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Morphology and Growth Characteristics of Epithelial Cells from Classic Wilms' Tumors

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The ability to establish cell cultures representing the epithelial component of Wilms' tumor was determined for 18 cases of classic Wilms' tumors. From these 18 cases only two resulted in the culture of epithelial cells. Although the tumors from both cases were composed of a prominent epithelial component, other classic tumors not producing epithelial cell cultures also possessed appreciable epithelial components. Likewise, heterotransplants of these two primary tumors failed to give rise to epithelial cell cultures, although cultures of the blastemal element were produced. This suggests that Wilms' tumors may be prone to differentiate in different directions at varying times during tumor growth, possibly dependent on local tumor environment. Epithelial cells from these two classic cases were grown in culture in basal medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium, supplemented with selenium, insulin, transferrin, hydrocortisone, triiodothyronine, and epidermal growth factor, on a collagen type I matrix with absorbed fetal calf serum proteins. One of the two cases also required the addition of bovine pituitary extract, ethanolamine, prostaglandin E1, and putrescine for optimum growth. Morphological analysis disclosed that the cultured cells were very similar to normal renal tubular cells in culture, except that the cells displayed little evidence for differentiated active ion transport and tended to grow in a multilayered arrangement. The culture of the epithelial cells from classic Wilms' tumors provides a model system for the study of tumor differentiation and progression. (Am J Pathol 1993, 142:893–905)

Wilms' tumor is a complex childhood neoplasm that resembles the embryonic kidney in its histological appearance. The classic Wilms' tumor is composed of the three components seen in normal kidney differentiation, ie, stroma, blastema, and epithelium. These components are believed to recapitulate the differentiation of the normal nephron unit, with variable degrees of expression.[1] Classic Wilms' tumors have been shown to exhibit considerable variability with respect to the predominate component and the degree of aberrant differentiation (ie, skeletal muscle, smooth muscle, cartilage, and bone). With treatment, classic Wilms' tumors have an excellent prognosis. To date, three separate genetic loci have been linked to the tumor. Two loci are located on chromosome 11, at p13[2,3] and p15.[4] The other locus, not linked to chromosome 11, has yet to be identified.[5,6]

Recently Beckwith et al[7] have subdivided Wilms' tumors and their precursor lesions, nephrogenic rests, into intralobar and perilobar types, with implications for their ontogenic origin and their potential for differentiation. They observed that the perilobar type of Wilms' tumors were commonly composed of blastema and epithelial cells, with sparse stroma. In contrast, the intralobar type contained a large amount of stroma, relative to the amount of epithelial or blastemal cells, and often demonstrated heterologous (eg, skeletal muscle) differentiation. In addition, patients with a deletion at 11p13 (the WAGR syndrome) were frequently associated with the intralobar type of Wilms' tumor and patients with a duplication at 11p15 were associated with the perilobar type.

Recent studies have also linked Wilms' tumors to a fetal growth factor, insulin-like growth factor (IGF). These tumors have been shown to have increased expression of IGF-2 messenger RNA,[8,9] increased content of IGF-2 protein,[10] and increased IGF receptor binding activity,[11] in comparison with adjacent normal tissue. These findings suggest that autocrine

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IGF production may be at least partially responsible for increased proliferation and inhibition of terminal differentiation of this neoplasm. Such a hypothesis is reinforced by the identification of a Wilms' tumor gene at 11p15, in close proximity or identical to the IGF-2 gene also mapped to p15 on chromosome 11.[12,13] These features of arrested differentiation and abnormal autocrine growth control render Wilms' tumors a potentially excellent model system for the study of solid tumor differentiation and progression.

Towards development of such a model system, it has been demonstrated that the majority of Wilms' tumors can be heterotransplanted into Balb/c nude mice with retention of original histological characteristics.[14–16] Additionally, from these heterotransplants and original tumors the cell culture conditions necessary for the chemically defined growth of cells comprising the blastemal,[17] skeletal muscle,[18] and stromal[18] elements of classic Wilms' tumors have been established. The present report details the cell culture growth and heterotransplantation of the epithelial component of classic Wilms' tumors. It is demonstrated that isolation of this component from primary tumors is rare, that the ability for isolation of the epithelial component is lost upon heterotransplantation, and that, when isolated in culture, the epithelial component does retain significant differentiation.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium, Ham's F-12 medium, Hanks' balanced salt solution (HBSS), fetal calf serum, trypsin, type IV collagen, and laminin were obtained from GIBCO Diagnostics (Grand Island, NY). Bovine type I collagen was obtained from the Collagen Corporation (Palo Alto, CA). Components and growth factors for defined growth media were obtained from Collaborative Research (Lexington, MA). Collagenase (C-0130, type I, 200 units/mg of dry weight) and deoxyribonuclease (D-0875, 400 units/mg of dry weight) were obtained from Sigma Chemical Co. (St. Louis, MO). Reagents for the preparation of buffers and other routine solutions were obtained from Fisher Scientific (Atlanta, GA). Tissue culture flasks were of the Corning trademark. Water for the preparation of all reagents and growth media was deionized water that was further treated by using a MilliQ system equipped with carbon, two ion exchange, and organic cartridges (Millipore Corp., New Bedford, MA). The mice used for the heterotransplantation of tumor specimens were male Balb/C nude mice (6 to 10 weeks of age) obtained from the National Cancer Institute (Bethesda, MD). For immunohistochemical staining, mouse monoclonal antibodies to the following antigens were used: 1) cytokeratin, from Sigma; 2) epithelial membrane antigen, from BioGenix (San Ramon, CA); 3) vimentin, from BioGenix; 4) Thy-1 antigen, prepared by A. C. Wang (Medical University of South Carolina, Charleston, SC); and desmin, from BioGenix.

Tumor Specimens

Cell culture was attempted with 18 primary isolates of classic Wilms' tumors, which exhibited a variety of histological patterns (Table 1). Of these, only two isolates (cases 1 and 2) gave rise to an actively proliferating epithelial component. The clinical characteristics of these two tumor isolates are briefly outlined below.

