Light- and Electron- microscopic and Immunohistochemical Studies of Human Rhabdomyosarcomas. Comparisons Among Primary Tumors, Heterotransplants in Nude Mice, and Cultured Cells from 13 Patients

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ABSTRACT

Eighteen human rhabdomyosarcomas (RMS) were transplanted into the subcutaneous space on the back of nude mice. Thirteen of the tumors gave rise to transplantable tumors that were further examined morphologically and immunohistochemically. The morphology of the transplanted tumors was similar to that of the primary tumors. Immunohistochemically, five primary tumors and six transplanted tumors were reacted with both desmin and myoglobin. However, in eleven cases cultured cells derived from the transplanted tumors, which showed elongated to strap-spindle-shaped cytoplasm resembling myotubules, reacted more intensely with both myoglobin and desmin. On ultrastructural examination, six primary tumors and seven transplanted tumors were found to have myofilaments or Z-bands. However, cultured cells showed myofilaments or Z-bands in their cytoplasm in all cases examined. We concluded that, on xenografting, the histologic characteristics of the primary tumor are essentially conserved, and that tumor cells under culture conditions undergo an increased differentiation of skeletal muscle. These human RMS strains in nude mice and in cell lines will provide an excellent model system for investigating the biology of RMS and for further study of the molecular events underlying the genesis of this tumor.

Key words : Rhabdomyosarcoma, Nude mouse, Immunohistochemistry, Ultrastructure

INTRODUCTION

Since Rygaard and Povlsen's report in 1969 (1), experiments on transplanta-

tion of various human tumors into nude mice have been carried out. Nude mice can support and grow a variety of human solid neoplastic tissues. There are several advantages compared to tissue culture techniques. It is easier to etablish long-term growth, and tumors grown subcutaneously are readily accessible for observation and measurement. There are a number of published reports on the biologic behavior of transplantable human tumors as well as changes in tumor markers and sensitivity to chemotherapy. Several reports have been published concerning the investigation of transplantable human rhabdomyosarcoma (RMS) in unde mice (2-4).

RMS is a highly malignant soft-tissue sarcoma that usually occurs in childhood. The diagnosis of RMS depends upon the identification of skeletal-muscle differentiation of the tumor cells on pathologic examination. However, with conventional histologic techniques it is sometimes difficult to distinguish RMS from other soft-tissue tumors, such as malignant fibrous histiocytoma, liposarcoma, and small-cell tumors of childhood (i. e., extraskeletal Ewing's sarcoma, neuroblastoma, and malignant lymphoma). We have studied 13 primary RMS that were transplanted into nude mice, as well as cultured cells derived from these xenografts. Our purpose in the present study was to investigate the morphologic, and immunohistochemical characteristics of the tumor cells in 13 cases of RMS.

MATERIALS AND METHODS

Specimens from 17 patients were obtained at surgery, before chemotherapy or radiation therapy, and one specimen was obtained at autopsy after chemotherapy. Thirteen (67%) of the 18 tumors gave rise to serially transplantable tumors for more than 2 passages. Clinical data on the patients are listed in Table 1.

Heterotransplantation into nude mice and cell culture

The tissue specimens, sampled under sterile conditions, were minced into small fragments with scissors. With a #18 needle, 0.3 ml of minced specimen was inoculated into the subcutaneous space on the back of BALB/c AJc-nu mice (Nihon Clea, Tokyo, Japan). Serial transplantations were carried out with the same procedure when the transplanted tumors reached a diameter of approximately 20 mm. The large and small diameters of the tumors were measured once a week with vernier calipers.

The specimens of the heterotransplanted tumors were finely minced with scissors for cell culture and suspended in 2 ml of 1.0% collagenase in RPMI-1640 at 37℃ for 20 min. The supernatant was removed and centrifuged for 5 min at 1,500 rpm. Plastic culture flasks (25 cm^2) were seeded with RPMI-1640 sup-

