Characterization of Two Human Cell Lines (TK-10, TK-164) of Renal Cell Cancer

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ABSTRACT

Two previously unreported cell lines of human renal cell carcinoma are presented. TK-10 and TK-164 have each been in culture for over 4 years. The epithelial nature of both cell lines has been documented by light and electron microscopy. The cells in each line contain a Y chromosome, have specific marker chromosomes, and a distinct flow cytometric histogram. Both lines grow in agar, albeit not in athymic mice.

INTRODUCTION

The in vitro establishment of cell lines of primary human RCC provides a valuable tool for studying the immunological and biochemical aspects of this most common renal malignancy. Although Richter first reported in 1957 the successful short-term cultivation of human RCC, it wasn't until 1962 that Ishihara and coworkers reported the maintenance of a human renal cell cancer in cell culture for longer than 1 year (1, 2). Subsequent reports of human cell lines have been sparse; in a review article, Williams noted only 13 cell lines of human RCC (3). However, for many of these cell lines, neither confirmatory clinical data nor characterization data is available thereby creating doubts as to the origin and individuality of these lines. Herein we describe two new cell lines of human RCC which are characterized with regard to: clinical origin, media requirements, karyotype, doubling time, morphological characteristics, biochemical characteristics, storage, and tumorigenicity.

MATERIALS AND METHODS

Clinical History. TK-10 was derived from a specimen of primary RCC obtained following a left radical nephrectomy in a 43-year-old male on August 21, 1980. Preoperatively, there was no evidence for metastatic disease. The kidney, which measured 18 × 17 × 11 cm, was almost completely occupied by a largely necrotic, soft-yellow tumor measuring 15 × 12 × 12 cm. The uninvolved area of kidney and ipsilateral adrenal gland appeared normal. The tumor was confined to the subcapsular area of the kidney indicating a Stage I or T2NXM0 neoplasm (4, 5). Microscopic examination of the tissue revealed that the tumor had invaded the renal vein and transgressed the renal capsule. There was no evidence of metastatic disease in the ipsilateral adrenal gland or in three removed lymph nodes (Stage IIIa or T3abNoMo (4, 5). On microscopic examination, the tumor showed a largely solid pattern of growth with focal glandular formations. The tumor cells were predominately of the clear type with a minor population of granular type. The cytoplasm contained abundant glycogen and the nuclei were irregular with prominent nucleoli (nuclear grade, 3). At 54 months following diagnosis, the patient is alive without evidence of metastatic disease; he has received no adjunctive therapy.

Tissue Culture Methods. Primary cultivation of TK-10 and TK-164 was accomplished largely by methods recommended and utilized by Williams et al. for the propagation of human RCC and nontumor kidney cells (7, 8). Tumor tissue was transferred from the hospital in either RPMI 1640 outgrowth medium (TK-10) or DF12 medium (TK-164). RPMI 1640 medium consisted of the base solution supplemented with 15% heat-inactivated FBS (KC Biological, Lenexa, KS), 10% tryptose phosphate broth (Difco Laboratories, Detroit, MI), penicillin 100 IU/ml (KC Biological), streptomycin 100 μg/ml (KC Biological), 2 mM L-glutamine (GIBCO, Grand Island, NY), insulin 0.5 units/ml (Elly Lilly and Co., Indianapolis, IN), and gentamicin 5 μg/ml (Elkinks and Sian Inc., Cherry Hill, NJ). DF12 outgrowth medium consisted of a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (KC Biological), supplemented with 10% FBS, 10 mM (2-hydroxyethyl)-1-piperazinethanesulfonic acid (Sigma Chemical Co., St. Louis, MO), sodium bicarbonate 1.1 mg/ml, 10 mM Na2SeO4·5H2O, and gentamicin 5 μg/ml. Triple distilled water was used for all media preparation.