In case 1, a 26-month-old girl was admitted with respiratory distress, hypertension, and an abdominal mass. Upon examination the patient had a blood pressure of 150/120, a left flank mass, and signs of congestive heart failure. Ultrasound analysis showed a large solid mass in the left upper quadrant, crossing the midline. A computerized tomography scan demonstrated a large left kidney mass. Serum renin levels were elevated. The patient underwent a left radical nephrectomy and lymph node biopsies. Postoperatively the blood pressure returned to normal levels. The excised left kidney contained an 8- × 15-cm mass in the upper pole that was encapsulated and did not extend beyond the capsule.

In case 2, an 18-month-old girl was noted to have a large, palpable, flank mass. Ultrasound analysis confirmed the presence of the solid left flank mass.

Table 1. *Characteristics of Classic Wilms' Tumors*

Case	Age	Sex	Histology*	Type†
1	26 months	Female	T, b, s	P
2	18 months	Female	t, b, s	P
3	6 months	Male	b, T, s	P
4	24 months	Male	T	P
5	10 years	Male	b, t, s	I
6	5 years	Female	B, t, s	P
7	10 months	Male	b, T, s	I
8	22 months	Female	B, t, s	I
9	30 months	Female	s, t, b	I
10	6 months	Male	s, t, b	I
11	4 years	Female	B, t, s	P
12	19 months	Male	b, t, s	U
13	24 months	Female	b, t, s	U
14	8 months	Female	B, t, s	U
15	7 years	Female	B, t, s	U
16	9 years	Female	b, t, s	P
17	2.5 years	Male	b, t, s	P
18	14 months	Male	b, t, s	P

* B, blastema; T, tubules; S, stroma; capitalization indicates prominent component, if any.
† P, perilobar; I, intralobar; U, undetermined subtype.

An exploratory laparotomy revealed a large irregular mass in the left kidney. A radical nephrectomy and lymph node sampling were performed. The excised left kidney contained a 7.5-cm multinodular mass confined to the lower pole. The renal hilum was not involved and the lymph nodes were negative.

Tumor Heterotransplantation into Nude Mice

The establishment of heterotransplants from primary tumors and their subsequent serial passage were performed as described previously.[14] Briefly, primary Wilms' tumors were placed into chilled saline immediately after surgical removal. Typically, 50% of the original surgical specimen was available for research protocols. From this specimen, tissue was excised in a nonselective manner. Portions of the tumor were minced into small fragments and injected subcutaneously, by means of a 16-gauge needle, into a series of male Balb/c nude mice. Approximately 1×10^8 cells were injected per site. After tumor growth, the tumor was serially passaged by aseptic excision of the cutaneous tumor nodules, with subsequent mincing and injection as described above.

Cell Culture

Both fragments from the original Wilms' tumor specimen and tumor nodules from heterotransplants were used to establish cell cultures, as follows. Original tumor fragments or subcutaneous tumor nodules excised from sacrificed mice were placed into chilled HBSS. The tissue was then minced into fragments of approximately 1 $cm.^3$ Fragments were chilled and maintained in sterile HBSS until the entire specimen was minced. These fragments were then randomly subdivided and explanted directly into culture flasks as described previously[17,19] or further digested using collagenase/DNase and then transferred to culture flasks. For digestion, the fragments were transferred to a sterile trypsinizing flask containing a stir bar. A solution of collagenase (1.4 mg/ml) and deoxyribonuclease (1.0 mg/ml) in HBSS was prewarmed to 37 C and added to the trypsinizing flask. The enzyme solution was added in the volume necessary to cover both the fragments and the stir bar. The dissociation process was accomplished by placing the flask on a submersible stirrer table in a 37 C water bath. The fragments were dissociated for 15 minutes. Dissociated cells in the enzyme solution were poured through a fabric filter (90-μm mesh diameter) into a 50-ml centrifuge tube. The fabric filter was rinsed with HBSS until the volume of cells, enzyme, and rinse HBSS measured 50 ml. The cells were pelleted by centrifugation for 8 to 10 minutes at 1000 rpm. The pelleted cells were resuspended in 15 ml of HBSS and held at 4 C. The dissociation process was repeated three or four times and the cells collected from each dissociation were pooled and recentrifuged. The final pellet was resuspended in growth medium and aliquots were added to a series of 25-cm^2 culture flasks containing the various growth formulations and matrices to be tested. Twenty-four hours after the cells were plated, debris and contaminating blood cells were removed from the cultures by gentle agitation of the growth medium before aspiration and refeeding of the culture. The cultures were then re-fed fresh growth medium every 2 days.

When confluent, the cell layers were subcultured by rinsing of the monolayer twice with phosphate-buffered saline (PBS), followed by the addition of 1.0 ml of 0.05% trypsin/0.02% EDTA. Cell detachment was monitored by light microscopy and, after cell detachment, further trypsin action was halted by the addition of an equal volume of fetal calf serum. The detached cells were added to a 15-ml centrifuge tube, brought to volume with PBS, and centrifuged at 1000 rpm for 5 minutes. The cell pellet was resuspended in PBS, recentrifuged, resuspended into culture medium, and distributed to new flasks at a 1:5 subculture ratio.

The tumors used in the present study were obtained over a period of several years. Each tumor culture was initiated routinely in both serum-containing medium (15% fetal calf serum in Dulbecco's modified Eagle's medium) and a serum-free medium (Dulbecco's modified Eagle's medium/Ham's F-12 medium) containing growth factors that had been previously shown to promote the long term culture of normal kidney epithelium[19] (see Table 2). The effect of growth media, growth factors, and matrices on cell growth was judged by daily observation of the cultures by using a Zeiss IM35 inverted microscope. The efficiency of the attachment of the cells after plating, the ability of the culture to reach confluency, and the subsequent ability of confluent cultures to continue to undergo subculture were the major parameters used to judge the success of growth medium formulations. Photomicrographs of cultures were recorded on 35-mm Kodak Panatomic X film (ASA 32) by using a Zeiss IM35 inverted microscope.

