The In Vitro *Growth and Characterization of the Skeletal Muscle Component of Wilms' Tumor*

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Skeletal muscle differentiation within a Wilms' tumor is a well-documented histopathologic entity thought to occur at a relatively low incidence and influence prognosis. A serum-free hormonally defined growth medium has been developed, allowing the long-term growth of the skeletal muscle component of Wilms' tumors. Eight Wilms' tumors have been grown under these conditions. Three cases grew a homogeneous population of cells which ultrastructurally displayed all stages of myogenesis through myotubule formation. They also possessed immunoreactivity for skeletal muscle myosin and myoglobin and synthesized the M and B subunits of creatine kinase. Of interest was the finding that the ability to yield skeletal muscle cultures was limited to those cases which exhibited skeletal muscle fibers *in vivo*. This technique is also a very sensitive marker for identifying Wilms' tumors possessing a myoid component. A second serum-free hormonally defined medium has also been developed that supports the long-term culture of a unique cell type from Wilms' tumors which contain a myoid component. These cells are spindle-shaped and exhibit all of the characteristics of early myoblasts. (Am J Pathol 1985, 121: 298–310)

SKELETAL muscle differentiation within a Wilms' tumor is a well-documented histopathologic entity. The skeletal muscle is believed to arise from the differentiation of the mesenchymal or stromal component of the tumor. This differentiation has been thought to occur at a relatively low incidence and influence prognosis. A detailed study of the myoid component has been hampered by the lack of both a cell culture or animal model system. Wilms' tumor has been known to develop in many animal species *in vivo*, but no convenient, repeatable model system for study has resulted.[1] Attempts to establish the myoid component in cell culture have likewise demonstrated only marginal success. Short-term primary or organ cultures of Wilms' tumors have been reported to yield blastemal or epithelial elements with many cultures described as possessing bizarre or altered morphologic features.[2–6] In all cases the cultures either lost proliferative capability or were overgrown with fibroblastlike cells.

To overcome these limitations our research group sought to develop a serum-free hormonally defined tissue culture medium which would facilitate the selective explantation of the myoid component of Wilms' tumors. As documented in a preliminary report, the culture conditions for the primary explant of the myoid component of a single case of Wilms' tumor was achieved.[7] The selective growth medium developed allowed the expression of myoid differentiation, as noted by the presence of long, multinucleated myotubes clearly observable at the light-microscopic level. Ultrastructural examination of the explanted cells confirmed the above findings with the demonstration of all stages of early myogenesis from myoblasts to myotubes and the abundant presence of skeletal muscle myosin at various stages of maturity. A combination of ultrastructural analysis and examination with antibody to skeletal muscle myosin confirmed the purity of these explant cultures and an absence of fibroblast contamination.

In this report, the repeatability of the above system and the further refinement of culture for the long-term growth of the myoid component and an undifferentiated

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myoblast cell will be documented. Of interest is the finding that only Wilms' tumors possessing an *in vivo* differentiated skeletal muscle component can give rise to skeletal myotubes and myoblast cells in culture. In addition, preliminary data suggest that skeletal muscle may be more common in Wilms' tumors than originally observed by routine histologic examinations.

Materials and Methods

Reagents

Dulbecco's modified Eagles' medium (DME) and Ham's F-12 Medium (F-12) were obtained from Gibco Diagnostics (Grand Island, NY). Fetal calf serum (FCS) and trypsin-versene solution (0.05%:0.02%) were obtained from Biofluids (Rockville, Md). Components for serum-free growth medium were obtained from Collaborative Research (Lexington, Mass). Bovine Type I collagen was obtained from Flow Laboratories (McLean, Va). 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 and tissue culture plates of the Costar trademark. Triple-distilled water was utilized for the preparation of all cell culture reagents. Myoglobin antibodies were purchased from Cappel Laboratories (Westchester, Pa).

Specimens of Wilms' Tumors

Eight Wilms' tumors were utilized in this study, and all experimental protocols were performed with approval of the Institutional Review Board for Human Research of the Medical University of South Carolina. The surgical specimens were received fresh from surgery in 0.9% sterile saline and accessioned for diagnosis in the Surgical Pathology Laboratory. Each tumor was photographed and processed for frozen section diagnosis. For each specimen, several small portions of the tumor were fixed in 3% cacodylate-buffered glutaraldehyde for electron-microscopic examination. This tissue was rinsed in 0.1% cacodylate buffer, postfixed in 1% OsO_4, dehydrated through graded alcohols, and embedded in Epon 812. Ultrathin sections were examined following staining with uranyl acetate and lead citrate in a JEOL 100S electron microscope. Additional portions of tumor were randomly selected and placed into sterile DME medium with sterile technique and transported to the tissue culture facility. The remaining kidney and tumor were fixed overnight in 10% neutral buffered formalin and Carnoy's fixative; and multiple portions of the tumor, kidney, lymph nodes, veins, and ureter were processed in a routine fashion into paraffin blocks, sectioned, and stained with hematoxylin and eosin (H&E). Additional sections from selected blocks were stained with phosphotungstic acid hematoxylin (PTAH) or Masson's trichrome for connective tissue elements and immunostained for skeletal muscle myosin with the use of the biotin–avidin technique. The antibody to skeletal muscle myosin was a gift from Dr. Mark Willingham of the National Institutes of Health and has been characterized as to its specificity for skeletal muscle myosin.[8]

Explant Culture of Wilms' Tumors

Upon arrival at the cell culture facility, the tumor tissue was transferred into a sterile 100-mm Petri dish inside a laminar flow hood. The tissue was sectioned with scissors and forceps into fragments of approximately 1 cu mm. Nine tissue fragments were placed on the growth surface in each of a series of 25-sq cm T-flasks. The flasks were placed in an inverted position for 30 minutes at room temperature for facilitation of fragment adhesion to the growth surface. The flasks were righted, and 2.5 ml of growth medium was added to each flask. The explant flasks were placed at 37 C in a 5% CO_2:95% air atmosphere and left undisturbed for 3 days. The cultures were fed fresh growth medium every 3 days until confluency was reached.

Subculture of Cells

When confluent, the cell monolayers were subcultured by rinsing the monolayer twice with phosphate-buffered saline (PBS) followed by the addition of 1.0 ml of trypsin–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 FCS or 0.1% soybean trypsin inhibitor. The detached cells were transferred to a 15-ml centrifuge tube, brought to volume with PBS, and centrifuged at 800*g* for 5 minutes. The cell pellet was resuspended in PBS, recentrifuged, and resuspended into culture medium and distributed to new flasks at a 1:3 subculture ratio.

Development of Improved Growth Medium Formulations

To develop improved serum-free growth medium formulations, we took two approaches. The first involved explanting the myoid component in the serum-free medium described previously[7] and allowing cell proliferation to proceed to confluency. These cells were then subcultured as described above except that after subculture the cells were equally distributed among a se-

ries of 24-well tissue culture plates (1 sq cm growth area per well) with duplicate wells containing each growth medium modification to be assessed. With this technique, a 25-sq cm T-flask of confluent cells can be utilized for subculture, at a 1:3 ratio, into three 24-well plates. This allows the assessment of 36 growth medium formulations in duplicate from a single flask of explanted cells. The second approach for testing improvements in growth medium formulations arises because of the large mass of most Wilms' tumors. This large size allows sufficient explant tissue to initiate over 100 primary explant flasks, and thus growth medium alterations can be tested at the explant stage of growth.