Table 1 *Clinical Data and Growth of Xenografts*

No.	Age (yrs)	Sex	Histologic subtype	Anatomic location	Patient's outcome from diagnosis	No. of xenograft passage	Passage interval time (mean days)	Tumor doubling time (mean days)	Chromosomal translocation of t(2 ; 13)
1	31	F	E	left hand	death**	101	32.9	6.6	—
2	26	M	E	right buttock	dead with disease 2 months	89	30.6	8.5	—
3	8	F	E	right forearm	dead with disease 10 months	5	42.7	12.0	ND
4	16	F	A	right forearm	dead with disease 17 months	10	112.9	20.5	+***
5	35	F	A	right thigh	dead with disease 34 months	17	78.6	15.3	—
6	9	M	A	left leg	dead with disease 29 months	11	117.6	26.5	+****
7	68	M	P	right leg	dead with disease 9 months	4	57.8	15.4	—
8	28	M	A	left upper arm	dead with disease 48 months	2	152.0	23.0	ND
9	7	F	A	left forearm	dead with disease 32 months	6	84.4	14.5	—*****
10	9	M	E	left popliteal reagion	alive without disease 48 months	7	97.7	19.6	—
11	42	M	E	left leg	alive without disease 41 months	5	79.6	17.0	—
12	78	M	P	left thigh	dead with disease 11 months	3	70.0	17.5	—
13	13	M	A	left thigh	alive without disease 29 months	2	163.5	24.5	ND
14	37	M	E	right hand	alive without disease 74 months	NT			ND
15	12	F	A	left thigh	alive without disease 72 months	NT			+
16	19	M	E	left buttock	dead with disease 4 months	NT			—
17	9m*	M	E	right shoulder	dead with disease 6 months	NT			ND
18	24	M	E	right upper arm	alive without disease 72 months	NT			ND

E, embryonal type ; A, alveolar type ; P, pleomorphic type
—, absent ; +, present ; ND, not done ; NT, not transplantable
* months
** autopsy case
*** case described in Nojima et al. (1990)
**** complex translocation of t(1 ; 2 ; 13)
***** t(2 ; 15)

plemented with 10% heat-inactivated fetal calf serum and were incubated at 37℃ in humidified 5% CO_2 in air. The culture medium was replaced with fresh medium twice weekly, and the subpassage was carried out at 90-100% confluence.

Pathology studies

Specimens of the primary tumors and of the tumors transplanted into nude mice were fixed in neutral 10% formalin and stained with hematoxylin and eosin, as well as periodic acid-Schiff (PAS) with and without diastase. The tumors were classified as embryonal, alveolar, or pleomorphic RMS according to standard criteria (5). For transmission electron microscopy, the tissue sections of primary tumors, transplanted tumors, and monolayers of cultured cells were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-800 electron microscope. For immunohistochemical studies, we used the standard three-step indirect avidin-biotin-peroxidase method (6) on paraffin sections of primary tumors, transplanted tumors, and cultured cells. The antibodies used were myoglobin (Dako, Denmark, at a dilution of 1:200) and desmin (Dako, 1:100).

RESULTS

Clinical data

The patients were twelve men and six women, with an average age of 25.7 years (range; 9 months to 78 years). All tumors had arisen in the soft tissue, with eight of the tumors in the lower limb, seven in the upper limb, two in the buttock, and one in the shoulder. Twelve patients died of pulmonary metastases, and five patients are alive without disease (Table 1).

Transplantability in nude mice

The results are listed in Table 1. Thirteen RMS were successfully grafted and transplanted for two to 101 generations. The mean passage interval ranged from 30.6 to 152.0 days. The tumor doubling time of these strains was from 6.6 to 26.5 days, with a mean of 16.4 days.

Light-microscopic findings

The thirteen cases of primary RMS were classified into three histologic subtypes on the basis of light microscopy. Five of these were diagnosed as embryonal type, six were alveolar, and two were pleomorphic. In all cases, glycogen granules were found in many tumor cells. There was massive necrosis, but no cross-striations were seen in any of the cases.