Tissue Processing

For histological characterization, primary tumor and heterotransplants were cut, immediately after exci-

sion, into 0.5- to 0.8-cm pieces and fixed for 12 to 18 hours at 4 C in Carnoy's solution or calcium acetate-buffered formaldehyde. After fixation, tissues were stored overnight in 70% ethanol and on the next day were dehydrated through graded ethanols, cleared in xylene, and embedded in paraffin. For ultrastructural characterization, the primary tumor and heterotransplants were minced into 1-mm^3 fragments and fixed in 2.5% glutaraldehyde overnight at 4 C. The tissue was postfixed in 1% osmium tetroxide in 0.1 mol/L PBS for 1 hour at room temperature. The tissue was dehydrated and embedded in Epon 812. Ultrathin sections were generated by using routine procedures. Cell cultures were fixed *in situ* with 2.5% glutaraldehyde in PBS (pH 7.4) for 1 hour at room temperature. The cell cultures were then processed routinely as described above. After polymerization of the Epon 812, the plastic was removed and ultrathin sections were cut parallel and perpendicular to the surface of the flask. Ultrathin sections from all samples were stained with uranyl acetate and lead citrate and examined in a JEOL 100S electron microscope. Freeze-fracture analysis was performed as described previously.[20]

Cell Preparation for Immunohistochemistry

Normal kidney tubule cells and Wilms' tumor cells were passaged to Lab-Tek chamber slides (Nunc, Inc.) and were fed using the optimal medium as determined in the present study. Cultures were fed until confluent. The cultures were then rinsed with PBS and fixed for 10 minutes in either Carnoy's solution or calcium acetate-buffered formalin. After fixation the chamber slide with attached cells was rinsed for a minimum of 30 minutes in PBS. The slides were then used in routine immunostaining protocols. Chamber slides were prepared throughout the series of subcultures to compare the effects, if any, of prolonged passaging on immunoreactivity for various antigens.

Immunohistochemistry

Immunohistochemical staining was performed on Wilms' tumor tissue sections as well as cell culture preparations as described previously.[17,21] The indirect avidin-biotin-peroxidase procedure was used to visualize antibody binding, by using a Vectastain kit (Vector Laboratories, Burlingame, CA). Controls consisted of the use of normal kidney cell cultures as positive controls, omission of the primary antibody from the staining sequence, and substitution of spent medium from mouse myeloma cells (SP-2) in place of the primary antibody for Thy-1 (HB-2S-1).

Thymidine Incorporation

Cells were plated onto collagen-coated 96-well plates. At confluence, the medium was replaced with basal medium without added peptide growth factors. Two days later, cells were re-fed with basal growth medium and the added factors. Eight hours later, the cells received a 12-hour pulse of [*methyl*-^{3}H]thymidine (0.5 μCi/well). The cells were detached with trypsin-EDTA, and the DNA-associated radioactivity was immobilized on glass fiber filters by using a semiautomatic cell harvester (Skatron Inc., Sterling, VA). Tritium was quantitated by liquid scintillation counting and the mean values of triplicate wells were expressed as a percentage of control (basal medium only) values.

Results

Epithelial Cell Culture from Primary Tumors

Previous studies in this laboratory demonstrated that a serum-free growth formulation was effective for establishment of the growth conditions for the blastemal and skeletal muscle components of Wilms' tumors[17,18] and the proximal tubules of normal human kidneys.[19] For that reason these growth medium formulations, as well as a serum-containing medium, were used in the present study for the potential isolation of the epithelial components of the classic Wilms' tumors listed in Table 1. Regardless of growth medium formulation or method of tumor tissue presentation (explantation or dissociation), two primary tumors (cases 1 and 2) gave rise to a rapidly proliferating epithelial cell culture that reached confluency within 3 to 4 days of initiation. The other isolates failed to yield a substantial epithelial component, although occasional foci of epithelial cells were noted that were rapidly overgrown by blastemal elements. Of interest is the fact that neither of the two isolates giving rise to the epithelial component produced primary cultures of blastemal cells.

Beyond the primary culture, in both cases, a basal serum-free growth medium composed of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), and triiodothyronine (4 pg/ml), in conjunction with a collagen type I matrix, allowed the stable subculture of cells from the pri-

mary culture. Use of collagen type IV and/or laminin failed to enhance subculture capabilities. Under basal growth medium conditions, cells attached with a plating efficiency of approximately 70%, exhibited a constant morphology without vacuolation, and remained viable, but nonproliferating, for at least 7 days. No cells with a fibroblast-like morphology were present. For each individual case, this formulation was used as the basal medium for the testing of further additions to the growth formulation. A summary of the final formulation for each case is provided in Table 2.

The testing of growth factor additions to the primary cultures derived from case 1 demonstrated that only epidermal growth factor (EGF) (10 ng/ml) and the addition of fetal calf serum proteins to the collagen type I matrix were needed to achieve optimum cell growth and retention of morphology. The absorption of fetal calf serum components onto the collagen type I matrix resulted in >95% cell attachment after subculture, compared with approximately 60% on collagen alone. The addition of EGF allowed both primary and secondary cultures to proliferate to confluency. The addition of both factors allowed the serial passage of primary cultures of the cells at a 1:3 subculture ratio for at least 30 serial passages. The cells exhibited a doubling time of 40 to 60 hours. The cells were assessed for their response to the addition of the components of the serum-free medium through the determination of thymidine incorporation. Initial determinations demonstrated that selenium, transferrin, hydrocortisone, and triiodothyronine had a marginal effect on thymidine incorporation by the cells. Therefore, the serum-free growth medium including these four components was used as the basal medium for the testing of additional factors. Of the remaining factors, only insulin and EGF were identified as being mitogenic for the cells. Insulin and EGF elicited an increase in thymidine incorporation of 166% and 145%, respectively, compared with the basal growth medium. The simultaneous addition of both factors demonstrated no additional increase in thymidine incorporation (170%) over that noted individually (Figure 1).