In a petri dish (Corning Glass Works, Corning, NY) containing cold phosphate-buffered saline, pH 7.2, the tumor tissue was trimmed of perirenal fat, necrotic areas, and any fibrous capsular tissue. Next it was washed with phosphate-buffered saline in order to remove red blood cells. The tumor tissue was minced with a scalpel and a tissue forceps into 1-mm3 pieces, and placed in a stirring flask (Bellco Glass Inc., Vineland, NJ) with 40 ml 0.25% warm (37°C) trypsin solution (Grand Island Biological Co., Grand Island, NY), and stirred for 45 min on a Magnestir for an additional 45 min. The trypsin-cell suspension was decanted to remove any remaining red blood cells. Forty ml of fresh, warm trypsin was added to the remaining tissue mince, and replaced on the Magnestir for an additional 45 min. The second trypsin-cell suspension was added to the outgrowth medium and centrifuged for 10 min at 200 x g. The supernatant was decanted and the cells were resuspended in 10 ml of outgrowth medium, pipetted into 25-cm2 culture flasks, and incubated in a humified atmosphere of 5% CO2 at 37°C. The cells were observed with an inverted phase microscope and the media changed three times weekly.

When the cultures reached a monolayer, they were subcultured. First, the medium was reduced and the cells were washed twice with 4-ml trypsin. The trypsin washes were decanted into a 50-ml centrifuge tube containing 5-ml outgrowth medium. Versene (1:5000) 4 ml was added to the flask and incubated for 5 min. The additional dislodged cells were added to the centrifuge tube and spun at 200 x g for 10 min. The resulting cell pellet was resuspended in outgrowth medium and the cells were again plated on 25-cm2 flasks. When large quantities (104–105) of cells were needed (i.e., for animal inoculation), cultures were grown on 490-cm2 plastic roller bottles (Corning Glass Works, Corning, NY),...
The amount of FBS in the RPMI 1640 growth medium was reduced from 15 to 10% after both cell lines had been subcultured several times. Growth Kinetics. Growth curves were established for each cell line by seeding 2.5 x 10⁴, 5.0 x 10⁴, and 7.5 x 10⁴ cells onto 25-cm² flasks. Triplicate flask samples were harvested and counted after 24 h of incubation to determine the seeding efficiency and at 3, 5, 7, and 9 days. Cells were stained with neutral red dye and counted using a Bright-line hemocytometer.

Cells were also tested for growth on K-1, a chemically defined, serum-free medium developed by Taub and coworkers for the growth of Madin-Darby canine kidney cells (9). TK-10 and TK-164 cells were first plated on FBS-containing media. On day 1, the plates were washed twice with plain RPMI base media (5 ml) to remove any remaining FBS and the K-1 medium was added. Growth curves were established as previously described. Attempts to develop a cloning growth assay system were unsuccessful with TK 10 and TK 164.

Cell Storage. Cells were subcultured onto 75-cm² flasks and grown to confluence. Cells were removed using trypsin and versene, counted, and diluted in RPMI 1640 growth medium with 30% FBS and 10% dimethyl sulfoxide to a concentration of 2-3 x 10⁴ cells/ml. The cell suspension (1.8 ml) was pipetted into 2.0 ml Nunc freezing vials (GIBCO Laboratories, Grand Island, NY). Vials were put on ice in a cooler (4°C) for 1 h, transferred to a styrofoam container in a −80°C freezer for 24 h, and then placed in a liquid nitrogen (LN₂) freezer (vapor phase) for long-term storage. Cells were revived after 6-24 months (TK-164 and TK-10, respectively) by the method of Elliott and coworkers and plated onto 25-cm² flasks (10).

Mycoplasma Testing. Cells were tested for mycoplasma by direct growth on agar (read at 4 and 21 days), Hoechst staining, and BTS-7 staining methods (Flow Laboratories, Rockville, IL). Cells were also examined for the presence of viral or mycoplasmal contamination during electron microscopy.

Karyotype. Chromosome analysis was performed on TK-10 at passages 30 and 48 and on TK-164 at passages 10 and 20. Confluent monolayers of cells were treated with colcemid (0.14 g/ml) for 2-6 h. Trypsin was used to harvest single cell suspension. The cells were then treated with 0.075 M KCl and fixed in methanol/acetic acid (3/1, v/v). Slides were prepared by air drying and G-banded by the method of Wang and Fedoroff (11). For each cell line, 20-25 metaphases were analyzed.