Histologically, the transplanted tumors were similar to the primary tumors, but there were several differences. In transplanted tumors of the alveolar type, the tumor was composed of nests of cells separated by fibrous septa, forming the characteristic alveolar pattern. However, the alveolar spaces were occupied and filled with tumor cells more compactly than were those of the primary tumors (Fig. 1a, b). In the embryonal type, transplanted tumor cells had a less irregular shape and more cellularity than did those of the primary tumor. In the pleomor-

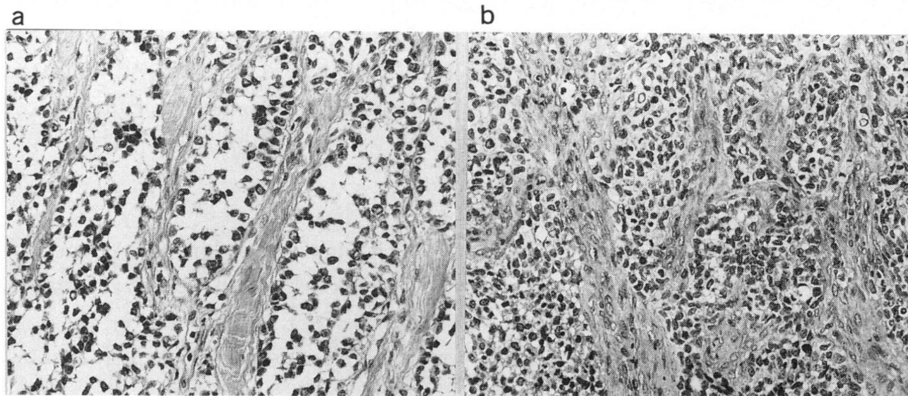

Fig. 1 Micrographs of alveolar RMS (case 4).
a. The primary tumor shows loosely textured aggregates of tumor cells separated by irregular-shaped fibrous septa. (HE stain, ×100)
b. The transplanted tumor shows solid cellular nests surrounded by fibrous septa. (HE stain, ×100)

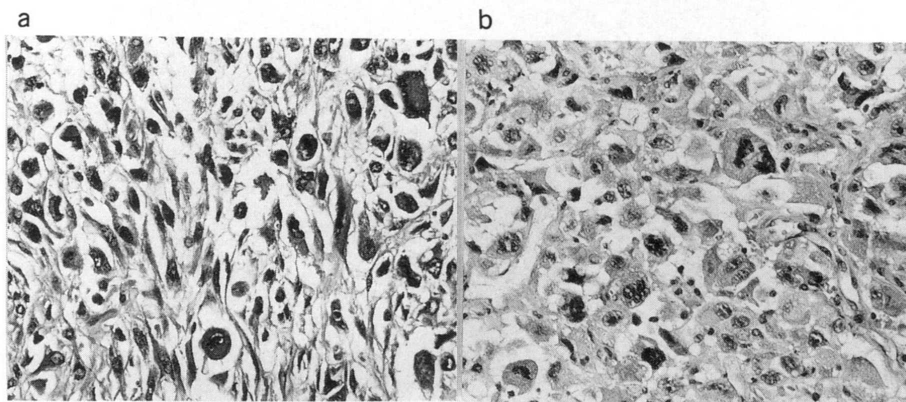

Fig. 2 Micrographs of pleomorphic RMS (case 7).
a. The primary tumor shows large, irregularly shaped cells with abundant eosinophilic cytoplasm. (HE stain, ×100)
b. The transplanted tumor shows pleomorphic cellular arrangements with multinucleated giant cells. There is much less collagen between cells than in the primary tumor. (HE stain, ×100)

phic type, the xenografts showed less collagen and more cells with bulky eosinophilic cytoplasm with multiple nuclei than did the primary tumors (Fig. 2a, b). No cross-striations were demonstrated.

The cultured cells derived from the transplanted tumors showed pleomorphic histologic features, including the size and shape of cells which were loosely attacted to the bottoms of the plastic flasks. Mononucleated polygonal or stellate cells predominated, whereas elongated to spindle-shaped cells resembling myotubular structures could be identified (Fig. 3). In addition, a small number of large multinucleated cells were present. Moderate numbers of mitotic figures were found. The cultured cells of cases 1, 2, and 7 were subpassaged more than 20 times. However, continuous growth of cells did not occur in cultured cells derived from the other ten strains.

Ultrastructural findings

Electron-microscopic examination was performed on 10 primary tumors, 13 transplanted tumors, and 11 sets of cultured cells. Minimal ultrastructural features judged essential for establishing the diagnosis of RMS differentiation of the neoplasms included cytoplasmic myofilaments and Z-bands. The principal ultrastructural features of the RMS are summarized in Table 2.