The primary cultures isolated from case 2 required both EGF (10 ng/ml) and an absorbed fetal calf serum/collagen type I matrix for subculture, proliferation, and retention of original light microscopic morphology. However, between passages 5 and 10 (1:3 subculture ratio) the cells could be noted to rapidly lose proliferative capacity and undergo senescence. The further testing of growth factor supplements disclosed that putrescine (10 ng/ml), bovine pituitary extract (30 μg/ml), prostaglandin E_1 (10 ng/ml), and ethanolamine (1.2 μg/ml), when added alone, were able to extend the proliferative capacity of the cells by several passages. Deletion studies demonstrated that all four factors were required to extend the life span of the cells to at least 25 passages. Under these conditions, the cells proliferated as a monolayer with a doubling time between 24 and 36 hours. Deletion of any one of the four factors decreased the life span of the cells to <25 passages. Thymidine incorporation studies gave results identical to those for case 1, with insulin

Table 2. *Effects of Culture Conditions on the Growth of Epithelial Cultures*

Components*	Case 1†	Case 2
Basal medium		
15% DME	+	+
DME/F-12‡	+/++	+/++
Matrix		
Collagen I	60%§	70%§
Collagen IV	NE	NE
Laminin	NE	NE
Absorbed FCS	95%§	95%§
Growth factors		
EGF	+++	+++
BPE	NE	+++
PGE_1	NE	+++
ETH	NE	+++
PTE	NE	+++

* DME, Dulbecco's modified Eagle's medium; F-12, Ham's F-12 medium; FCS, fetal calf serum; BPE, bovine pituitary extract; PGE_1, prostaglandin E_1; ETH, ethanolamine; PTE, putrescine.

† NE, no effect; +, allowed primary culture; ++, allowed stable subculture; +++, promoted continued subculture for ≥25 passages.

‡ Including insulin, transferrin, selenium, hydrocortisone, and triiodothyronine.

§ Plating efficiency with basal Dulbecco's modified Eagle's/Ham's F-12 medium.

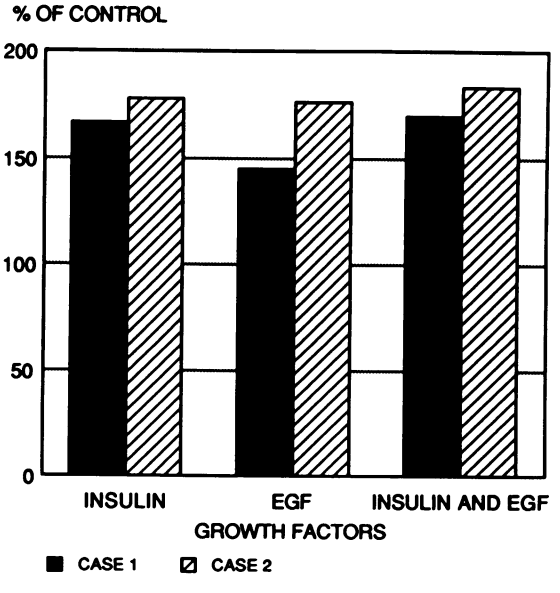

Figure 1. *Effects of insulin and EGF on thymidine uptake, expressed as a percentage of the control value of uptake in basal medium.*

(178%) and EGF (176%), alone and combined (183%), increasing thymidine incorporation over basal levels (Figure 1). The other additions were without effect.

Epithelial Cell Culture from Heterotransplanted Tumors

In this laboratory, no Wilms' tumor heterotransplant has ever given rise to an epithelial cell culture. In the case of the two primary tumors described above, tissue from each case was placed into culture after initial heterotransplantation into nude mice and, although identical procedures were used, neither case gave rise to cultures of epithelial cells. However, these heterotransplants did give rise to rapidly growing cultures of blastemal cells identical to those described previously by this laboratory.[17]

Primary Wilms' Tumors and Heterotransplants

The light microscopic features exhibited by the primary tumors from cases 1 and 2 were similar. Each contained blastema, stroma, and a prominent epithelial component. However, other primary tumors had similar histological profiles and failed to give rise to epithelial cell cultures. The epithelial component present in these two cases represented a major portion of both tumors and formed both tubules and glomeruloid bodies (Figures 2 and 3). The tumors demonstrated no heterotopic elements. These tumors had the appearance of the perilobar type of Wilms' tumor. Ultrastructurally the tubules contained central lumina of varying sizes. A discrete basal lamina was seen around the tubules. Microvilli on the surface of the lining cells were sparse, short, and irregularly distributed. Fully developed brush borders were not identified. In the apical regions most of the epithelial cells were attached by junctions with parallel cytoplasmic densities of approximately 0.6 μm in length but were separated by a gap of approximately 25 nm. Rare junctions suggested membrane fusion characteristic of tight junctions, but definite tight junctions were not identified. Adjacent cells were attached by short desmosomes along their lateral borders. Although there was some cytoplasmic organization, the quantity and orientation of the organelles were not those of fully differentiated renal tubular cells. (Figure 4).

Heterotransplants from cases 1 and 2 contained the same components as the primary tumors. However, in contrast to the primary tumors, blastema constituted the majority of the heterotransplanted tumors. Epithelial tubules and stroma were sparse, and there were rare glomeruloid bodies identified in the heterotransplants. The ultrastructural features of the epithelial cells from cases 1 and 2 were maintained in the heterotransplants in spite of their smaller number, relative to the primary tumors.

Immunohistochemical Characterization of Wilms' Tumors and Cultures

Previously reported immunohistochemical studies of Wilms' tumors by this laboratory[17] indicated that blastemal elements within classic Wilms' tumors were characteristically immunoreactive for vimentin. Tumor blastema did not immunoreact with any of the other antibodies used. However, epithelial tubules within the tumors were immunoreactive for cytokeratin, epithelial membrane antigen, and Thy-1 protein.[21] The same pattern of immunoreactivity was confirmed in tumors used in the present study (Table 3). Similarly, epithelial cultures derived from cases 1 and 2 stained positively for the presence of cytokeratin, epithelial membrane antigen, and Thy-1 protein, confirming the epithelial differentiation in the cultures. This immunoprofile parallels that observed in normal kidney tubule cultures. Vimentin staining was also noted in these cultures and is consistent with the development of vimentin immunoreactivity in a variety of cells in culture. Staining characteristics were not observed to change with increasing cell subculture numbers.