Electron Microscopy

Monolayers were fixed *in situ* with 2.5% glutaraldehyde in pH 7.4, 0.1 M cacodylate buffer for 1 hour at room temperature. The monolayer was postfixed with 1% OsO_4 in 0.1 M cacodylate buffer for 1 hour at room temperature. The monolayers were dehydrated, and the flask was embedded in Epon 812. After polymerization, the plastic was removed, and ultrathin sections were cut parallel and perpendicular to the surface of the flask. The ultrathin sections were examined with uranyl acetate and lead citrate staining in a JEOL 100S electron microcope.

Immunohistochemistry

The avidin–biotin system was utilized for the immunohistochemical demonstration of skeletal muscle myosin, smooth muscle myosin, and myoglobin. A 75-sq cm confluent flask of cells was fixed for 3 minutes with Carnoy's fixative and rinsed three times with 70% ethanol. At this stage, the cells were rolled off the plastic with the aid of a rubber policeman and centrifuged. The cell pellet was routinely dehydrated and paraffin-embedded. Sections of cells, 6 μ thick, were deparaffinized and rehydrated prior to staining. In all cases, the antiserum was at a 1:500 dilution. Controls included staining of serial sections with nonimmune serum and staining of sections of Carnoy-fixed smooth and skeletal muscle.

Creatine Kinase (CK) Isoenzyme Determination

The cells under study were grown to confluency on 100-mm tissue culture dishes, the monolayer of which was rinsed twice with 10 ml PBS and then evacuated. Two milliliters of PBS were added to each dish, and the cells were scraped off with a rubber policeman. The cells were disrupted in a homogenizer, and the suspension was centrifuged at 10,000 rpm for 20 minutes at 4 C. Total CK activity was measured with the use of creatine phosphate and ADP as substrates by the modification of Szasz.[9] For isoenzyme separation, the supernatant was placed on agarose gel; and after electrophoresis, visualization was accomplished with a fluorometric substrate (Beckman's Paragon electrophoresis system). Quantitation of isoenzymes was accomplished by densimetric scanning with reference controls.

Results

Characteristics of Wilms' Tumors Which Yield Growth of the Myoid Components

In a previous report from the laboratory, the serum-free cell culture conditions necessary for the selective growth of the myoid component from a single case of Wilms' tumor was described.[7] To determine whether growth *in vitro* of the myoid component was a general property of all or only certain morphologic types of Wilms' tumor, we evaluated 7 additional cases for their ability to yield viable cultures of the myoid component. The characteristics of these cases of Wilms' tumors are summarized in Table 1 (Case 8 is the original case cited in Sens et al[7]); and of the 8 cases, only case 8 was noted to possess skeletal muscle differentiation during routine diagnostic procedures. Likewise, only Case 8 was noted to possess skeletal muscle differentiation upon subsequent examination by NWTS. No evidence of smooth muscle differentiation was noted on histologic evaluation.

Of the 8 cases of Wilms' tumors assessed for growth of the myoid component *in vitro*, 3 cases gave rise to rapidly proliferating cultures of skeletal muscle cells. The identification of these cultures as skeletal muscle relied on the unmistakable appearance of long, multinucleated myotubes upon light-microscopic examination (Figure 1a and b). The skeletal muscle cultures derived from each of the 3 cases of Wilms' tumor appeared identical at the light-microscopic level and were indistinguishable from that depicted in Figure 1. The 3 cases of Wilms' tumors yielding skeletal muscle cell cultures were Cases 1, 4, and 8. An inspection of the characteristics of these tumors (Table 1) demonstrated that tumors composed of prominent blastema, with tubular differentiation, and possessing a prominent fibromyxomatous stroma appear to yield skeletal muscle cell cultures. Wilms' tumors lacking any of the above three characteristics failed to yield cultures of skeletal muscle cells. The presence of a prominent fibromyxomatous stroma was the single most striking feature of tumors yielding skeletal muscle cell cultures.

Because 2 of the 3 Wilms' tumors yielding skeletal muscle cell cultures were thought not to possess a my-

Table 1—Characteristics of Wilms' Tumors Tested for Growth of the Myoid Component in Cell Culture

Case	Sex	Age (mos)	Side	NWTS prognosis	Blastemal component	Fibromyomatous stroma	Tubular differentiation	Other features
1	M	16	L	Favorable	+ + +	+ + +	+ + +	
2	F	55	R	Favorable	+/−	−	+	Papillary pattern
3	M	4	L	Favorable	+ + +	+/−	+/−	
4	M	10	B	Favorable	+ + +	+ + +	+ + +	
5	M	23	L	Favorable	+/−	−	+ + +	
6	F	58	R	Favorable	+ + +	+/−	+/−	
7	F	81	R	Unfavorable	−	+/−	+/−	Papillary pattern
8	M	9	R	Favorable	+ + +	+ + +	+ + +	

The characteristics of the eight Wilms' tumors tested for growth of the myoid component in cell culture. The growth medium utilized and conditions for explant have been described previously.[7] Abbreviations: *b*, bilateral; *R*, right; *L*, left; *NWTS*, National Wilms' Tumor Study. All patients are presently alive and well.

oid component on the basis of routine histologic examination, these tissue blocks were extensively reexamined for the presence of a myoid component. In both cases, extensive reexamination confirmed the presence of a myoid component in these 2 tumors first thought not to contain skeletal muscle. It should be noted that in 1 case, skeletal muscle was noted quite quickly on reexamination; however, in the remaining case, many tissue blocks were examined before a small area of skeletal muscle differentiation was visualized, and this was found in samples taken for an unrelated experimental study. The five remaining tumors, which failed to yield skeletal muscle cell cultures, were likewise reexamined, and no evidence of a myoid component was found.

The specificity of this growth medium was also assessed with regard to normal skeletal muscle for determination of whether it would support the growth of either adult or fetal skeletal muscle cells. For this, thigh and abdominal muscle was obtained from five adults and four infants during postmortem examinations. In