Flow Cytometry. Both TK-10 and TK-164 were analyzed for DNA content and growth kinetics by flow cytometry. In order to compare the DNA histograms of the ongoing cell cultures to the same cell cultures which had recently been frozen, 2 x 10⁶ cells of TK-10 and TK-164 were frozen in liquid nitrogen for 10 days and subsequently thawed using the methods described previously. Each cell suspension was placed back into culture at a seeding concentration of 5 x 10⁴ cells 4 days before being analyzed by flow cytometry. Also, an earlier passage of TK-10 (p70) was cultured for cytometric comparison with the most recent passage (p171). Each cell line was harvested in the previously described manner and prepared for immediate analysis by the methods of Stone et al. (12). Briefly, the cells were stained for 1 h in two changes of a modification of Krishan's reagent (13) which contained 0.005% propidium iodide (w/v, Calbiochem-Berin, San Diego, CA), 0.02% ribonuclease A (w/v) and 0.3% Nonidet P-40 (v/v) (Sigma Chemical Co., St. Louis, MO) in 0.1% sodium citrate (w/v), pH 8.3. Fresh chicken erythrocytes and trout erythrocytes were added at approximately 7.5 x 10⁴ cells per each 10⁴ tissue culture cells prior to staining. The resulting nuclei were examined in an EPICS V flow cytometer (Coulter Electronics, Inc., Hialeah, FL) equipped with an argon laser. Propidium iodide bound to DNA was excited at 488 nm and measured through a 515-nm long-pass interference filter, a 515-nm long-pass absorption filter, a 550-nm dichroic filter, and a 590-nm long-pass absorption filter. Histograms were analyzed on a TERAK LSI/1123 microcomputer utilizing a software program (PARA 1.0) written by Dr. C. B. Bagwell (Coulter Electronics Inc.) The relative positions of the chick and trout nuclei which have known DNA contents allows identification of position of the unknown tumor cell peaks in relation to the expected position of normal human diploid cells using the relationship developed by Vindelov et al. (14).

Tumorigenicity. Male, nu/nu, athymic NIH Swiss mice (5-7 weeks) received 500 rads of total body irradiation. One to three days following irradiation each of five mice was inoculated s.c. with 10⁶ cells of TK-10 or TK-164 suspended in 0.3 ml RPMI 1640 growth medium. The injection site was observed for 10 weeks, following which the mice were killed to assess tumor growth. This process was repeated twice at later passages for both cell lines (total of 15 mice inoculated for TK-10 and TK-164, respectively).

Two groups of five irradiated athymic mice were also inoculated beneath the renal capsule with 2 x 10⁶ tumor cells of TK-10 (passage 88) and TK-164 (passage 41), respectively. Mice were killed at 3, 6, and 9 weeks. The previously inoculated kidney was removed and sectioned for histological examination.

In addition, male C57/BALB/c 4-week-old athymic nude mice were injected intrasplenically with TK-10 or TK-164 at a concentration of either 5 x 10⁴ or 5 x 10⁵ cells in 100 μl of RPMI complete media by the method of Kozlowski et al. (15). Mice were sacrificed at 6 and 12 weeks. The lungs and liver were placed into 10% buffered formalin. Sections were stained with hematoxylin and eosin and microscopically examined for metases.

Tumorigenicity was also tested by attempting to grow each cell line on soft agar. A lower, feeder layer of 0.5% agar was prepared with RPMI 1640 Complete media (37°C-40°C) and 3.0% Bacto-Noble agar (1:1). Three ml aliquots were placed into 35- x 10-mm sterile petri dishes (Costar, Cambridge, MA) and allowed to solidify. The plates were incubated for 24 h at 37°C in a 5% CO₂ atmosphere. Cells were harvested from 75-cm² flasks as described previously. Viability counts were done with Neutral Red dye on a Bright-Line hemocytometer. Bacto-Noble Agar (3%) was added to the single-cell suspension to form a 0.5% agar solution. One ml of the agar/cell solution containing 5 x 10⁴ cells was placed over the lower feeder layer of agar, allowed to solidify, and incubated at 37°C in a 5% CO₂ atmosphere. Colony-sized clusters (diameter ≥ 50 μm) were scored by automated image analysis with an Omnicon FAS-II scanner (Bausch & Lomb) 1 and 9 days after the cells were seeded. Final colony counts were determined by subtracting cluster counts (day 1), and values were expressed as the mean count of nine dishes.

