examinations and have maintained an unchanged appearance through some 60 passages. The cells in vitro had a similar morphology to those in the nephrectomy specimen. The cells grew to confluence in vitro within one week. By light microscopy they generally had a fusiform shape with occasional polygonal configurations (Fig. 3). Nuclei were equidistant from cellular poles and contained a single prominent rounded nucleolus. Few mitoses were visualized, and aberrant or atypical mitoses were not identified. Both lines grew well in serum free medium and in primaria (TM) culture dishes. Cells maintained

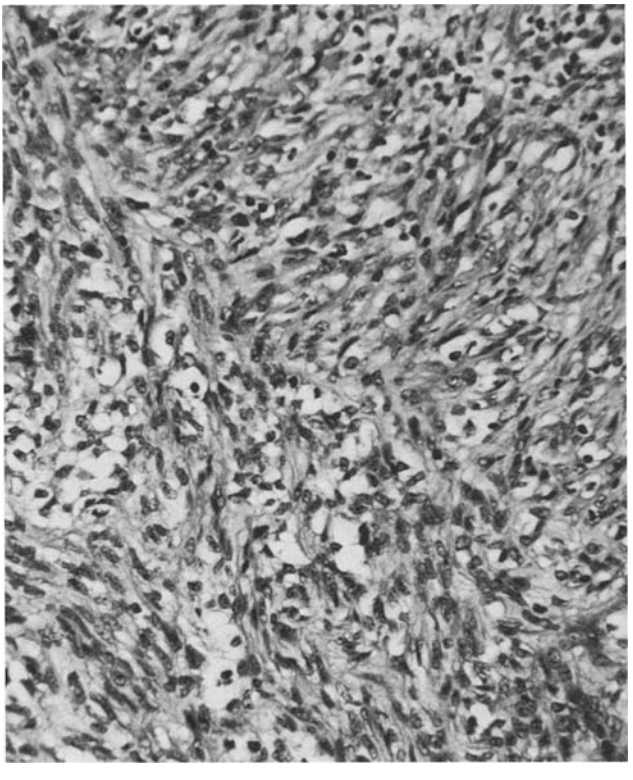


Fig. 2. Light microscopy photomicrograph of primary neoplasm demonstrating spindle cells, nuclear pleomorphism, and variable density of the cytoplasm. Hematoxylin and eosin stain, ×250 magnification

beyond confluence demonstrated lack of contact inhibition with syncytial or multilayered growth. Dome formation, as seen in cultured cells with origin from distal nephron segments, was never visualized in either line.

Scanning electron microscopy demonstrated spindle cells with frequent cell-to-cell contacts, occasional small apical microvilli and rounded protrusions of cytoplasm overlying the nuclei (Fig. 4). Thin, delicate cytoplasmic processes were occasionally observed.

Transmission electron microscopy of monolayers demonstrated rhomboid to cuboid cells, short intercellular junctions and a few short apical microvilli (Fig. 5). The cell junctions, often located apically toward the incubation media, were similar to the leaky apical junctions of proximal tubular cells. Basolateral membranes contained very few infoldings. Focally cellular multilayers were present as evidence of lack of contact inhibition. Mitochondria were scarce and did not have a tendency towards polar localization. Rough endoplasmic reticulum was present in moderate amounts, and few lysosomes were observed. The nuclei were irregular in shape with peripherally clumped chromatin and a single large nucleolus.