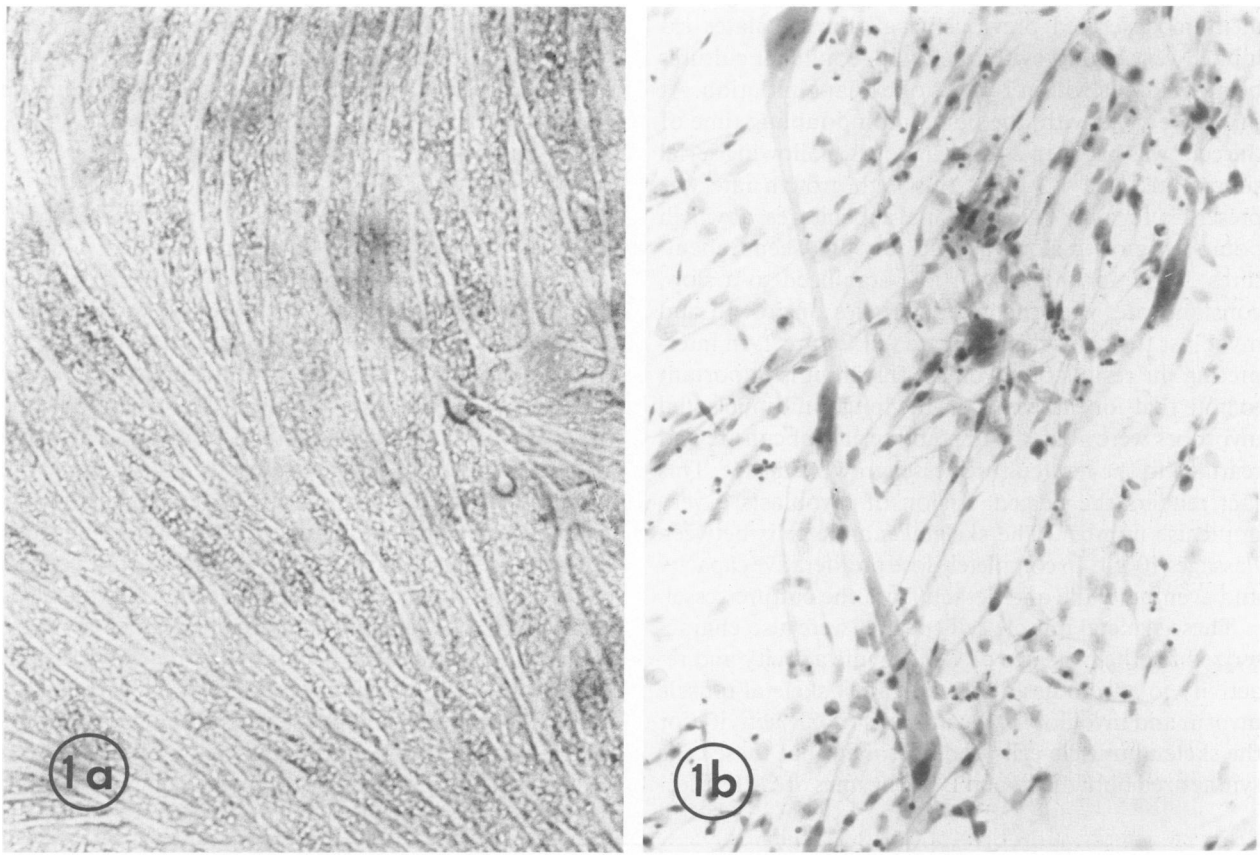


Figure 1a—A phase-contrast micrograph of the Wilms' tumor explant on serum-free medium showed cells with a tubular configuration. (×400) **b**—Fixation and staining with H&E of the Wilms' culture demonstrated the linear multinucleation and long cytoplasm characteristic of myotubes. (×100)

all cases, the tissue was obtained within 6 hours *post mortem.* Two of the infants were full-term, and the other two were between 6 and 8 months of gestation. In no case were viable cultures of skeletal muscle cells obtained. Several other types of pediatric tumors were assessed for their ability to yield viable cultures of skeletal muscle cells. This included 2 cases of congenital mesoblastic nephroma, 1 case of pediatric renal cell carcinoma, 3 cases of neuroblastoma, and 1 case of the "clear cell" sarcoma variant of Wilms' tumor; and, as expected, none gave rise to viable skeletal muscle cell cultures.

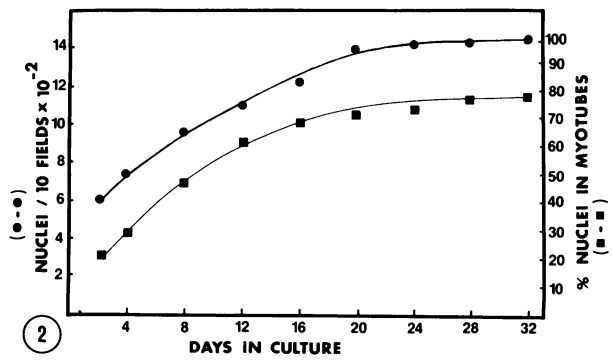


Figure 2—The rate of cell growth and myotube formation were measured for the skeletal muscle cell cultures over a 32-day period of growth. Both the growth and myotube formation were assessed, in triplicate, by examination of 10 microscopic fields (×200) for each time point. The cells were examined at Passage 5 after subculture at a 1:4 ratio. The two other skeletal muscle cell cultures isolated from Wilms' tumors with a myoid component yielded similar results.

Cell Culture Characteristics of the Skeletal Muscle Cells Isolated From Wilms' Tumors

Growth

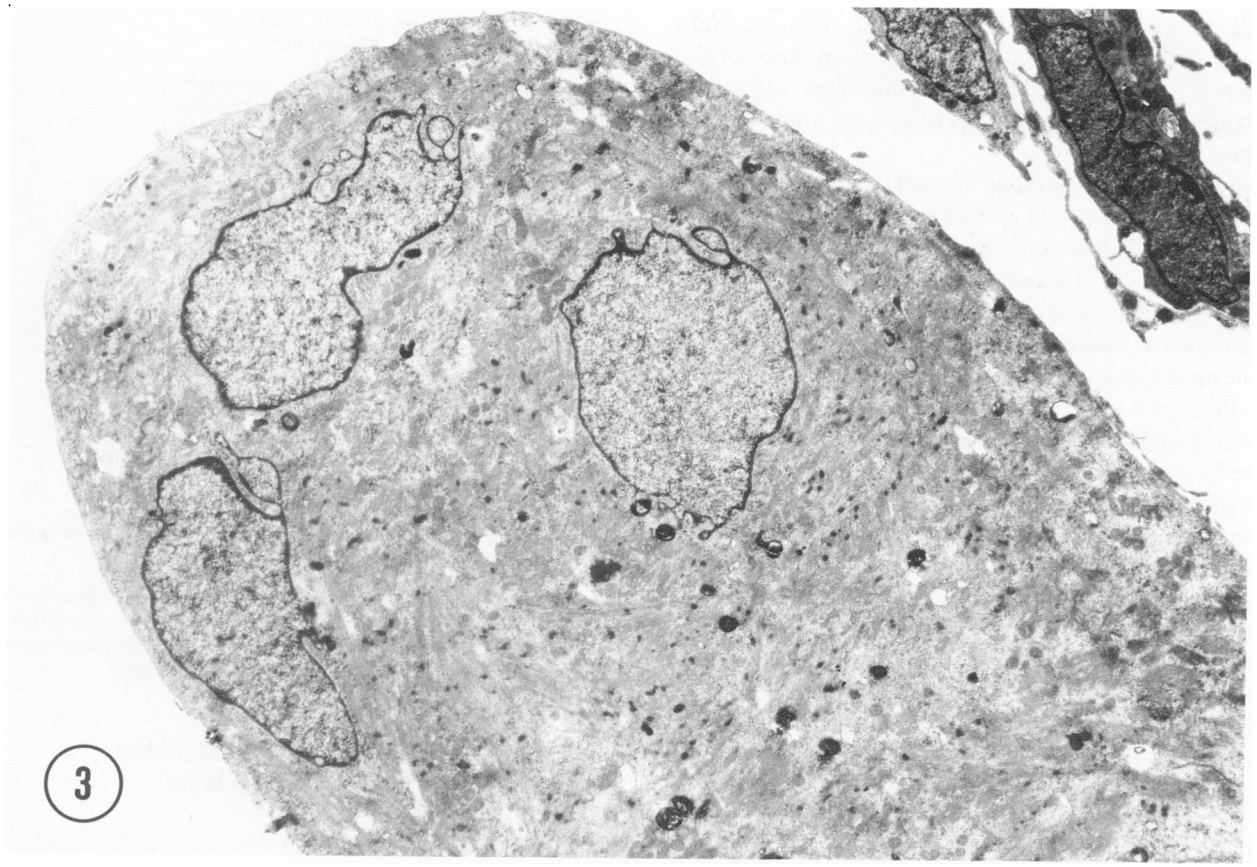
The skeletal muscle cell cultures, isolated from the 3 cases of Wilms' tumors possessing a myoid component, were characterized according to their growth characteristics *in vitro.* At both the explant and serial subculture stages, all three isolates exhibited identical growth characteristics; and, thus, the results presented will be shown for only a single isolate. At the explant stage of growth, with the use of the serum-free growth medium described previously,[7] all three isolates exhibited very high growth rates, with confluent cultures being obtained within 2 weeks of explant initiation. At this stage of growth, the population doubling time of the cells was between 24 and 36 hours. Following serial subculture, at a 1:3 ratio, this high growth rate was preserved for only 1–2 additional subcultures, at which point cell growth slows appreciably. Between Subcultures 3 and 10, the growth rate stabilized to a slow, constant rate, with the characteristics of growth and myoblast fusion as demonstrated in Figure 2. In interpreting the results on myoblast fusion, it is important to note that for these isolates the long, multinucleated myotubes were able to withstand serial subculture and reattach to the new culture vessel growth surface. This fact renders the percent fusion of myoblasts a very imprecise number. The skeletal muscle cells between Passages 10 and 16 completely lose proliferative capacity and eventually die and detach from the culture vessel.