Morphology. Both cell lines were subcultured into a Leighton tube (Belco Glass) containing a 9-mm x 35-mm glass cover slip and 1.5 ml RPMI 1640 growth medium. When the monolayer was confluent, 95% ethanol or 10% formalin was added to fix the cells. Subsequently, the cover slips were removed and stained with hematoxylin and eosin (H & E), oil red 0, Sudan black, and PAS with and without diastase pretreatment. The slides used for the oil red O and the Sudan stains were processed without using ethanol or other lipid solvents.

Electron microscopy was also done on TK-10 and TK-164. Cells were cultured on 25-cm² culture flasks until they attained a monolayer and harvested as described previously. The media was replaced with 2% glutaraldehyde in cacodylate buffer. Next, the cell pellet was postfixed with osmium tetroxide, stained en bloc with uranyl acetate, dehydrated with alcohol, and embedded. Sections were stained with uranyl acetate and lead citrate.

Biochemical Studies. Samples of TK-10 and TK-164 were prepared for the determination of total cholesterol, free cholesterol, HMG-CoA reductase activity, ACAT, and cholesterol esterase activity. Protein was measured by the method of Lowry et al. (16). Aliquots of cells were extracted for total cholesterol determination by the method of Abell et al. (17). Free cholesterol was extracted in the same manner, except KOH was not added during the procedure. Cholesterol was measured by gas-liquid chromatography (Hewlett-Packard 5830A chromatograph). Cholesterol, added in the extracting solvents, served as the internal standard. HMG-CoA reductase activity was measured by the method of Shapiro et al. (18). Measurement of the microsomal enzyme ACAT was performed by a modification of the method of Rothblat et al. (19).
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Growth Kinetics. The primary culture of TK-10 attained a monolayer within 2 weeks and was subcultured at a split ratio of 1:1. Subsequently, cells were subcultured in greater split ratios and at decreasing time intervals. Currently, the split ratio is 1:20 every 7–10 days. TK-10 is presently in its 190th passage after 59 months in culture and has a mean doubling time of 25 h (passage 155). Seeding efficiency varied slightly between the three cell concentrations which were analyzed. The mean seeding efficiency of TK-10 was 63 ± 9%. When tested for growth on K-l, TK-10 showed an increase in cell number only during the first 5 days of the study (Fig. 1). At each time point, growth on K-l was less than growth on the serum-supplemented medium.

Initial growth of the primary culture of TK-164 was slow. Tumor cells grew in patches and the culture did not attain a complete monolayer for 5 months; at this time a subculture was done at a split ratio of 1:2. After 11 months in culture, TK-164 was changed from DF12 to RPMI 1640 medium. On RPMI 1640, the growth rate improved. By passage 10, subcultures were necessary every 10–14 days with a 1:20 ratio. TK-164 on RPMI 1640 is presently in its 65th passage after being frozen in liquid nitrogen from March 1984 to November 1985. TK-164 has a mean doubling time of 43 h depending slightly on the seeding concentration. The mean seeding efficiency was 43 ± 4% and the cells require subculturing every 9–10 days. These cells did not grow on K-1 (Fig. 1).

Liquid Nitrogen (LN₂) Storage. When revived from LN₂ storage, TK-10 cells took several days before attaching to the flask whereas TK-164 cells attached readily. After the first passage, the revived cells of TK-10 and TK-164 had histological characteristics similar to the cells in continuous culture.

Contamination. Results of laboratory tests for mycoplasma (Flow Laboratories) and electron microscopy for viral contaminants were negative for both cell lines.

Karyotype. The chromosome number for TK-10 ranged from 47 to 59 with a modal number of 51. Four different marker chromosomes, M1(1p+), M2(1p+), M3(t3;5), and M4(6q-), were identified in every metaphase spread analyzed (Fig. 2). The Y chromosome was also present in all karyotypes. The chromosome number for TK-164 ranged from 80 to 82 with a modal number of 82. Three different marker chromosomes, M1(t2;5), M2(3p-), and M3(6q-) were identified in 100% of the cell population (Fig. 3). Again a Y chromosome was present in all karyotypes. Each karyotype was distinct from that of other cell lines maintained in our laboratory.