In primary tumors, the majority of the cells were round or polygonal, and the nuclei were usually irregular and often deeply indented. The cytoplasm was

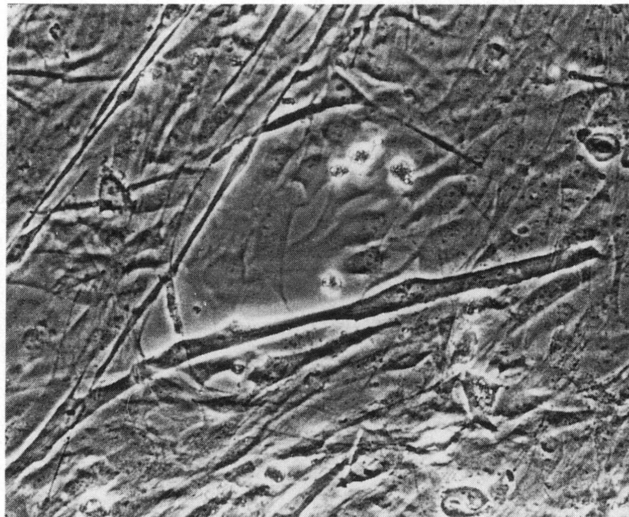

Fig. 3 Phase-contrast micrograph of the cultured cells (case 3). Myotube-like structures and multiple nuclei are evident. (×300)

Table 2 *Comparison of Results of Electron Microscopy*

No.	Primary Tumor		Transplanted Tumor		Cultured Cells	
	M	Z	M	Z	M	Z
1	ND	ND	−	−	+	−
2	ND	ND	−	−	+	+
3	+	+	+	+	ND	ND
4	+	−	+	−	+	+
5	−	−	−	−	+	+
6	ND	ND	+	−	+	−
7	+	+	+	+	+	+
8	−	−	−	−	ND	ND
9	+	−	+	−	+	+
10	−	−	−	−	+	+
11	+	+	+	+	+	+
12	+	+	+	+	+	+
13	−	−	−	−	+	−

M, myofilaments
Z, Z-bands or Z-band-like material
ND, not done
+, present
−, absent

occupied by polyribosomes, rough endoplasmic reticulum, Golgi apparatus, mitochondria, and variable amounts of glycogen. Very few instances of intercellular primitive junctional apparatus were noted in one case (case 10). Parallel arrays of alternating thick filaments, measuring 13 to 16 nm in diameter, and thin filaments, 5 to 7 nm in diameter, of varying length were found in six cases. In four of these, such filaments (myofilaments) formed focal densities of Z-bands or Z-band-like material in various stages of formation.

The ultranstructure of the transplanted tumors bore a striking resemblance to that of their parent tumor. In six cases, electron microscopy showed undifferentiated small mesenchymal cells without myofilamentous fibrils (Fig. 4a). In the remaining seven cases, intracytoplasmic bundles of myofilaments were found, and Z-bands or Z-band-like material were observed in four cases in which they were present in the primary tumor (Fig. 4b).

In all 10 cases of cultured cells studied ultrastructurally, the cells were elongated with oval nuclei containing prominent nucleoli, and the cytoplasm contained various amounts of filaments. These filaments aggregated into linear bundles along the peripheral cytoplasmic membrane. In eight cases, there were tumor cells with intracytoplamic bundles of myofilaments arranged in parallel with Z-bands or Z-band-like densities (Fig. 5a, b).