These skeletal muscle cell cultures were also characterized for the presence of CK enzyme activity and reactivity to antibodies directed against skeletal muscle myosin and myoglobin. An analysis of CK activity for the skeletal muscle cells demonstrated that these cells synthesized both the M and B isoenzymes of CPK. Electrophoretic analysis demonstrated that the isoenzyme distribution for both the cells and conditioned growth medium was 25.3% BB, 43.4% MB, and 31.3% MM. The skeletal muscle cell cultures also reacted positively to antibodies directed against skeletal muscle myosin and myoglobin. The staining intensities of both were at +2, based on a 0 to +3 scale, when compared with the staining of normal skeletal muscle tissue. In both cases, the skeletal muscle cells failed to stain when exposed to normal rabbit serum in place of immune serum.

Ultrastructure

The ultrastructural characteristics of a single case of Wilms' tumor, explanted on serum-free growth medium, have been described previously.[7] Without exception, the 2 additional cases of Wilms' tumor with a myoid component reported herein, when placed into cell culture employing identical methods, demonstrated at the explant stage of culture identical ultrastructural characteristics. In brief, ultrastructural examination of these cultures revealed two populations of cells. The first type of cell was small, with a single oval nucleus and few cytoplasmic organelles. Areas of attachment between adjacent cells could readily be identified, and various stages of fusion and disappearance of the plasma membrane between these cells were common findings. These cells, which presumably were in the process of fusion, had more pronounced nucleoli and nuclear irregularities. The cytoplasm contained myofilaments measuring 5–7 nm and 13–16 nm in diameter, arranged in bundles with dense bodies along their course. The other

Figure 3—The tubular cells present in the Wilms' tumor showed peripherally located, multiple nuclei. The abundant cytoplasm was filled with randomly oriented arrays of thick and thin filaments. (×6180) **Figure 4**—The cytoplasm of the myotubes contained skeletal muscle myosin and actin with Z and I-band delineation. Glycogen was interspersed between the contractile filaments. (×19,250)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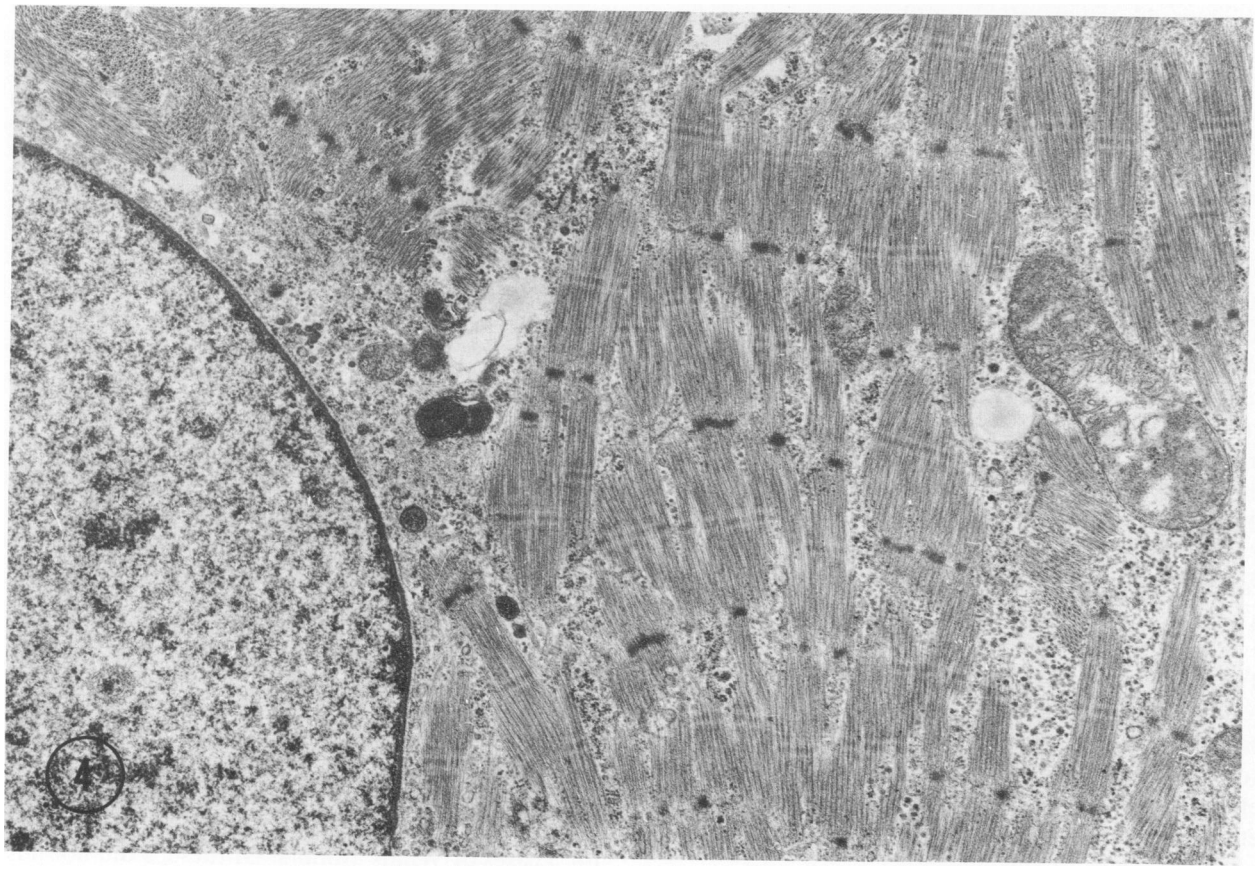
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cell population consisted of long, tubular, multinucleated structures possessing an abundance of filaments, 5–7 nm and 13–16 nm in diameter, with all the organizational characteristics of skeletal muscle myotubules.

