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Heterogeneity of Cloned Cell Lines Established from a Transplantable Rat Malignant Fibrous Histiocytoma

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Four cloned cell lines, MT-7, MT-8, MT-9 and MT-10, were established from a transplantable malignant fibrous histiocytoma (MFH) of F344 rats to investigate the histogenesis of the tumor. Cells of MT-7, MT-9 and MT-10 had fine structures characteristic of histiocytes, such as numerous cell processes, many lysosomes and well-developed cytoplasmic organelles. They stained positively for histiocytic lysosomal and antigenic markers. In addition, MT-9 cells contained microfilaments and well-developed RER in their cytoplasm, suggesting that they may be facultative fibroblasts. MT-8 cells stained weakly for histiocytic markers and had scant cytoplasmic organelles. They were identified as undifferentiated mesenchymal cells. The tumors induced in syngeneic rats by inoculating MT-7 or MT-10 consisted of a mixture of the pleomorphic, myxoid and storiform types of MFH, and those by MT-9 were of the storiform type. Cells forming these tumors stained positively for histiocytic markers. Tumors induced by MT-8 consisted of undifferentiated cells negative to these stainings. The histogenesis of MFH is surmised to be related to various differentiation stages shifting from undifferentiated cells to histiocytic cells capable of acting as facultative fibroblasts.

Key words: Cloned cell — F344 rat — Malignant fibrous histiocytoma — Morphology — Transplantation

Malignant fibrous histiocytoma (MFH) consists of histiocytic and fibroblastic cells in varying proportions and shows a broad spectrum of histologic patterns.[1-5] Based on immunohistochemical and electron microscopic examinations, histiocytes,[4, 6-9] fibroblasts[10, 11] or undifferentiated mesenchymal cells[3, 5, 11-14] have been presumed to be possible progenitor cells of MFH. In an attempt to clarify the histogenesis of MFH, neoplastic cell lines have been isolated from human MFH and examined in detail.[7, 8, 11] As a result, neoplastic histiocytes with a potential for multidirectional differentiation have been proposed as the origin of MFH by Iwasaki *et al.*[7] and Shirasuna *et al.*[8] On the other hand, Roholl *et al.*[11] concluded that the group of MFH is heterogeneous and is probably derived from more than one progenitor cell. Iwasaki *et al.*,[15] in a subsequent study using monoclonal anti-MFH antibodies, reported that MFH originates from perivascular mesenchymal cells having the capability to form various types of cells. It thus appears that the histogenesis of MFH still remains to be determined.

In rats spontaneous MFH has rarely been detected,[16, 17] although soft-tissue sarcomas resembling human MFH have been experimentally induced by various agents.[12, 13, 18-20] To our knowledge no cell line established from spontaneous rat MFH has been described. Recently, we established a transplantable tumor (MFH-MT) in syngeneic rats from a spontaneous subcutaneous MFH arising in a 15-month-old male F344 rat.[21] Neoplastic cells originating from MFH-MT were successfully passaged *in vitro*, and the cell line thus obtained was named MT-P.[22] In the present study four neoplastic clones were isolated from MT-P and examined by light and electron microscopy as well as enzyme/immunocytochemistry. In addition, tumors induced in rats by inoculating the cloned cells were examined histologically. This paper describes the results obtained in these studies.

MATERIALS AND METHODS

Cell culture The derivation of MT-P from MFH-MT has been described.[22] MT-P at passage 21 was cloned twice consecutively by a limiting dilution technique as described.[15] Four cloned cell lines, MT-7, MT-8, MT-9 and MT-10, were established. Cloned cells were cultured in a humidified, 5% CO_2 atmosphere at 37°C. The growth medium used was Dulbecco's minimum essential medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO), streptomycin (100 μg/ml), and penicillin (100 U/ml). Confluent cell sheets were subcultured by dispersing cells with a mixture of 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid in phosphate-buffered saline at 7- to 10-day intervals.

Observations of cloned cells Growth of cloned cells was observed at passage level 5 by the methods described.[23]

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The doubling time was determined from the cell numbers at 2 and 5 days after seeding. Cells grown on tissue culture chamber slides (LAB-TEK, Miles, IL) were fixed in Bouin's fluid or 4% formol calcium at 4°C for 1–2 h. They were stained with hematoxylin and eosin (HE), periodic-acid Schiff (PAS) with and without diastase digestion, and oil red 0. They were also stained for acid phosphatase (ACP) (pH 5.0), nonspecific esterase (NSE) (pH 7.4) and alkaline phosphatase (ALP) (pH 9.0), and cells fixed in acetone were reacted with anti-rat monocytes/macrophages monoclonal antibody (MAB-1435) (Chemicon International Inc., CA), as described.[23] Immunorosette formations for Fc- and C3-receptors, and phagocytic activities to latex particles (0.78 μm; Cosmo Bio. Co., Ltd., Tokyo) or sheep red blood cells (SRBC) were examined by the conventional methods as described.[22, 23]

For electron microscopy, cloned cells were pelleted by centrifugation, fixed in 2.5% buffered glutaraldehyde and postfixed in 1% buffered osmium tetroxide. They were embedded in epoxy resin and sectioned. Thin sections were stained with uranyl acetate and lead citrate and examined in a JEM-100B electron microscope at 80 kV.