Morphological Characteristics of the Cultured Cells

The cells isolated from case 1 demonstrated a differentiated epithelial morphology and proliferated as a monolayer until confluency was reached. Upon reaching confluency, the monolayers did not form multilayers in a typical fashion but, rather, the monolayer persisted while elaborate networks of "cords of cells" formed that traversed the underlying mono-

Figure 2. *Case 1. The primary tumor was composed primarily of tubules (arrow) and glomeruloid bodies (arrowhead) (×100).*
Figure 3. *Case 2. The primary tumor contained tubules (arrow), blastema (arrowhead), and occasional glomeruloid bodies (not shown) (×100).*
Figure 4. *Case 1. Ultrastructural examination of the primary tumor showed tubules surrounded by a distinct basal lamina (arrowheads). Short microvilli were on the apical surface and the cytoplasmic organelles did not show organization (×10,250). Inset, the apical regions were joined by junctions with parallel cytoplasmic densities and were separated by a gap (×21,000).*

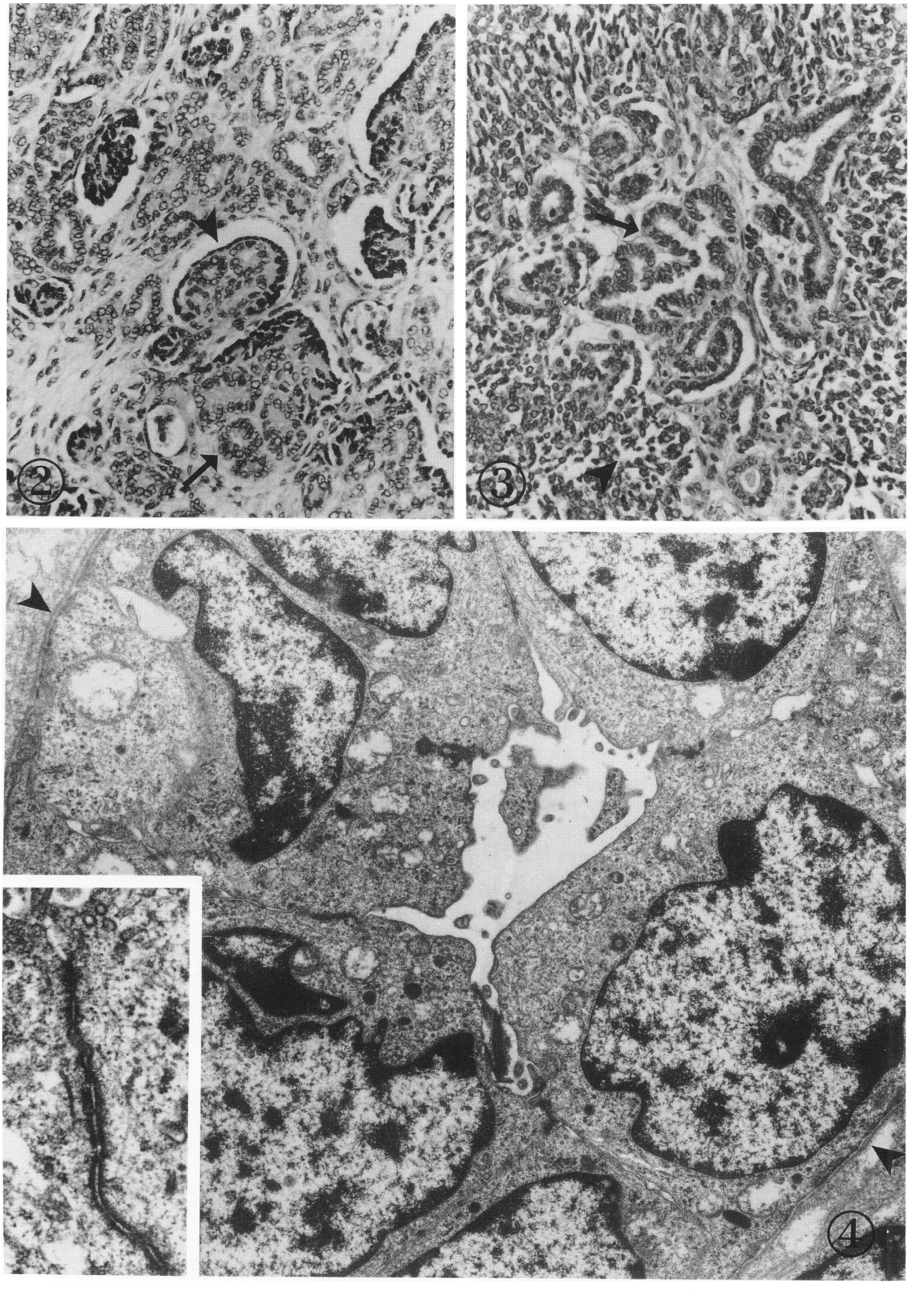
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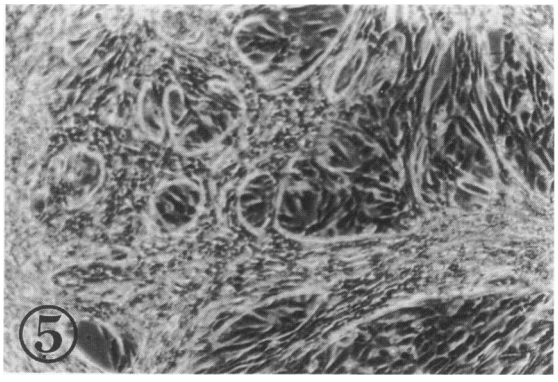
Table 3. *Immunohistochemical Profile of Wilms' Tumors and Epithelial Cultures*

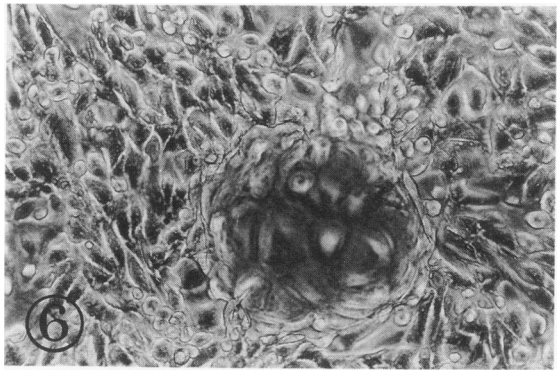
	Immunohistochemical stains*				
	CYTO	EMA	VIM	Thy-1	Desmin
Wilms' tumor					
Blastema	–	–	+/–	–	–
Tubules	+	+	–	+	–
Wilms' tumor epithelial cultures	+	+	+	+	–

* CYTO, cytokeratin; EMA, epithelial membrane antigen; VIM, vimentin.

layer (Figure 5). These cords could be clearly noted to branch extensively and intermix with other cords, forming a complex multilayered network. The cells from case 1 were also demonstrated to occasionally form "domes," structures indicative of retention of differentiated active ion transport capability (Figure 6). Light microscopic examination disclosed that the cells isolated from case 2 exhibited a poorly differentiated epithelial morphology and proliferated largely as a monolayer, with only occasional focal patches of multilayer growth (Figure 7). The cells from case 2 were never observed to form domes. The continued feeding of confluent cultures for an additional 10 days did not result in an increase in the number of areas of multilayered growth.