Flow Cytometry. Histograms obtained from the cytometric analysis of TK-10 and TK-164 displayed distinct differences between the cell lines. The calculated DNA index (the ratio of the modal G0/G1 peak of the cell line to that calculated for normal diploid standards) for TK-10 was 1.5 and there were no significant variations in the DNA pattern between the early passage population of cells and the late passage of cells (Fig. 4). Likewise, no variations in the histograms could be observed.
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Fig. 3. Karyotype of TK-164 (passage 10). Note the presence of the Y chromosome and three marker chromosomes.

Fig. 4. DNA histograms derived from flow cytometric analysis of: A, TK-10 (passage 171) and B, TK-164 (passage 42) illustrating the difference in DNA index of the two cell lines. Peaks, chick RBCs (C), trout RBCs (T), G0/G1, aneuploid cells (G0/G1), and G2/M aneuploid cells (G2/M).

between the ongoing culture and the culture which had been recently revived from liquid nitrogen. The calculated DNA Index for TK-164 was 2.0 and there were no differences in the histograms from the ongoing and the recently frozen cell cultures (Fig. 4).

Both TK-10 and TK-164 appear to have a large percentage of cells in the S-phase of the mitotic cycle, as evidenced between the G0/G1 and G2/M peaks on the respective histograms. The calculated values were 22.3% for TK-10 and 16.5% for TK-164.

Tumorigenicity. Inoculation of 2 x 10⁶ to 1 x 10⁷ cells of TK-10 or TK-164 s.c. and beneath and renal capsule failed to produce tumors in athymic mice. Neither 5 x 10⁴ nor 5 x 10⁵ cells injected intrasplenically produced pulmonary or hepatic metastases in any of the athymic mice.

Both TK-10 and TK-164 produced colonies on soft agar. The mean colony counts for TK-10 and TK-164 on the ninth day after seeding were 198 ± 86 and 64 ± 20, respectively.

Morphology. TK-10 cells had an epithelial appearance and scant intracytoplasmic vacuoles. TK-164 cells also had an epithelial appearance but were larger in size and contained more vacuoles. The histology of both cell lines was consistent with their origin from RCC (Fig. 5). Both cell lines contained large amounts of material positive for PAS staining. Because diastase pretreatment removed the majority of the PAS positive material, we believe the PAS positive material is glycogen. The oil red O and the Sudan black stains were only weakly positive indicating a paucity of neutral fat.

Electron microscopic analysis of both cell lines confirmed their epithelial origin. Desmosomes, and tight, intracellular junctions and surface microvilli were identified in both TK-10 and TK-164. Also evident were intracytoplasmic vacuoles, multiple nucleoli, and intranuclear entrapments. There was no evidence of intracytoplasmic viral particles or cell surface budding in either cell line (Fig. 5).
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Fig. 5. Comparison of the original histology (left) of tumors TK-10 (upper) and TK-164 (lower) (300×) with their appearance in cell culture by light microscopy (middle) (300×) and electron microscopy (right) (42,000× and 15,000× respectively). Arrows, desmosomes.

Table 1 Lipid biochemical studies

<table>
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<tr>
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<th>TK-10 (passage 110)</th>
<th>TK-164 (passage 20)</th>
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<tr>
<td>Total cholesterol (µg/mg protein)</td>
<td>27.9</td>
<td>32.7</td>
</tr>
<tr>
<td>Free cholesterol (µg/mg protein)</td>
<td>20.5</td>
<td>28.9</td>
</tr>
<tr>
<td>Ester cholesterol (µg/mg protein)</td>
<td>7.4</td>
<td>3.8</td>
</tr>
<tr>
<td>HMG-CoA reductase (pmol/mg⁻¹ min⁻¹)</td>
<td>8.9</td>
<td>30.2</td>
</tr>
<tr>
<td>ACAT (pmol/mg⁻¹ 20 min⁻¹)</td>
<td>453</td>
<td>104</td>
</tr>
<tr>
<td>Cholesterol esterase (pmol/mg⁻¹ min⁻¹)</td>
<td>0</td>
<td>0</td>
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Biochemical Studies. In both lines, high levels of cholesterol were noted when compared to normal kidney (Table 1). The cholesterol within the tumor was predominantly in the ester form. Levels of HMG-CoA reductase, the rate-limiting enzyme for cholesterol production, were low. Interestingly, levels of ACAT, the enzyme responsible for cholesterol esterification, were high while cholesterol esterase levels were depressed.