Since the above studies were all performed at the explant stage of culture, we reexamined the three isolates from the 3 Wilms' tumors with a myoid component at a later passage to ascertain whether these initial characteristics had been maintained. All three isolates were examined at Passage 8, and all yielded consistent results. The most common ultrastructural finding of these cells at Passage 8 was the presence of long myotubules possessing an abundance of skeletal muscle myosin (Figure 3). This is in marked contrast to the earlier findings at the explant stage of growth, where the most common cell profiles were single, small cells undergoing fusion with adjacent cells. These profiles were a rare finding in cultures at Passage 8. The cells at Passage 8 possessed an abundant amount of skeletal muscle myosin with clear Z- and I-band elaboration (Figure 4). Adjacent to and interdispersed with these bundles of skeletal muscle myosin were concentrated areas of polyribosomes (Figures 3 and 4). The clear areas in the cell profiles presumably represent areas of glycogen accumulation which were lost during the fixation process (Figure 3). Thus, at the explant stage of culture, the most common ultrastructural cell profiles were small cells undergoing active fusion with adjacent cells. At later stages of culture growth, this population became sparse and was replaced by fused cells possessing abundant skeletal muscle myosin.

Improved Growth Medium Formulation

The fact that the cultures isolated from Wilms' tumors all lost proliferative capacity between Passages 10 and 16 suggests that improvements in the cell culture conditions might increase culture life span. The most logical area for improvement in culture life span centers on the possible improvement of the serum-free growth medium and provision of an extracellular matrix coating on the culture vessel surface. Thus, we tested the components listed in Table 2 to ascertain whether they would increase the culture life span of these cells. The results of this study demonstrate that no single component, when added to the growth medium, had a positive effect on cell growth or culture life span. However, this study did demonstrate that a bovine collagen Type I matrix followed by absorption of FCS proteins resulted in an expansion of cell life span to between Passages 18 and 24. The presence of this matrix had as its sole effect an increase in culture life span and had no effect on cell population doubling times. Likewise, no effect on cell ultrastructure was noted to occur because of the presence of this matrix.

Table 2—Growth Supplements Tested as Serum-Free Medium Components

Prostaglandin E_1	1–25 ng/ml
Prostaglandin F_2	1–25 ng/ml
Hydrocortisone	1–100 ng/ml
Triiodothyronine	1–15 pg/ml
Progesterone	2–20 ng/ml
Putrescine	0.1–1.0 mg/ml
Glucagon	50–250 ng/ml
Aldosterone	1–20 μg/ml
Testosterone	0.1–5 ng/ml
Dihydrotestosterone	0.1–5 ng/ml
Estradiol	1–5 ng/ml
Calcium	0.4–4 μg/ml
L-glutamine	15–1500 μg/ml
Fibroblast growth factor	1–25 ng/ml
Epidermal growth factor	10–100 ng/ml
Fibronectin	1–10 μg/sq cm
Collagen Type I	1–5 μg/sq cm
Collagen Type IV	1–5 μg/sq cm
Laminin	1–5 μg/sq cm
Collagen Type IV/laminin	Same concentrations as above
Collagen Type I/FCS proteins	200 μl FCS/sq cm

The growth medium supplements were obtained from Collaborative Research except collagen Type IV and laminin, which were obtained from Bethesda Research Laboratories. The supplements were prepared according to the product specifications provided by the company.

Cell Culture Characteristics of a Myoblast Cell Population From Wilms' Tumors

Isolation and Growth

During the attempt to improve the growth medium formulation for skeletal muscle cells, a new growth medium was developed which supported the growth of a new, morphologically distinct cell type. This growth medium consisted of a 1:1 mixture of DME and F-12 growth medium supplemented with selenium, (5 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and epidermal growth factor (10 ng/ml). The growth surface of the culture flask contained a bovine collagen Type 1 matrix with absorbed FCS proteins. This cell type possessed at the light-microscopic level a spindle shape strongly resembling that of a typical human fibroblast in culture (Figure 5). This new morphologically distinct cell type was first noted when we were subculturing confluent skeletal muscle cell cultures to smaller growth chambers containing the various growth factors listed in Table 2. Thus, this new cell shape may have resulted from a conversion of the skeletal muscle cells to a new, distinct form or by proliferation of a previously unrecognized contaminating cell type present in the skeletal muscle cell cultures. To begin to separate these two possibilities, we assessed the ability to isolate this cell type, utilizing the new growth medium formulation, at the explant stage of culture.

The ability of this growth medium to selectively propagate this cell type, at the explant stage, was assessed for 5 cases of Wilms' tumors. Of these, 3 did not pos-

Figure 5—A phase-contrast micrograph of the long-term culture of Wilms' tumor on serum-free medium showing the spindle shape of the cells. (×400)

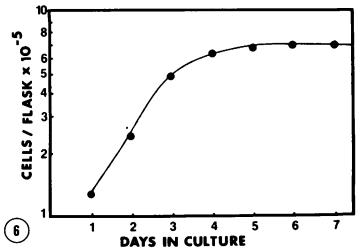


Figure 6—The rate of cell growth for the spindle-shaped cell cultures isolated from Wilms' tumors possessing a myoid component over a 7-day period of growth. The rate of growth was determined, in triplicate, with the use of point-counting on a Zeiss Videoplan image analysis system. The cells were examined at Passage 7 after subculture at a 1:3 ratio.