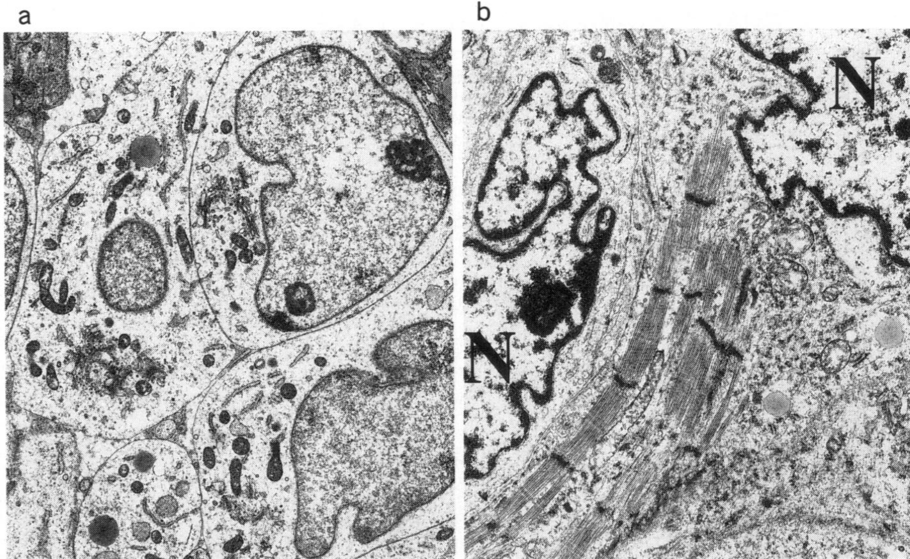

Fig. 4 Electron micrographs of transplanted tumors in nude mice.

a. The tumor cells are closely packed, with smooth surfaces. Golgi apparatus, mitochondria, and rough endoplasmic reticulum are seen scattered in the cytoplasm, but there are no myofilaments or Z-bands (×5,600, case 5).

b. Abundant thick and thin filaments form well-developed Z-bands in the cytoplasm. N, nucleus (×8,600, case 12).

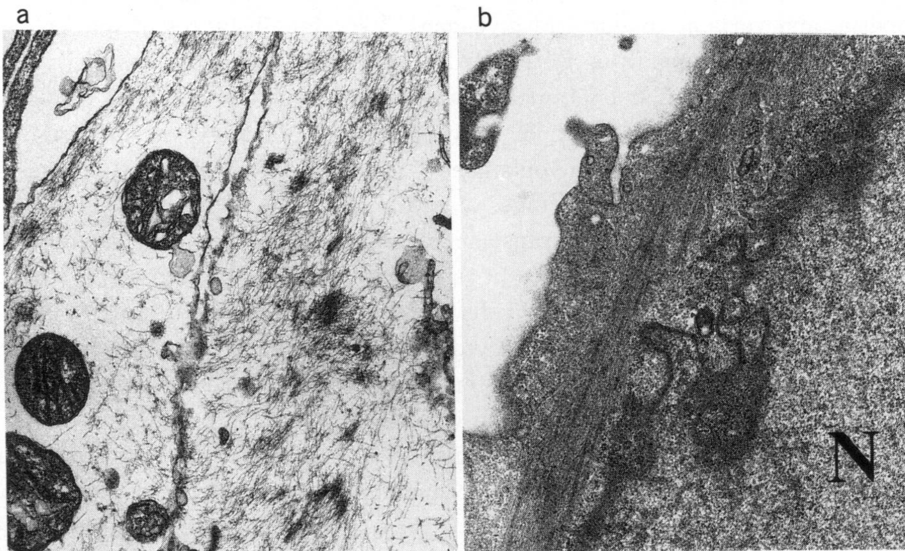

Fig. 5 Electron micrographs of cultured cells.

a. There are several aggregates of electron densities in disarray, forming poorly differentiated Z-band materials (×15,400, same case as Fig. 4a).

b. Aggregates of filaments with focal densities form adjacent to cell membrane. N, nucleus (×10,500, case 7)

Immunohistochemistry

The results of the immunohistochemical staining are summarized in Table 3. The tumor cells of primary RMS were positive for myoglobin in six cases (46%) and for desmin in 13 cases (100%). There was diffuse, cytoplasmic staining of individual cells or in focal collections of tumor cells of various shapes, including round and strap cells. Normal muscle fibers were stained with both of these

Table 3 *Results of Immunostaining*

Marker	stain grade				
	0	1	2	3	4
primary tumor					
myoglobin	7	2	1	3	0
desmin	0	2	4	3	4
transplanted tumor					
myoglobin	6	2	2	3	0
desmin	3	0	7	1	2
cultured cells					
myoglobin	2	0	3	3	5
desmin	0	0	3	0	10

The results are presented as number of cases in each grade.
0 : no staining ; 1 : individual cells positive ;
2 : 10-20% of cells positive ; 3 : 20-50% of cells positive ;
4 : 50-100% of cells positive.