An ultrastructural examination of cultured cells derived from the two cases largely confirmed the light microscopic observations and revealed further differences in the degree of epithelial differentiation between the different epithelial cell cultures. Cultures derived from case 1 exhibited multilayers of cells consisting of four layers. The most differentiated cells appeared to be located in the upper layer, with good lateral contact between adjacent cells and well formed microvilli oriented at the apical membrane. A full complement of organelles and cell junctions was noted. Rough endoplasmic reticulum appeared to be more abundant in these cultures than in case 2 (Figure 8). A unique feature observed in case 1 involved the network of cord-like structures described above. An ultrastructural analysis of cross-sections through cords revealed that each structure was actually a tube with a well defined lumen. The polarity of the cells comprising the tube was entirely reversed from that observed in a normal kidney tubule, in that the microvillar (apical) border of the cell appeared to face outward while the basal region of the cell and the basement membrane bordered the center of the cord or "lumen" (Figure 9). Freeze-fracture examination confirmed the presence of tight junctions, with each junctional profile being composed of at least





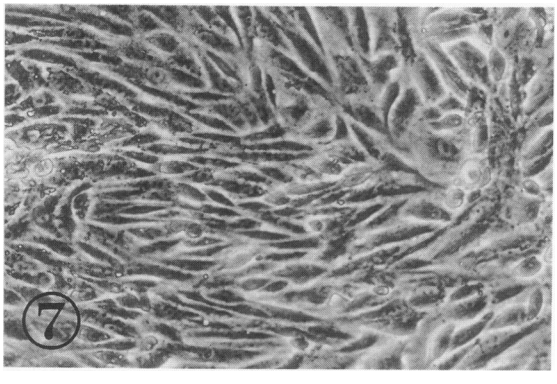


Figure 5. *Case 1. Inverted phase microscopy of the epithelial cells in culture showed cords of cells growing over the underlying monolayer (×100).*
Figure 6. *Case 1. Inverted phase microscopy of epithelial cells demonstrated dome formation in these cultures. Domes appear in the out-of-focus regions (×125).*
Figure 7. *Case 2. Inverted phase microscopy of the cells in culture showed monolayer growth with a poorly differentiated epithelial morphology and no multilayering (×125).*

three sealing strands (Figure 10). When these cells were plated on Millicell filter inserts, multilayering was enhanced and the cord-like network of cells persisted.

Cultures derived from case 2 revealed epithelial cells that were less differentiated. In flasks, the cultures were minimally multilayered, in that two layers of cells predominated throughout the culture. As in case 1, the upper layer of cells appeared most well differentiated, with good lateral contact between