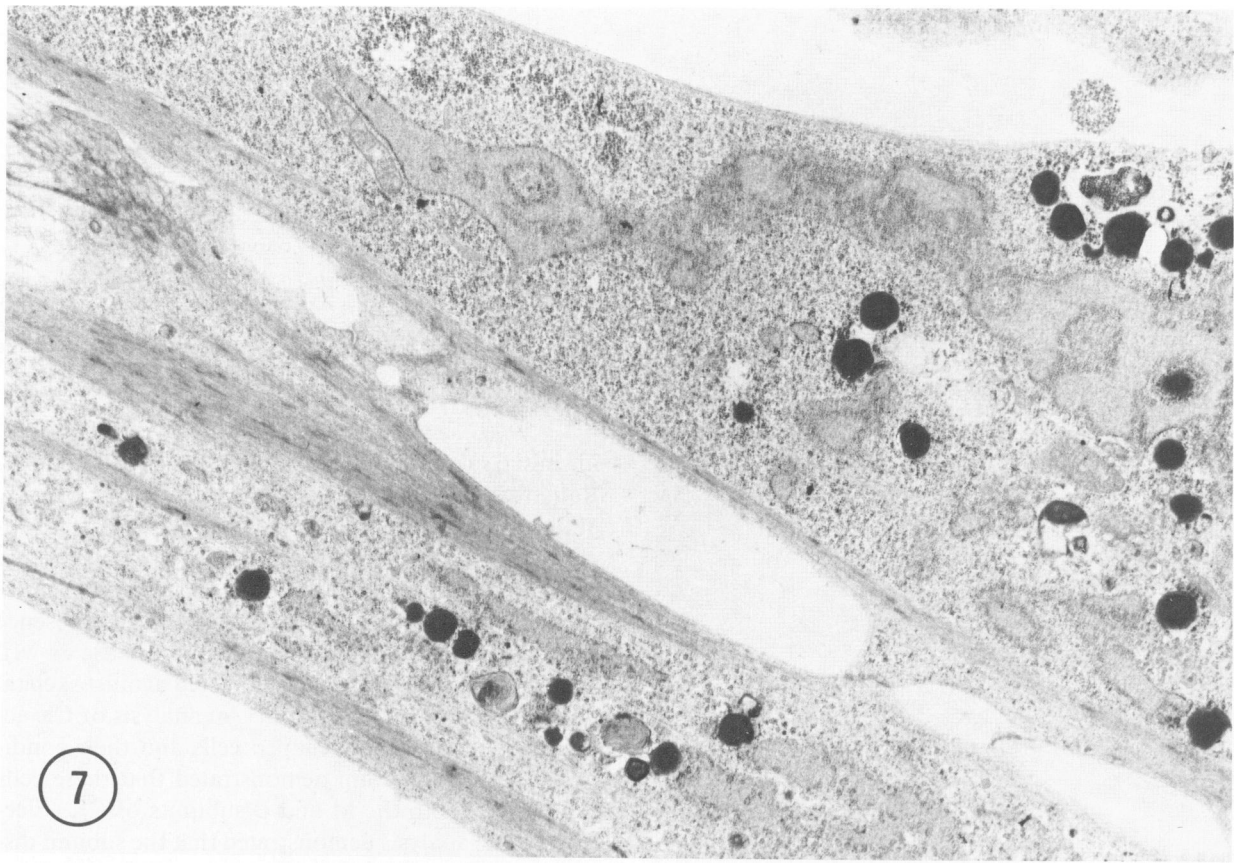
sess a myoid component (Cases 5, 6, and 7; Table 1); the remaining 2 did possess a myoid component (Cases 1 and 4; Table 1). In the case of the 3 tumors without a myoid component, no cultures were isolated which possessed spindle-shaped cells. In contrast, the two cases of Wilms' tumor possessing a myoid component gave rise to a rapidly proliferating culture of cells which possessed light-microscopic morphologic features indistinguishable from those noted previously for skeletal muscle cells (Figure 1a and b). However, within one to two subcultures at a 1:3 ratio, these explanted cells had undergone a complete change to a spindle shape (Figure 5). This spindle shape was then preserved for at least 25 additional subcultures. A reversal in the growth medium formulation back to that utilized for the growth of skeletal muscle cells, after three to five subcultures on this new growth medium, resulted in cell death within three subcultures and not in a change back to a skeletal muscle cell structure.

After explantation and two to three additional subcultures, these spindle-shaped cells were characterized with regard to their *in vitro* properties. Whereas the skeletal muscle cells described previously underwent a large reduction in population doubling times following initial subculture, these spindle-shaped cells did not. Rather, the high rate of proliferation, noted previously at the explant stage of culture, was preserved after subculture (Figure 6) and remained at this high rate throughout 20 additional subcultures. These spindle-shaped cells were also characterized for the presence of the isoenzymes of creatine phosphokinase as well as the reactivity to antibodies directed against skeletal muscle myosin and myoglobin. An analysis of CK activity for these spindle-shaped cells and their conditioned growth medium demonstrated that these cells synthesized both the M and B subunits of CK. Electrophoretic analysis demonstrated that the subunit distribution for both cells and conditioned medium was 29.7% BB, 46.1% MB, and 24.2% MM. These spindle-shaped cells were also slightly positive to antibodies directed against skeletal muscle myosin and myoglobin. The staining intensity for skeletal muscle myosin and myoglobin was a +/− to +1 reaction, indicating only a slight reactivity for these antigens. In all cases, these spindle-shaped cells failed to react when exposed to normal rabbit serum in place of immune serum.

Ultrastructure

Because the spindle-shaped cells only became apparent as a unique entity after one to two subcultures of the primary explants (which initially yielded a cell population identical to the skeletal muscle cells described previously), these cells were examined for their ultrastructural features at various stages of culture growth. At the explant stage, the ultrastructural features of confluent cultures were indistinguishable from those described previously for skeletal muscle cell cultures (see Sens et al[7] and Results). However, after three subcul-