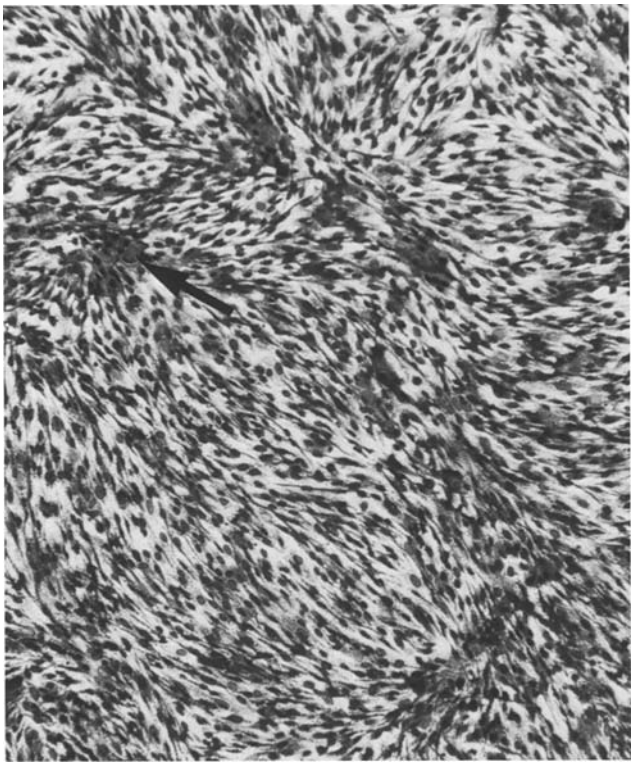


Fig. 3. Light microscopy photomicrograph of cultured cells obtained from seeded metastasis. The cells are generally spindle-shaped and focally overgrow the monolayers to form syncytia (→). Giemsa stain, ×250 magnification

Karyotypic analysis

Both RCC and RCSC lines of cultured cells were found to be hypodiploid, XX, with C-1 and C-3 monosomy. The cells from both lines had 40 or less chromosomes with a modal number of 39. While no consistent chromosomal rearrangement was identified, there appeared to be a consistent monosomy for chromosomes 1 and 3.

Heterotransplantation

None of the cell preparations injected into athymic, nude mice appeared to survive and no neoplasms in the inoculated mice were identified. This was in spite of the fact that cells injected through a similar caliber needle into tissue culture plates grew readily and the viability testing did not uncover significant cell death.

Viral receptor analysis

Similar viral receptors were identified in both RCC and RCSC lines. Herpes simplex virus types 1 and 2, adenovirus 5, echovirus 11, and Coxsackievir-

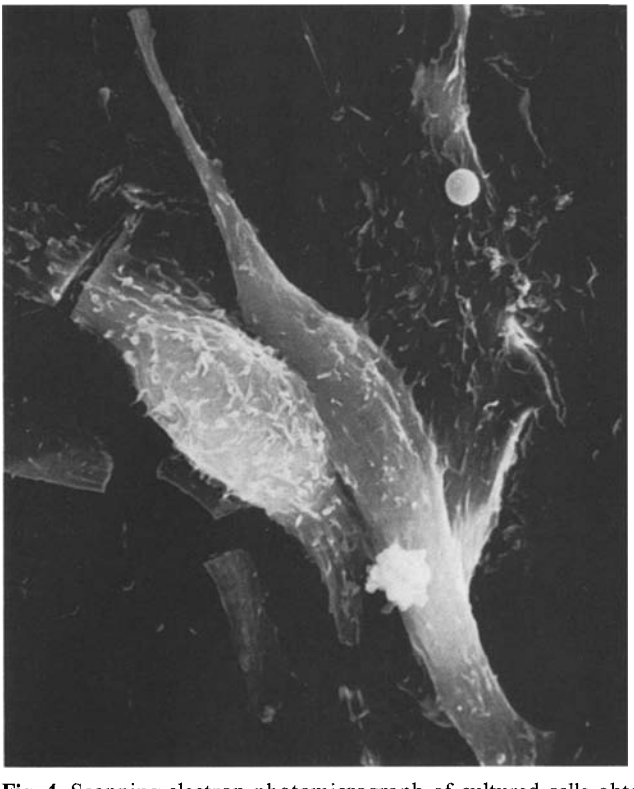


Fig. 4. Scanning electron photomicrograph of cultured cells obtained from seeded metastasis. The spindle-shaped cells have short microvilli protruding from their surfaces. × 2,000 magnification

us B4 grew readily in both cell lines showing typical CPE as observed in other susceptible epithelial cell lines.