***In vivo* observations of cloned cells** Specific-pathogen-free male and female F344/DuCrj rats either obtained from Charles River Japan, Inc. (Kanagawa) or produced in the authors' laboratory were used. They were maintained in a barrier room conditioned to 23±2°C, 50±20% relative humidity, and a 12-h light-dark cycle.

One ml of a cell suspension containing 10^7 cloned cells at passage levels 1, 7, and 21 was inoculated subcutaneously into syngeneic male rats, 10–25 weeks old. The diameters of all tumors that developed in inoculated sites were measured once weekly with calipers. Tumors developed by inoculating cloned cells at *in vitro* passage 7 were minced into small pieces, <2 mm in diameter, with scissors and transplanted subcutaneously at the interscapular region of syngeneic male rats through a trocar with a diameter of 2 mm. Thereafter tumors were serially transplanted in syngeneic male rats when their diameter reached >30 mm, about 5 to 6 weeks after transplantation.

Tumors removed from all the inoculated animals were weighed and fixed in 10% neutral buffered formalin. They were embedded in paraffin, sectioned and stained with HE, PAS with and without diastase digestion, Watanabe's silver impregnation for reticulin, azan Mallory and alcian blue (pH 2.5). Frozen sections from formalin-fixed tissues were stained with oil red 0. Sections from fresh specimens were examined for ACP, NSE and ALP by the methods described.[21, 23] Paraffin-embedded sections were also stained by the peroxidase antiperoxidase technique using commercial kits (Universal Immunoperoxidase Staining Kit, Cambridge Research Laboratory (CRL), MA or DAKO PAP Kit, DAKO Corp., Santa Barbara, CA). Rabbit antisera to α_1-antitrypsin (CRL), S-100 protein (CRL), lysozyme (CRL), keratin (CRL), factor VIII-related antigen (DAKO) and desmin (DAKO) were used as primary antibodies. Paraffin-embedded sections were reacted with MAB1435 as described.[23]

RESULTS

Characteristics of *in vitro*-passaged cloned cells Fig. 1 shows the growth curves of four cloned cell lines. The doubling times of MT-7, MT-8, MT-9 and MT-10 were 42.8, 27.7, 51.0 and 58.8 h, respectively.

In monolayer cultures of the cloned cells stained with HE, each clone appeared to be composed of only one type of cells. Predominant cells of MT-7 were round or polygonal in shape with abundant cytoplasm and a large round nucleus. MT-8 consisted of elongated or spindle-shaped cells with a fusiform nucleus arranged in a fascicular pattern. In MT-9 large, round cells with a lobulated nucleus were predominant. Cells of MT-10 had abundant cytoplasm with a few elongated cell processes and appeared dendritic in shape. Cells of all the clones often contained a PAS-positive, diastase-digestible material or oil red O-positive lipid droplets. Morphologic changes were not observed during *in vitro* serial passages up to the 21st generation.

Ultrastructurally, cells of MT-7 and MT-10 resembled each other. They had an irregular surface with many surface folds and their nuclei were oval or horseshoe-shaped. There were many lysosomes, slightly dilated

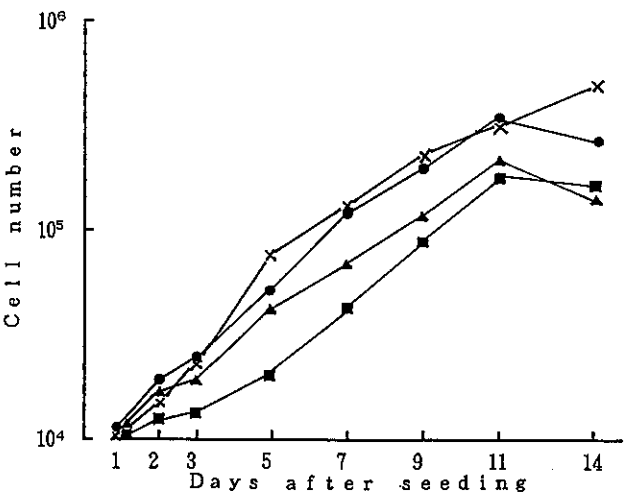

Fig. 1. Growth curves of four cloned cell lines, MT-7 (●), MT-8 (×), MT-9 (▲) and MT-10 (■), established from a transplantable rat malignant fibrous histiocytoma.