Figure 7—The long-term cultured cells of Wilms' tumor were elongated, with peripheral, longitudinally oriented, thin filaments with interspersed dense zones. The cytoplasm contained abundant rough endoplasmic reticulum. (×9520) **Figures 8 and 9**—Adjacent cells in the long-term cultures of Wilms' tumor demonstrated the partial or complete disappearance of the cell membrane. Residual thin filament concentrations were located in areas which, presumably, were at one time located at the periphery of the cytoplasm. (**Figure 8**, ×19,080); (**Figure 9**, ×9240)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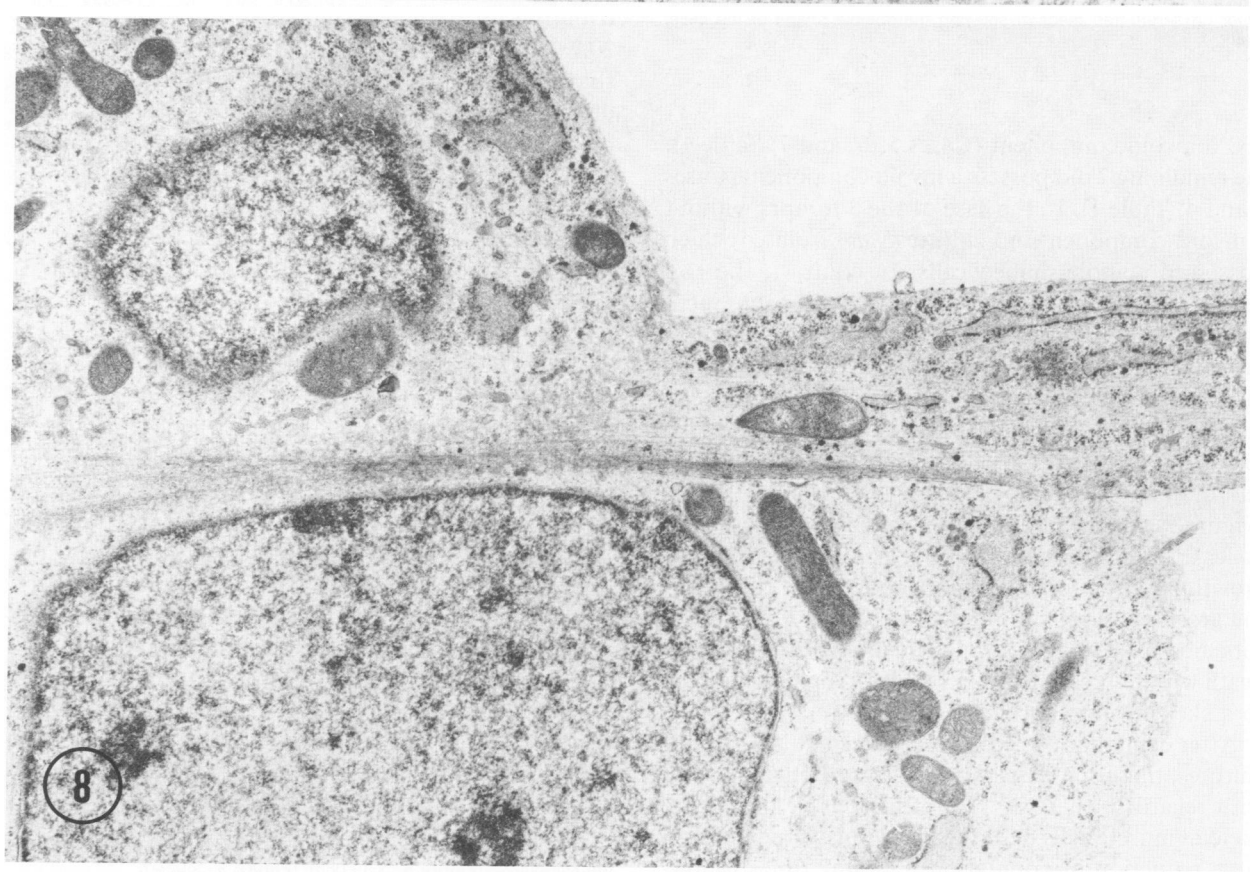
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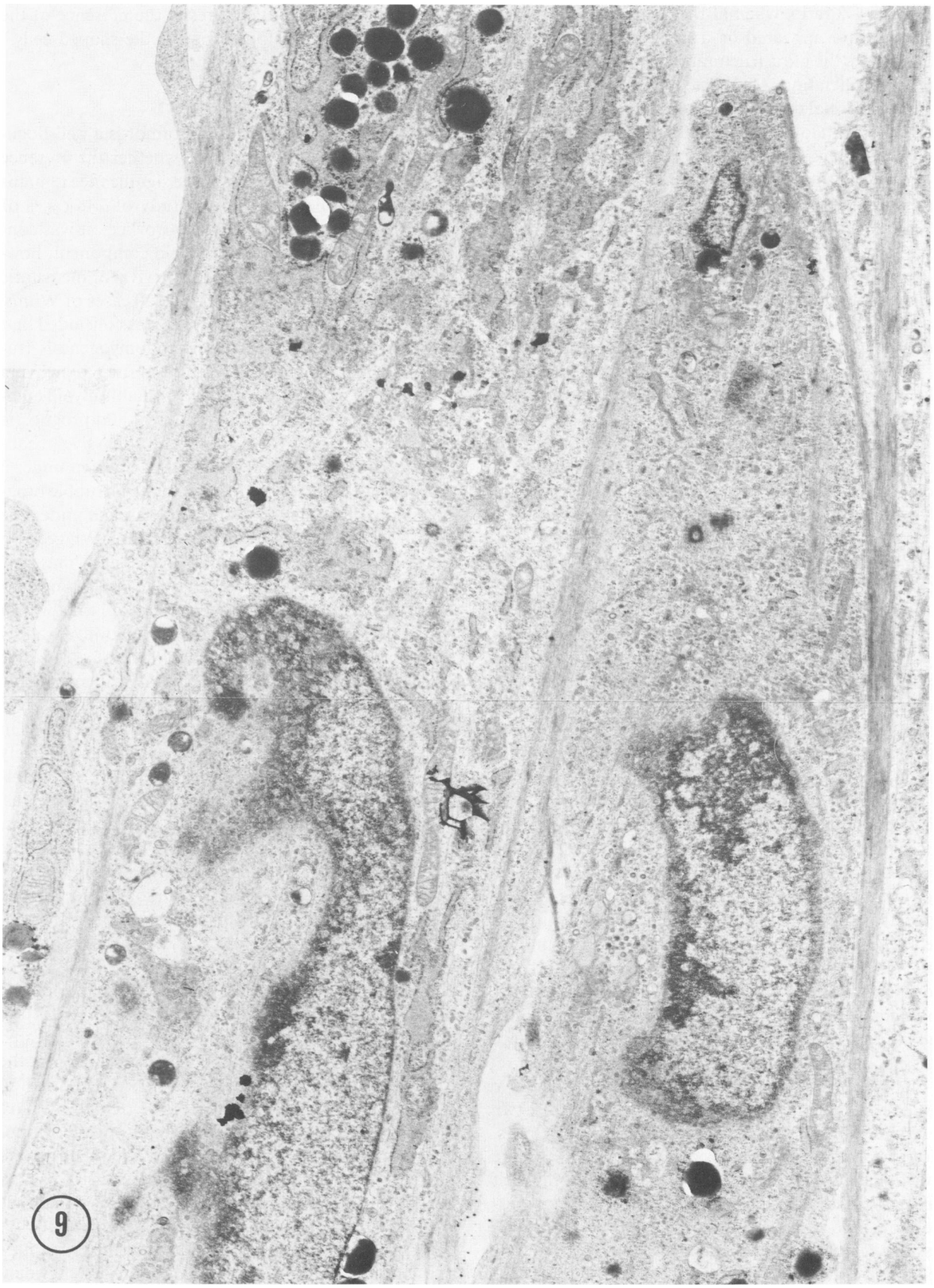
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tures at a 1:3 ratio, when at the microscopic level the entire culture appeared as a population of spindle-shaped cells, the ultrastructural features had undergone significant alteration. As depicted in Figure 7, these cells possessed several distinguishing features. The cells were elongated, with longitudinally oriented thin (7 nm) filaments with dense zones distributed peripherally within the cytoplasm. These cells also demonstrated abundant amounts of dilated rough endoplasmic reticulum (RER). An unexpected, but common, finding in approximately 20% of the cell profiles was the observation that these cells were capable of undergoing fusion with one another (Figures 8 and 9). An examination at higher magnification of these areas of fusion demonstrated the partial to complete disappearance of the cell membrane, as outlined by areas of thin filament concentrations which, presumably, were at one time located along the periphery of the cytoplasm of adjacent cells (Figures 8 and 9). The remaining cell profiles, although not fused, demonstrated areas of cell-to-cell interaction along the filamentous areas of the peripheral cytoplasm. The above ultrastructural characteristics were identified in over 95% of the cell profiles examined. The few contaminating cells were multinucleated, with abundant, well-developed skeletal muscle myosin. The ultrastructural features of these cells after 5, 10, and 20 subcultures were also examined, with the sole difference being the loss of the contaminating skeletal muscle myotubules; otherwise, all other features, including cell fusion, were preserved.

Because the thin filaments present in these spindle-shaped cells could easily be mistaken for the beginning of the skeletal muscle myosin elaboration, the purity of the primary explants of skeletal muscle cell cultures were reexamined by referrel to the original electron micrographs. Along with thin filament elaboration, the presence of dilated RER was also assessed, because this also was a distinguishing feature of the spindle-shaped cells. This reexamination revealed that, indeed, there were several cells present that contained both thin filament elaboration and dilated RER in the skeletal muscle explants. However, these profiles were rare and accounted for less than 5% of the cell profiles. At later subcultures, the number of these cells had decreased, but they were still present.