Electrical measurements

The electromotive forces existing across the apical plasma membranes of the RCC and RCSC cells, measured at 33° C in MEM with 10% calf serum, averaged -15.69 ± 0.5 mV SEM ($N = 125$), cell interior negative. No statistically significant differences were found between the cells of the two lines or between cells in passages 11 and 21 (Fig. 6 and Table 1). The cell potentials were found to depolarize (membrane potential less negative) significantly when 10^{-6}M amphotericin B was present in the incubation medium (see Table 1). While addition of 10^{-4}M ouabain to the incubation medium induced only a slight depolarization during the first 5 min (Table 1) a marked depolarization was observed in some experiments in which the exposure to ouabain was prolonged (Fig. 7). Cell potentials were also found to be depolarized by increases in extracellular potassium concentration as depicted in Fig. 8.

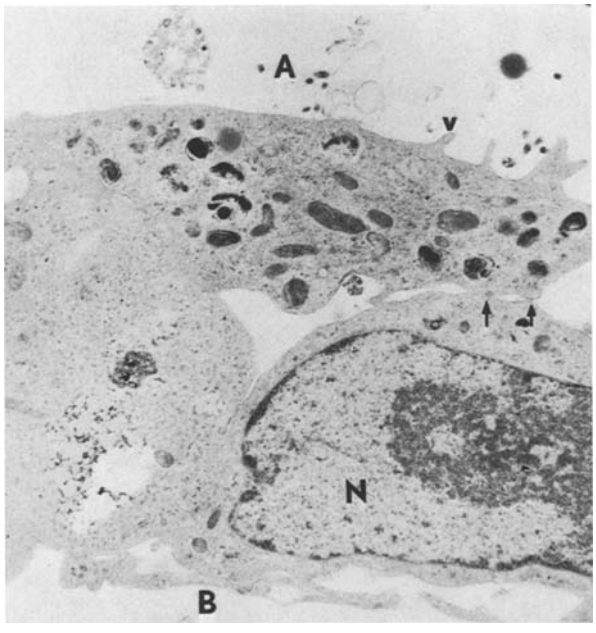


Fig. 5. Transmission electron photomicrograph of cultured cells obtained from seeded metastasis. Two cells depicted have short, loose, apical, intercellular junctions (→) and occasional microvilli (*v*). *A*, apical surface; *B*, basilar surface, *N*, nucleus. Lead citrate and uranyl acetate stain, ×4,000 magnification

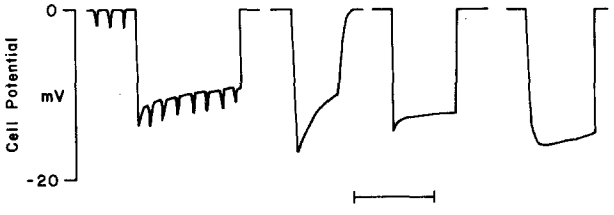


Fig. 6. Examples of the different types of intracellular potentials recorded from RCC cells within a confluent cellular monolayer attached to the bottom of a plastic petri dish. Base line is at the top. The step in voltage corresponds to the electrical potential difference recorded when the tip of the electrode is advanced across the cell membrane from the incubation media into the cytoplasm. The spikes on the first trace correspond to pulses of current being injected through the microelectrode. The rest are recordings obtained without the injection of current. The horizontal bar represents 60 s

Discussion

Although clear cell and granular variants of epithelioid renal cell carcinomas have been cultured for many years and the establishment of several cell lines have recently been reported [8, 18, 20, 30, 43], the establishment of a pure sarcomatoid variant cell line has never been reported. The RCC