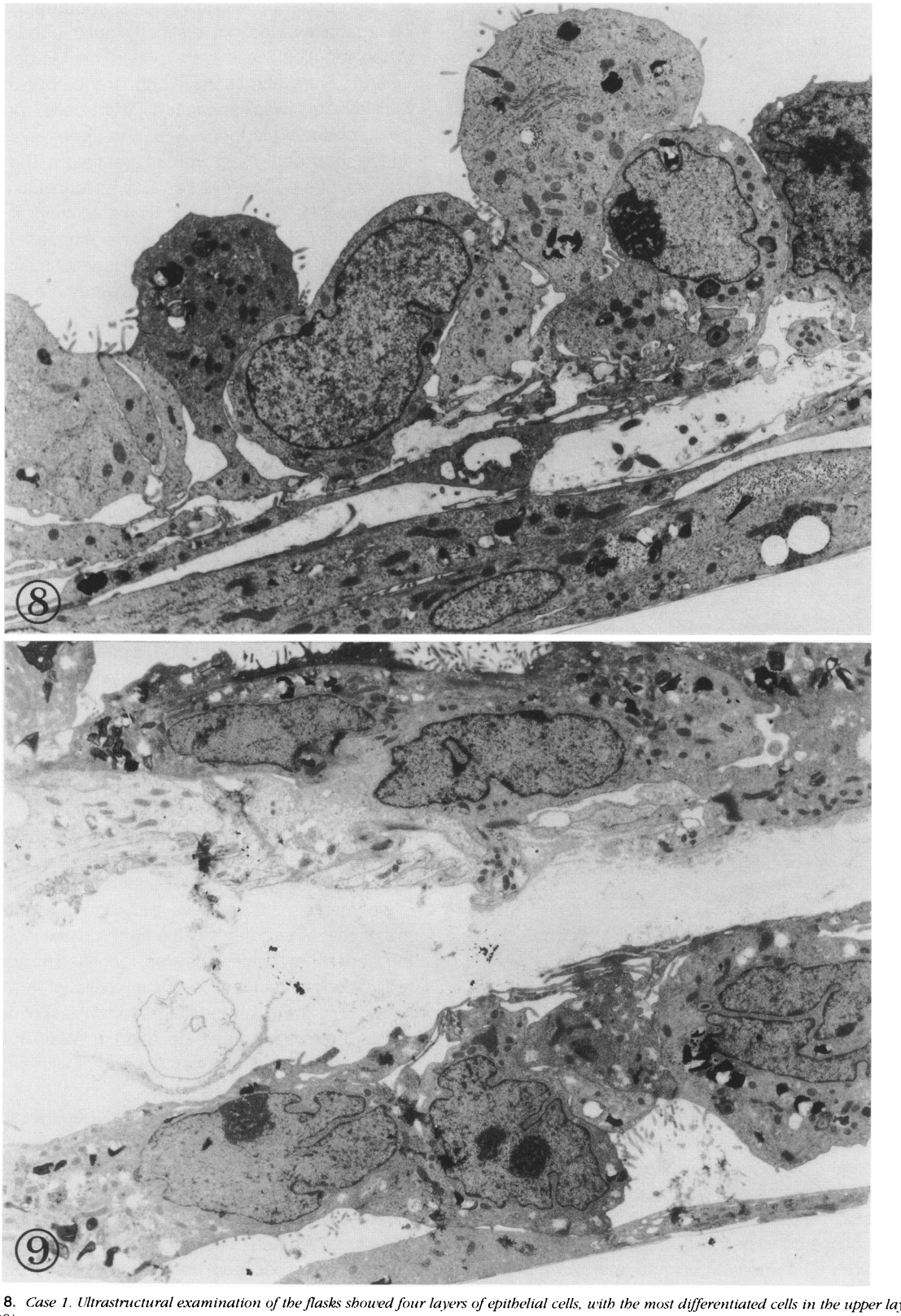


Figure 8. *Case 1. Ultrastructural examination of the flasks showed four layers of epithelial cells, with the most differentiated cells in the upper layer (×4300).*

Figure 9. *Case 1. A cross-section through one of the cords revealed that each was a tube with a lumen. The polarity of the cells comprising the tube was reversed, in that the apical border of the cells faced outward and the basal region of the cell and the basement membrane bordered in the center of the lumen (×4400).*

Figure 10. *Case 1. Freeze-fracture profiles exhibited networks of sealing strands, the structural components of tight junctions (×60,000).*

adjacent cells and well formed microvilli oriented at the apical membrane. Cell junctions were located at the apical portion of the lateral membranes. Clear evidence of membrane fusion, as observed in tight junctions, was not obtained at these sites. Instead, the junctions consisted of opposing cell membranes separated by a uniform gap and lateral cytoplasmic densities, as observed in the original tumor. Below these junctions were scattered desmosomes. Organelles observed included mitochondria, fragments of rough endoplasmic reticulum, myeloid bodies, and coated pits and vesicles. The arrangement of organelles was not distinctly polar (Figure 11). When identical cells were plated on Millicell filter inserts several alterations in appearance were noted. Multilayering was enhanced, with cells routinely being observed in four layers. The upper layer continued to appear most well differentiated. The complement of organelles observed was identical to that described for cells grown in flasks. Microvillar number and structure were somewhat enhanced, but no differences were observed in cell junctions and tight junctions were not noted by freeze-fracture analysis (Figure 12).

Discussion

Evidence that several different genetic loci may play a role in Wilms' tumor predisposition and progression continues to accumulate. Likewise, Wilms' tumor is an excellent potential model for demonstrating the relationship between malignancy and aberrant differentiation.[22] Clearly, cell cultures of the differentiated components of this tumor, such as the prominent epithelial component of these tumors, will be significant in studies designed to correlate gene expression with differentiation. Epithelial cells of the kidney originate from the blastemal (mesenchymal) cells of the mesoderm under the inductive effect of the ureter epithelium (ureteric bud). The morphogenesis or differentiation of these cells results in their ability to perform critical physiological renal functions. In Wilms' tumor, an embryonic renal tumor, the differentiation of epithelial cells has been hypothesized to recapitulate the stages of normal embryological development.[1] Immunohistochemical and lectin marker studies have provided evidence for this, in that all segments of the nephron unit including the collecting tubules may be identified in varying proportions within the epithelial cells of a Wilms' tumor.[23–25] Transport mediators (enzymes) of normal renal tubules have been identified in Wilms' tumors, and the enzyme profile was consistent with those of fetal tubules.[26]

In the present study, the culture of epithelial cells was attempted from 18 cases of classic Wilms' tumors, all of which possessed varying proportions of epithelial component. The finding that only two of these isolates gave rise to epithelial cell cultures was surprising. Whereas one of these tumors (case 1) did possess the most prominent differentiated epithelial component and glomeruloid bodies of all the tumors tested, the other possessed an epithelial component similar in abundance to those of several other tumors. Both cases were perilobar-type Wilms'