One additional study was performed with negative results which merits mention. Efforts to clone these two cell types were successful only for the myoblast cells. However, in no instance would the addition of the growth medium developed for skeletal muscle cells elicit a return of these spindle-shaped cells to a skeletal muscle morphology. In contrast, in all instances (regardless of the number of subcultures) the skeletal muscle cell cultures could be changed to spindle-shaped cells within two to three subcultures in the presence of the growth medium developed for spindle-shaped cells.

Discussion

Rhabdomyogenesis in Wilms' tumor is a well-documented clinical entity; however, whether the presence of this component has prognostic significance remains open to scientific debate. In a study of 248 cases of Wilms' tumor by Lemerle and co-workers, it was concluded that the presence of a myoid component, however abundant, did not influence survival or metastatic rates.[10] In contrast, in the study of 220 cases of Wilms' tumors by Crussi and co-workers, it was concluded that tumors possessing "massive" rhabdomyogenesis (tumors in which more than one-third of the parenchyma is composed of a histologically identifiable myoid component) carry definite positive clinical and prognostic implications.[11] Wilms' tumors with "massive" rhabdomyogenesis have been observed in children under 2 years of age, and thus are subject to the favorable prognostic qualifications that apply to patients under the age of 2 with this neoplasm.[12] In addition, Wigger also noted that these Wilms' tumors possessing "massive" rhabdomyogenesis features rarely yielded pulmonary metastases and proposed that these neoplasms were a distinctive clinicopathologic entity.[13] However, all of the above studies are hindered by lack of a sensitive method of clearly assessing which tumors in reality possess a myoid component. A classification of "massive" is a weak definition simply because, at some point in time, the myoid component may have been a relatively minor component. Likewise, limited histologic sampling in other studies may have easily missed Wilms' tumors possessing a limited myoid component. Thus, a sensitive methodology is clearly needed to assess which Wilms' tumors possess a myoid component.

The explant culture of Wilms' tumors on serum-free hormonally defined growth medium, carefully formulated to selectively propagate skeletal muscle cells, appears to provide an extremely sensitive method for identifying Wilms' tumors possessing a myoid component. The explant culture system developed is easy to perform and yields outgrowths of long, multinucleated myotubes which are unmistakable in appearance at the light-microscopic level. The sensitivity of this method is best exemplified by the fact that 2 of the 3 Wilms' tumors possessing an easily identifiable myoid component in explant tissue culture were not identified as possessing a myoid component on routine histologic examination. In both cases, an exhaustive histologic reexamination did confirm the presence of a small, but limited, myoid component. Thus, the employment of the above-described tissue culture explant system would

allow the accurate determination of which Wilms' tumors possess a myoid component and allow the determination of whether the presence of the myoid component has prognostic significance.

Of additional interest was the finding that all 3 tumors which yielded explants of skeletal muscle cells behaved identically in explant culture even though they contained vastly different amounts of the myoid component. In all instances, the explants attained confluency within 2–3 weeks, all the tissue fragments in each of the 20 explant flasks for each tumor yielded explants of skeletal muscle cells, and all were indistinguishable from one another at both the light-microscopic and ultrastructural levels. These findings suggest that even though 2 of the 3 tumors contained only rare mature, histologically identifiable muscle fibers, a common precursor cell must have been present throughout the tumor in significant quantities to yield rapidly proliferating cultures of skeletal muscle cells. We have previously observed *in vitro* myotube differentiation of a rhabdomyosarcoma grown under identical culture conditions.[14] However, the Wilms' tumors failed to show *in vitro* myoid differentiation in the absence of demonstrable muscle fibers *in vivo*. This suggests that blastema cannot be induced to differentiate into skeletal muscle cells under these *in vitro* conditions. A concerted effort has also been made to localize a precursor cell population for skeletal muscle differentiation in these tumors, without success. This effort has included lectin histochemistry with a battery of 12 lectins, enzyme histochemistry for 8 different enzymes, and immunohistochemistry for skeletal muscle myosin, keratin, uromucoid, and carbonic anhydrase. No cell population could be identified in sufficient quantity to explain the ability of these 2 tumors, with a limited myoid component, to rapidly explant skeletal muscle cells.

The 3 Wilms' tumors possessing a myoid component were histologically distinct from the other 5 tumors which possessed no myoid component. These tumors were composed of a prominent blastema with interdispersed tubules and a prominent fibromyxomatous stroma. The absence of any one of these features in the remaining tumors correlated well with the absence of skeletal muscle cell growth *in vitro*. Thus, the explant culture system described above is easy to perform and should provide an accurate determination of Wilms' tumors possessing a myoid component.

While the explant culture of Wilm's tumors for determination of the presence of a myoid component is easy to perform and applicable to most laboratories possessing minimal cell culture facilities, the same is not true for the long-term serial subculture of these cells. Although these skeletal muscle cell cultures are of human origin and undergo all the stages of myogenesis leading to myotube formation, they possess an extremely slow growth rate. This slow growth rate renders these cells unacceptable for scientific pursuits which require large populations of cells. Probably coupled to this slow growth rate, and further limiting their ease of usage, is an extremely high rate of fusion. This conclusion is drawn from observations that these cultures, when held at confluency, rapidly lose proliferative capacity, which suggests that the proliferating, precursor myoblasts are rapidly depleted through fusion. These cells are difficult to maintain for long periods of time, because careful attention must be paid to the subculture ratios and the resulting degree of confluency. Efforts were undertaken to improve the growth conditions and lessen the fusion frequency of these cells without success. Alterations neither in the extracellular matrix[15,16] nor in the serum-free growth medium[17,18] improved growth or lessened fusion frequency, as is reported to occur in rat myoblast cell lines.

As a fortuitous finding during efforts to improve the growth medium formulation for skeletal muscle cells, a new growth medium was developed which supported the long-term growth of a new, unique cell population. These cells possessed a spindle shape, a cytoplasm with peripherally distributed thin filaments, abundant amounts of dilated RER, and minimal immunoreactivity to skeletal muscle myosin. These ultrastructural characteristics agree closely with those found by Mendell and co-workers when explanting human skeletal muscle[19] for cells they termed "presumptive myoblasts." These cells were spindle-shaped mononucleated cells with undifferentiated cytoplasm but contained peripheral thin filaments. They observed these cells undergoing cell fusion and later differentiation into mature myotubes. A similar cell was described by Seidal and Kindblom in poorly differentiated alveolar rhabdomyosarcomas.[20] They described a spindle-shaped tumor cell with longitudinally arranged thin filaments found exclusively in the periphery of the cell, containing varying numbers of longitudinal densities on the filaments. Although the ultrastructural characteristics of this tumor cell gave resemblance to myofibroblasts, they were thought to be early myoblasts. To eliminate the possibility that this newly identified cell type was a myofibroblast, an extensive ultrastructural search was made of the Wilms' tumors and explant fragments for myofibroblasts, and none were found.

Thus, the growth conditions which favor the long-term growth of these cells appear to select for the growth of an early myoblast which contains minimal skeletal muscle myosin activity and isoenzymes (B, MB, and M) of CK. Although these cells show some evidence of cell fusion, myotube differentiation was not observed and cannot be induced by a return to the original medium.

Further efforts will have as their aim determination of the factors which inhibit the differentiation of this myoblast cell population.

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