Table 1. Values of intracellular electrical potential recorded from 156 renal cell carcinoma cells in culture. The value of cell potential from 37 RCC cells and 33 RCSC cells on passage 11 is compared to values obtained on 55 RCSC cells on passage 21. No significant differences were found. Also included are data concerning the depolarizing effects induced on RCSC cells by the addition to the incubation media of 10^{-4} ouabain or 10^{-6} M amphotericin B

Cell line	Passage	Experimental condition	Cell potential (mV ± S.E.M.)	Number of cells	Probability
RCC	11	Control	−14.92 ± 0.81	37	
RCSC	11	Control	−16.03 ± 1.61	33	N.S.
RCSC	21	Control	−16.14 ± 1.43	55	N.S.
RCSC	21	10^{-4}M ouabain × 5 min	−10.33 ± 0.99	12	$0.10 < P < 0.05$
RCSC	21	10^{-6}M amphotericin B	− 6.89 ± 0.93	19	$P < 0.001$

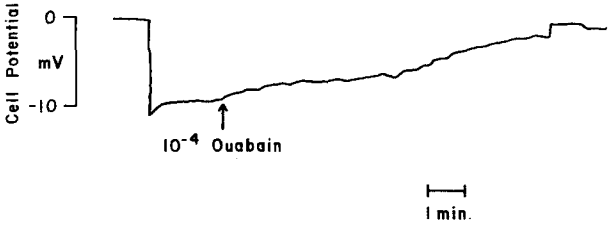


Fig. 7. Effect of 10^{-4} ouabain on the intracellular potential recorded from a RCC cell within a confluent monolayer attached to the bottom of a plastic petri dish. Base line is at the top. The step of voltage corresponds to the difference in electrical potential existing across the plasma membrane of the cell studied. Ouabain was added to the culture dish at the indicated time. A marked depolarization is in evidence as function of the time of exposure to the Na/K pump inhibitor

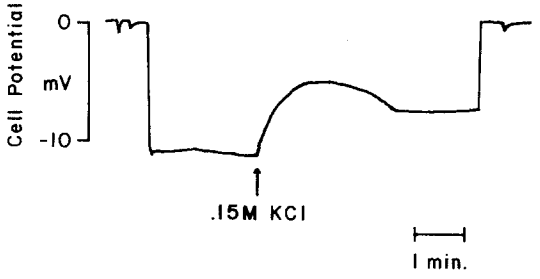


Fig. 8. Effect of increased extracellular potassium concentration on the intra-cellular potential recorded from a RCC cell within a confluent cellular monolayer attached to the bottom of plastic petri dish. Base line is at the top. The step voltage occurred when the tip of the electrode was advanced from the incubation media into the cell. At the indicted time a bolus of isotonic KCl was added to the petri dish (50 µl of 0.15 M KCl solution to 2 ml of incubation media). As the concentration of K became uniform, the initial sharp depolarization was partially reversed. The final potential is lower than the value prior to the addition of KCl and reflect the effect of change of extracellular potassium from 5 to 12.5 mM

and RCSC cell lines described here are thought to be the first two established cell lines of this rare and aggressive form of renal cell carcinoma. Both lines are similar cytologically, ultrastructurally, karyotypically and in both growth patterns and transport properties. Several pieces of evidence support the malignant nature of both lines. Both cell lines have been continuously grown in vitro for three years over 60 passages, which is much greater in survival time and number of passages than one would expect from either normal epithelial cells or fibroblasts derived from an elderly individual [21, 1]. Furthermore, both lines lack contact inhibition.

Potential contamination from other established cell lines is unlikely. Hela cells, while available in our cell culture laboratory, have not been grown for several years. The RCC and RCSC cell lines differ markedly from Hela cells morphologically. Karyotypically, the present lines are hypodiploid, while Hela cells are heterodiploid (hyperdiploid) with 80–90 chromosomes. Furthermore the RCC and RCSC lines do not grow in nude mice, while Hela cells grow readily. Finally, echovirus type II grew readily in the RCC and RCSC lines, while the same virus is reported to grow sluggishly, if at all, in Hela cells [33]. Established epithelioid cell lines that have been recently grown in our laboratory include MDCK, Vero, and LLC-MK_2. However, the RCC and RCSC lines differ from them morphologically, karyotypically, and by viral susceptibility.