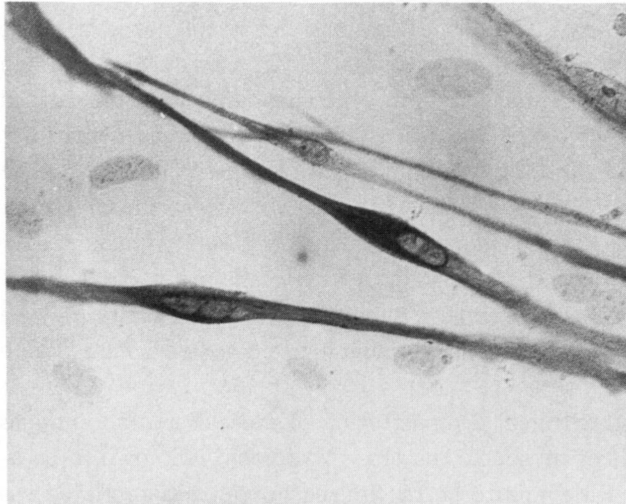

Fig. 6 Elongated-strap-shaped cultured cells showing positive staining for myoglobin in the cytoplasm (case 3). Adjacent polygonal cells show negative staining of cytoplasm. (ABC counterstained with hematoxylin, ×300)

markers, but stromal cells intermixed with tumor cells were negative. On immunohistochemical study of the transplanted tumors, there were slight changes in the marker expression of myoglobin and desmin compared with the primary tumors. Myoglobin was detected in seven strains (54%), of which six showed positivity in the primary tumors. Desmin was positive in 10 strains (77%), but the other three strains failed to be stained.

On the other hand, the staining for desmin was positive in all samples of cultured cells (100%), and 11 (85%) of the 13 cases showed myoglobin positivity. The number of positively staining cells was greater than that of primary or transplanted tumors. The positivity appeared strong in the large, elongated to spindle-shaped cells (Fig. 6).

DISCUSSION

Soft-tissue tumors are classified on the basis of their similarity to differentiated tissue or their histogenetic type. It is important to recognize myogenic differentation in determining the diagnosis of RMS. Routine light-microscopic examination rarely provides conclusive evidence of myogenic differention. The value of immunohistochemical techniques for the detection of muscle differentiation markers such as desmin, myoglobin, creatine kinase M subunit, and muscle-specific actin (7), and of ultrastructural observation of either specific myofilaments or Z-band material (8), has been proposed for confimation of a diagnosis of RMS.

There are several published reports on strains of soft-tissue tumors that were transplantable into nude mice. Most serially transplantable soft-tissue tumors resemble primary tumors histologically (2). In the cases of RMS presented here, we noted a striking similarity of the light-microscopic histologic findings between the primary tumors and the corresponding transplanted tumors. However, some changes in histology occurred after transplantation. The transplanted tumors showed more cellularity and less collagen. These differences between the primary tumors showed more cellularity and less collagen. These differences between the primary tumors and the xenografts may be explained by the fact that the stroma and vascular system in transplanted tumors are host-derived.

Immunohistochemical demonstration of muscle-specific antigens has facilitated the diagnosis of myogenic tumors. Myoglobin is known to be a good diagnostic marker for RMS and to be recognized in well-differentiated rhabdomyoblasts (9). The present findings showed that the pattern of staining for myoglobin in the primary tumors was similar to that in the transplanted tumors. On the other hand, in the cultures, more tumors were stained, and with stronger expression.

It is suggested that the change in expression of myoglobin in RMS is related to the state of differentiation.

Desmin in more useful in the diagnosis of RMS, and its sensitivity exceeds that of myoglobin, because it can be recognized in earlier and in poorly differentiated rhabdomyoblasts. All of the primary tumors in our cases reacted positively with anti-desmin; however, we failed to demonstrate desmin in three of the xenograft strains. These cells that were nonreactive with anti-desmin showed reactivity for desmin when grown in vitro. These strains seem to have a primitive character, with amounts of expression too minimal to demonstrate desmin.