DISCUSSION

The establishment of cell strains of human RCC can be accomplished in 75% of plated renal tumors. However, it is much more difficult to establish cell lines of RCC. In general, less than 3% of all renal tumors placed in tissue culture become an established cell line (7, 8).

Despite Jones’ report in 1967 of a long-term (greater than 1 year) culture of RCC, by 1980 Williams could identify only 13 lines of human RCC available for study (3, 20). Since then, five additional lines, not including TK-10 and TK-164, have been described (21–23). Of these 20 human RCC cell lines only six fulfill all of the following criteria for a human cancer cell line: (a) epitheliod, (b) no HeLa cell, (c) derived from a primary tumor, (d) in culture for greater than 1 year or more than 50 passages, (e) distinct human karyotype, (f) free of mycoplasma or other infectious agents, (g) defined doubling time, and (h) tumorogenicity. These cell lines are: OUR-1, 786-O, 769-P, SS-78, TK-10, TK-164, (3, 8, 21, 24).
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Electron microscopy is most helpful in establishing the epithelial nature of a cell line. Cell surface microvilli, tight intracellular junctions, and desmosomes are all recognized characteristics of epithelial cells. Likewise, electron microscopy can help rule out mycoplasmal or viral contamination.

Cell line contamination with HeLa cells is a prevalent problem. However, the presence or absence of HeLa cells can be evaluated from the mobility pattern of glucose-6-phosphate dehydrogenase isoenzymes, the presence of a Y chromosome, or the karyotype of the established cell line(s) in question (25). In addition, knowledge of the doubling time of the cell line is helpful, as it enables other investigators to better plan their use of the cell line and may further serve as an indicator of cell line contamination or alteration. Finally, the aggressive malignant nature of the host tumor may not be wholly transferred to the cell line; this can be tested by studying cell growth in agar or by injecting the cell line into a suitable animal host (26, 27). This information is necessary for other investigators planning to use established cell lines in their work.

The two cell lines reported herein, TK-10 and TK-164, are both derived from separate primary human renal tumors. Both have been in culture for greater than 4 years and more than 50 passages. The presence of a Y chromosome in both cell lines, rules out possible HeLa cell contamination. The karyotypes of TK-10 and TK-164 are distinct from other reported cell lines of human renal cancer, In both lines, there are unique marker chromosomes. Likewise, both cell lines have distinct aneuploid DNA patterns (histograms) which did not vary significantly among various passages of the cells or with freezing/thawing procedures.

The mean doubling times of 25 and 43 h for TK-10 and TK-164, respectively, make these cells a ready source of material for performing metabolic or immunological studies. Morphological examination by light and electron microscopy indicate both cell lines are epithelial in appearance. The tumorigenicity of TK-10 and TK-164 has been demonstrated by their growth on agar; however, neither cell line produced tumors in athymic mice despite subcutaneous, subrenal capsular, and splenic inoculations.

TK-10 and TK-164 provide the investigator with an unlimited supply of human renal tumor cells for immunological, biochemical and pharmacological studies. Recently, cell lines of RCC have been used to develop and test monoclonal antibodies. Indeed, the two cell lines reported herein have already been used in the study of several RCC monoclonal antibodies by others (28). Earlier immunological work with cell lines included the microcytotoxicity tests reported by Hakala, Ellhilali, Prout and coworkers (29-31). Second, cell lines are a valuable source of material for evaluating the unique biochemical and metabolic aspects of renal cancer. Sherwood and Goldwasser have used cell lines of human RCC to demonstrate the presence of a tumor derived erythropoietin (32). Similar biochemical studies in another RCC cell line have recently resulted in the isolation of a substance with parathormone-like activity (33).

In our work with cholesterol metabolism in renal cancer, cell lines of RCC and cell strains of nontumor human kidney have been used to demonstrate differences in the uptake and release of cholesterol by normal and malignant human renal cells (34). The lipid biochemical data presented in Table 1 are consistent with other data from our laboratory revealing high levels of cholesterol ester in human renal cancers studied immediately ex vivo.4 Finally, well-characterized cell lines of RCC can be used to test the effectiveness of putative chemotherapeutic regimens for renal cell cancer.

In summary, TK-10 and TK-164 are two well-characterized cell lines of human RCC. They should be of value to immunologists and biochemists alike as they provide a readily available and reliable source of material for the study of human renal cancer.

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