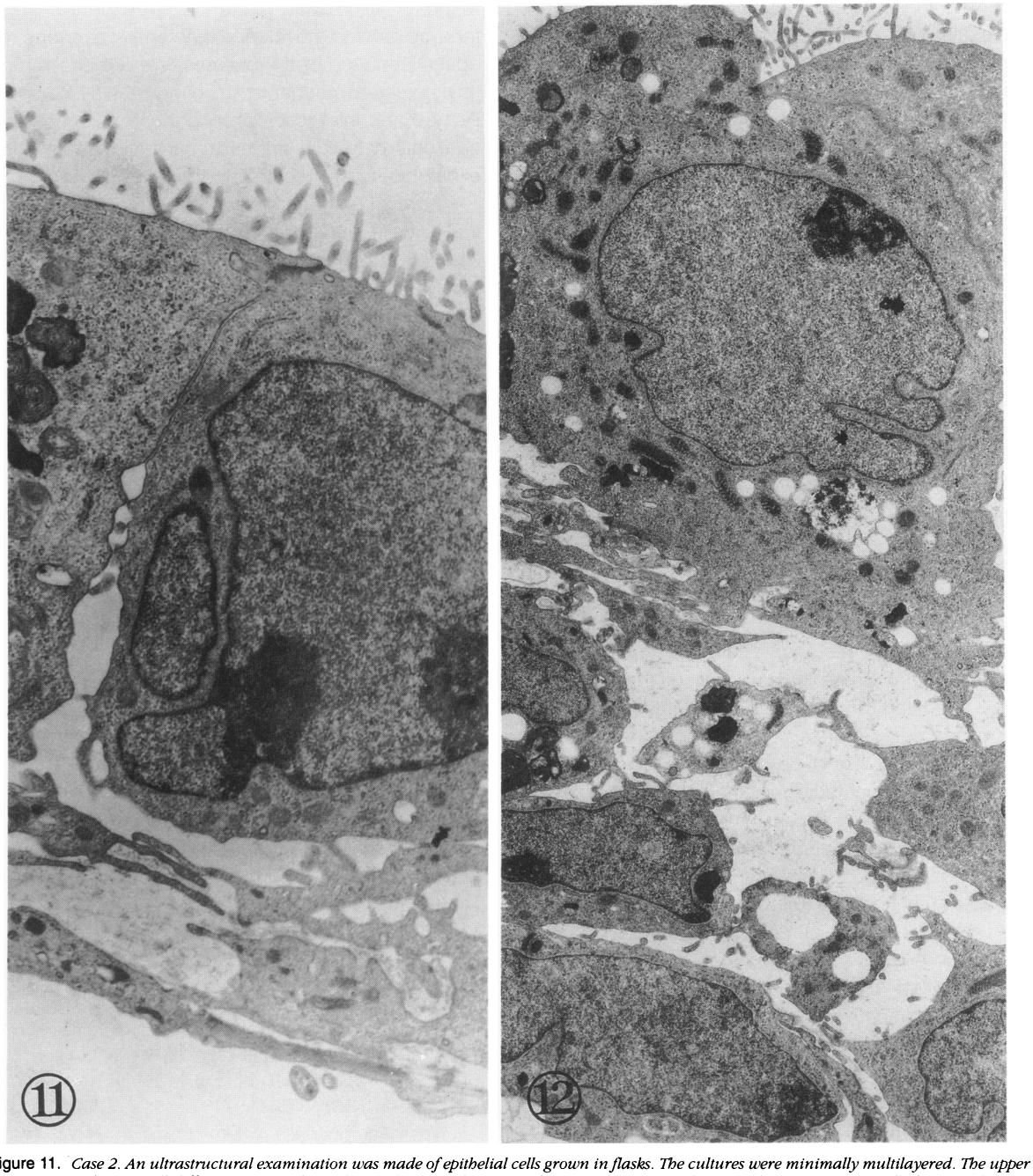


Figure 11. *Case 2. An ultrastructural examination was made of epithelial cells grown in flasks. The cultures were minimally multilayered. The upper layer appeared most well differentiated, with good lateral contact between adjacent cells, no cytoplasmic polarity, and microvilli (×10,000).*
Figure 12. *Case 2. Ultrastructural examination of epithelial cells grown on Millicell filter inserts showed that multilayering was increased, compared with cells grown in flasks (Figure 11), and the upper layer appeared the most well differentiated (×5000).*

tumors, which are usually composed predominately of blastema and epithelial cells. However, it should be pointed out that many of the tumors that failed to produce epithelial cultures were also of the perilobar subtype. In the two cases where epithelial cell cultures were initiated, the growth of these cells was rapid, producing a confluent primary culture within 48 hours. The cultures were composed of a homogeneous population of cells, as judged by light microscopic morphology. Additionally, the other primary tumors placed into culture under identical conditions acted similarly, except that these yielded rapidly proliferating cultures of blastemal cells, with one exception. This exception was an intralobar type of Wilms' tumor that yielded confluent cultures of skeletal muscle cells. These findings would be consis-

tent with the concept that different Wilms' tumors have variable ontological potential, possibly dependent on local tumor environment or specific genetic defect. This concept is reinforced by the findings that both primary tumors giving rise to epithelial cell cultures subsequently failed to produce similar cultures after initial heterotransplantation into nude mice. Rather, blastemal cultures were produced, even though the tumor still possessed an epithelial component, albeit one reduced in prominence.

The successful culture of the epithelial component from these two cases provides a comparison of the morphology and growth of these cells with the features of normal human renal tubule cells in culture, as previously characterized by this laboratory.[19,20] Two striking differences were noted between the cultured Wilms-derived epithelial cells and normal renal tubule cells. The first was the fact that only the tumor-derived cells became multilayered in culture. The second was the fact that the tumor-derived cells either rarely formed (case 1) or failed completely to form (case 2) domes in culture. Normal renal tubules in culture form tight junctions between adjacent cells and selectively transport fluid to their basal surface.[19] This causes the cells to lift off the flask and form fluid-filled domes that are visible by phase microscopy. This reduced capacity for differentiated transport of the Wilms'-derived epithelial cells was consistent with the finding of no tight junctional sealing strands between the epithelial cells from case 2 and only occasional junctional profiles between cells from case 1. Thus, with respect to differentiated transport function, the Wilms' tumor-derived epithelial cells appeared to be only marginally active.

Otherwise, the Wilms-derived epithelial cells appeared to possess many characteristics of normal renal epithelial cells in culture, including similar immunohistochemical profiles. These cells from the Wilms' tumor cases grew well in a growth medium similar to that which promotes the growth of normal proximal tubule cells.[19] In agreement with this, pulse labeling of the tumor-derived epithelial cells with thymidine demonstrated a mitogenic response to insulin and EGF that was identical for both tumor isolates. Although addition of EGF to basal growth medium has been previously shown to improve long term propagation of Wilms' tumor cells,[17] the present study indicates that EGF does not augment the acute mitogenic effect of basal medium with insulin. Insulin (or IGF) and EGF are highly synergistic cell cycle "progression" factors for fibroblastic cells.[27,28] In contrast, synergistic or even additive mitogenic effects of the two factors are not observed with many types of normal and neoplastic epithelial cells that respond to either factor independently,[29,30,31] thereby providing evidence that further supports the epithelial nature of the cultures. We are unaware of any molecular mechanism to explain the tissue specificity of the interaction among these mitogens and can only note that normal renal tubules in culture exhibit a similar response to these growth factors.[32]

Likewise, both the normal and tumor-derived epithelial cells demonstrated increased differentiation when grown on permeable supports that allow apical and basolateral exposure to nutrients. Lastly, both tumor-derived isolates of epithelial cells displayed senescence, losing proliferative capacity at approximately 40 to 60 generations. A practical aspect of this finding would be the apparent need to immortalize such cultures to assure a constant supply. Thus, the epithelial cells isolated from the two cases of classic Wilms' tumors displayed many characteristics of normal renal epithelial cells. Such cultures of the epithelial component should provide a valuable part of a model system in which to further study the differentiation and progression of Wilms' tumors at the cellular and molecular levels.

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