The cell origin of these two malignant cell lines is here postulated to be the proximal tubular epithelial cells. Evidence for this assumption is as follows: 1) The two present cell lines demonstrate cell membrane compartmentalization, or polarity, with two domains, apical and basolateral, formed by intercellular junctions. 2) The apical membranes, in direct contact with the bathing culture media domonstrate epithelial specialization in terms of microvillar formation, as visualized in normal proximal tubular apical membranes and as demonstrated by Oberling et al. [32] and Tannenbaum [38] in epithelioid variants of renal cell carcinoma. 3) The morphological observation of leaky type cell to cell junctions as previously described for proximal tubular epithelia [11]. 4) While dome formation has been observed in cultured cells of distal nephron origin [9] and other epithelia having tight junctions [2, 29], monolayers of the present lines grown without dome formation. 5) The presence of lipid droplets and particulate glycogen in these lines as often observed in proximal tubular cells [32, 38]. 6) Cytoplasmic tonofilaments and myofilaments were absent as is usual in proximal cells. 7) Spindle variant of squamous cell carcinoma can be excluded by the absence of desmosomes and keratohyaline granules [28]. Finally, 8) fibroblasts can be excluded by the above criteria and by the fact that the RCC and RCSC lines grow readily in defined serum-free medium and in Primaria plates, which do not support the growth of fibroblasts.

The failure to grow the RCC and RCSC cells in athymic, nude mice is difficult to explain. However, absence of growth, as in this case [20], or regression of growth [30] has been observed in several epithelial renal cell carcinoma lines.

Although there are at least six different epithelial cell types along the

nephron, all renal cell carcinomas reported so far in the literature, appear to be derived from proximal segments. Several possibilities may be invoked to explain the peculiarity of the origin of neoplasms within the kidney. The first deals with the fact that, contrary to other segments of the nephron, proximal tubular cells are known to contain a great number of various transport mechanisms for both organic and inorganic substances [16, 35]. Maximal reabsorption of carcinogens or tumor promoters is, therefore, likely to occur within this relatively long segment of nephron. The second possibility deals with a potentially auspicious cytoplasmic red-ox state. However, as recently demonstrated by Burch and her associates, the cellular concentration of glutathione is greater in the renal cortex and gradually decreases towards the deeper regions of the kidney [5]. Likewise, the activity of glutathione-S-transferase or ligandin, an enzyme that catalyzes the reaction of glutathione with a large number of compounds bearing electrophilic properties, or binds a great variety of anions, is mainly located in renal proximal tubule cells [23, 24]. Both glutathione and ligandin have been shown to provide a powerful cellular defense mechanism against tumor induction [10]. Thereby, differences in the red-ox state of proximal tubular cells with respect to other cells within the kidney, is an unlikely explanation. A third and related possibility is the existence of favorable metabolic conditions for the development of tumors at the renal cortex. For example, while renal cortical PO_2 is of 80–90 mmHg, the PO_2 in the renal papilla is only 15 mmHg [27]. Accordingly, the type of metabolism along the nephron varies greatly as aerobiosis is predominant in proximal cells and other cortical segments, and anaerobiosis predominates in the distal segments and in papillary cells [26]. However, since tumor cells are mainly glycolytic, this possibility is also an unlikely one. The fourth possibility relates to differences in osmolarity within the kidney: cortical segments are generally isotonic while the medulla and papilla are hypertonic. Although, at present, there is no evidence to support or refute the role of osmolarity on renal tumor induction it is of importance to note that not only proximal tubules but glomeruli, distal tubules, and cortical collecting tubules all exist within a similar range of osmolar environment. Therefore, this explanation seems also unlikely, at present.