Non-pecific myoglobin positivity has been demonstrated in reactive histiocytes and in several tumors derived from non-muscle cells, e. g., breast carcinoma, melanoma, and lymphoma (10). Desmin positivity also was shown in smooth-muscle and myofibroblastic lesions. The most recently reported muscle markers are α-sarcomeric actin and MyoD1. Alpha-sarcomeric actin was found to have a high degree of specificity and sensitivity, and immunohistochemical staining was observed in primitive rhabdomyoblasts. However, Carter et al. (11) investigated 52 cases of RMS by using immunohistochemistry for α-sarcomeric actin as compared to desmin, and they reported that α-sarcomeric actin was present in 40 (77%) of the cases, but desmin was expressed in 45 (82%). Furthermore, variable reactivity was demonstrable in necrotic tissue, macrophages, and in the muscle coats of blood vessels (11). Although desmin is a broad-spectrum marker for RMS, α-sarcomeric actin antibody is also valuable because it is present in more poorly differentiated RMS cases which are negative for desmin (9, 11).

MyoD1 is a myogenic regulatory protein and its expression is known to be restricted to skeletal muscle. Immunoreactive MyoD1 protein was detected in the nuclei and cytoplasm of all RMS cases examined, including tumors that failed to express desmin (12). The sensitivity and specificity of anti-MyoD1 staining thus are excellent; however, staining for MyoD1 in formalin-fixed paraffin sections is unsuccessful, and a commercial monoclonal antibody against MyoD1 has not yet become avaiable.

There are several transplantable cell lines in nude mice that increase or decrease in differentiation during passage (13, 14). Rohall *et al.* (14) investigated 12 transplanted soft-tissue sarcomas, and they found that two transplanted leiomyosarcomas and one transplanted malignant schwannoma showed a more pronounced differentiation compared to its primary tumor when the ultrastructure and immunophenotype were compared. Our heterotransplants gave a pattern of morphologic and immunohistochemical features essentially identical to that of the

primary tumor.

On the other hand, under cell culture conditions, most tumor cells underwent increased differentiation. Garvin *et al.* (15) reported a case of undifferentiated sarcoma which demonstrated myogenic differentiation in vitro. They observed that the tumor in vivo showed no evidence of differentiation by light microscopy, electron microscopy, or immunohistochemical staining for myoglobin and skeletal- muscle myosin. However, they found that the tumor cells in vitro showed multinucleated myotube-like structures by light microscopy and that, ultrastructurally, the cytoplasm contained myofilaments forming focal densities that mimicked Z-bands. For their cell cultures, they used serum-free medium (DEM 15), which did not permit continuous growth (15). In our laboratory, we used RPMI-1640 with 10% heat-inactivated fetal calf serum for cell culture, and an increase of myogenic differentiation was observed. Although we do not know the mechanism which induced myogenic differentiation, the application of tissue culture techniques for inducing this differentiation seems to be a useful supplementary method in the differential diagnosis of soft-tissue tumors.

Cytogenetic analysis of alveolar RMS has shown a non-random chromosomal translocation between chromosomes 2 and 13, t(2;13)(q37;q14) (16-18), but the genetic basis is unknown. This translocation has been observed in the embryonal and undifferentiated types as well as in the alveolar type (19). In our cases, three cases of alveolar RMS had t(2;13), and one alvolar RMS had t(2;15). This may indicate that the breakpoint at 2q37 is more important than that at 13q14 in the development of RMS. Furthermore, the *RBI* suppressor gene, which is located at 13q14, is not directly involved in this translocation (20). Molecular investigations of RMS with probes for chromosome 11 have demonstrated loss of heterozygosity in the region of the short arm of the chromosome (21, 22); however, most cytogenetic studies failed to identify karyotypic abnormalities involving 11p (23). Although the myogenic gene *MYODI* was mapped to chromosome band 11p15.4, this rhabdomyosarcoma-associated locus at 11p and the locus for *MYODI* were not the same (24). It would be worthwhile to investigate the relationship between these regions of chromosomes 2 and 11 and the mechanism of carcinogenesis.

In summary, 13 of 18 RMS were xenografted into nude mice with a successful tumor take, yielding rate of 72%. Our xenografts essentially retained the characteristics of the primary tumor and can provide a resource for repeatable samples over an extended period. We consider that these transplanted tumors will become a useful experimental model for investigations of their morphology and for studying the mechanism of carcinogenesis at the molecular level.

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