A few comments concerning the development of a renal cell carcinoma within a hydronephrotic kidney are in order. Renal cell carcinomas are known to occur within both hereditary and acquired renal cysts [22, 42]. Further-more, a Mayo Clinic retrospective study of 1,007 cases of renal cell carcinoma revealed an incidence of 1% of coexisting renal cysts and neoplasms [12]. While the mechanisms of cystic dilation of the kidney is a matter of current study [15], the development of renal cell carcinoma in a hydronephrotic kidney is not surprising since prolonged contact time between potential tumor promoters and the epithelium is likely to be causally related.

Concerning the karyotypes of the RCC and RCSC cells it is interesting that both lines are hypodiploid with C-1 and C-3 monosomy. The finding of a similar chromosomal number and karyotype through numerous pas-

sages suggests that this chromosomal change was probably a primary alteration within the original neoplasm rather than random deletions occurring during growth in vitro. Furthermore, the deletion of chromosomes 1 and 3, is in agreement with recent observations on epithelioid variants of renal cell carcinoma [20, 43].

The characterization of electromotive forces in these cultured cells is instructive. The cellular electromotive forces of both lines were found to be similar and significantly lower than those of normal proximal mammalian renal cells studied in situ [14] and in vitro [8, 25]. Several factors may be involved in this phenomenon. First, under the existing cell culture conditions, the Na/K ATPase is in a cryptic situation as the pump is probably working within a restricted diffusional space formed by the cell membranes, the cell junctions, and the bottom of the culture dish. The magnitude of this phenomenon is presently unknown but most likely is directly proportional to the degree of membrane permeability, the leakiness of cellular junctions and the magnitude of the unstirred layer effect. Second, in the normal proximal tubular cell, the cell transmembrane electrical potential difference (P.D.) is generated mainly by the ratio of sodium to potassium conductance and the intracellular and extracellular concentrations of sodium and potassium as expressed in the general equation:

$$\text{P.D.} = \frac{gK}{gNa + gK} E_K + \frac{g\,Na}{gNa + gK} E_{Na}.$$

Where E is the Nerstian equilibrium potential and g the membrane conductance.

In normal renal proximal tubular cells, the main determinants of cell P.D. are the potassium conductance (gK) and the ratio of its intercellular to extracellular concentration. This situation is most likely not operative in malignant cells, or in proliferating cells, where increased sodium influx through sodium channels, sodium-glucose and sodium-amino acid cotransport systems have been demonstrated [7, 19, 31, 40, 41]. The lower cellular P.D. found in these cells may, therefore, simply represent an increased influx of sodium into the cell, and is in agreement with what other authors have found in other neoplastic cells [7]. The marked depolarization (less negative cell potential) induced by amphotericin B tends to indicate, however, that the potassium ion contributes to the genesis of the transmembrane potential. Further support to this possibility is given by the depolarization observed when extracellular potassium was increased. As normal proximal renal cells depolarize rapidly in the presence of 10^{-4} M ouabain with a $t(1/2)$ of 2.2 min [39], the delayed effect of ouabain (10^{-4} M) on this proximal renal cell line was somehow unexpected, and perhaps indicative of lower affinity for ouabain by the Na/K pump of these cells. Reuss et al. have already reported a similar phenomena in non-tumor cells [34].

Finally, in view of the significant incidence of renal cell carcinoma (8.6 new cases per 100,000 persons per year in the U.S.A.) [37], and of the lack of effective chemotherapy for these neoplasms, the establishment of

these two cell lines might prove useful for the study of the biology of this aggressive neoplasm. As a corollary, therapeutic approaches may hopefully follow.

In summary, this in vitro study of a pure sarcomatoid variant of renal cell carcinoma provides evidence to support the concept that this variant also originates from proximal tubular epithelial cells.

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