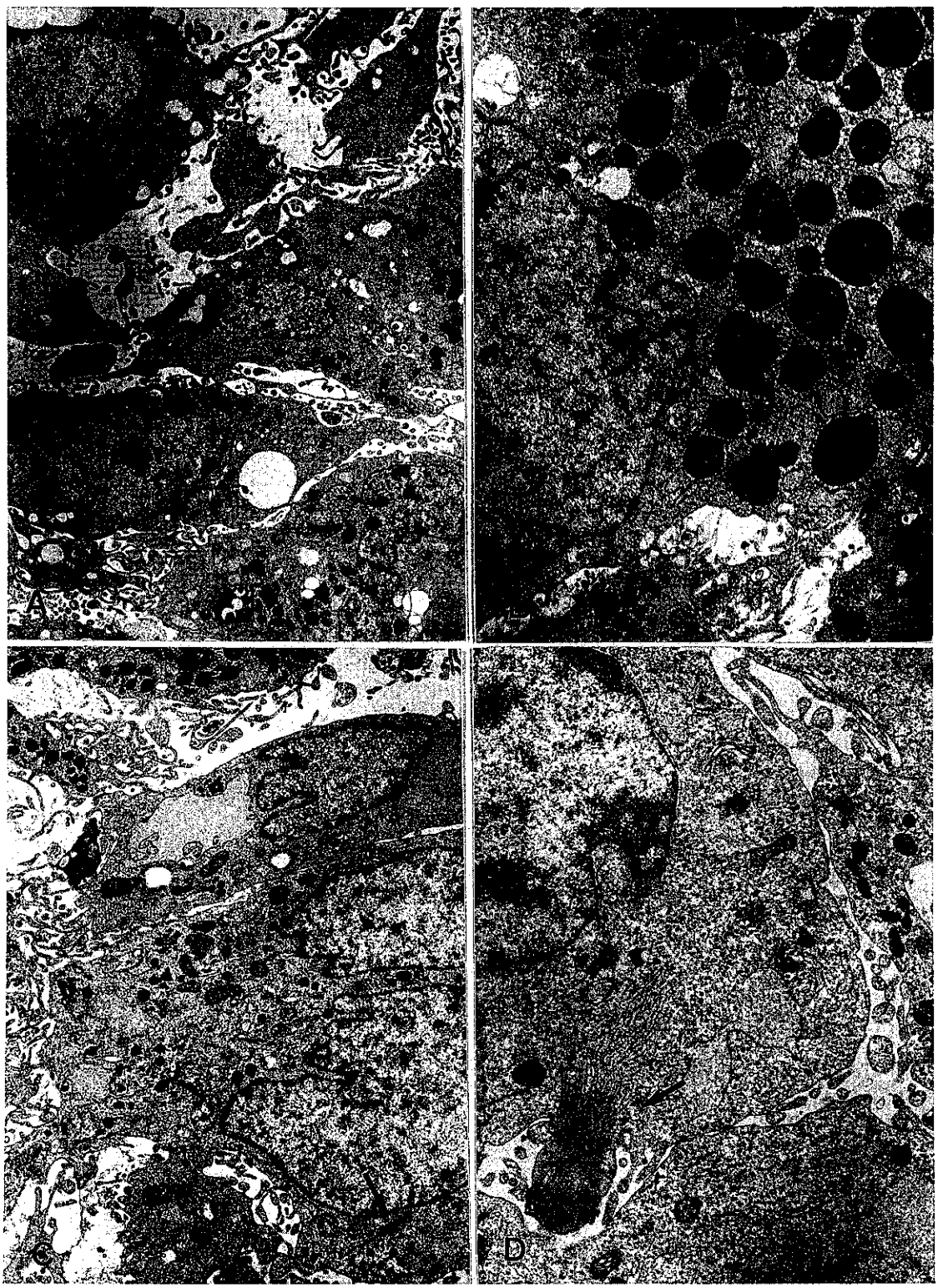

Fig. 2. Electron micrographs of MT-7, MT-9 and MT-10. MT-7 (A) and MT-10 (B) have a histiocytic appearance with numerous surface folds and many lysosomes in their cytoplasm (A, ×3,500, B, ×9,000). MT-9 (C and D) contains well-developed dilated RER, many small lysosomes and microfilaments (arrow) (C, ×7,000, D, ×9,000).

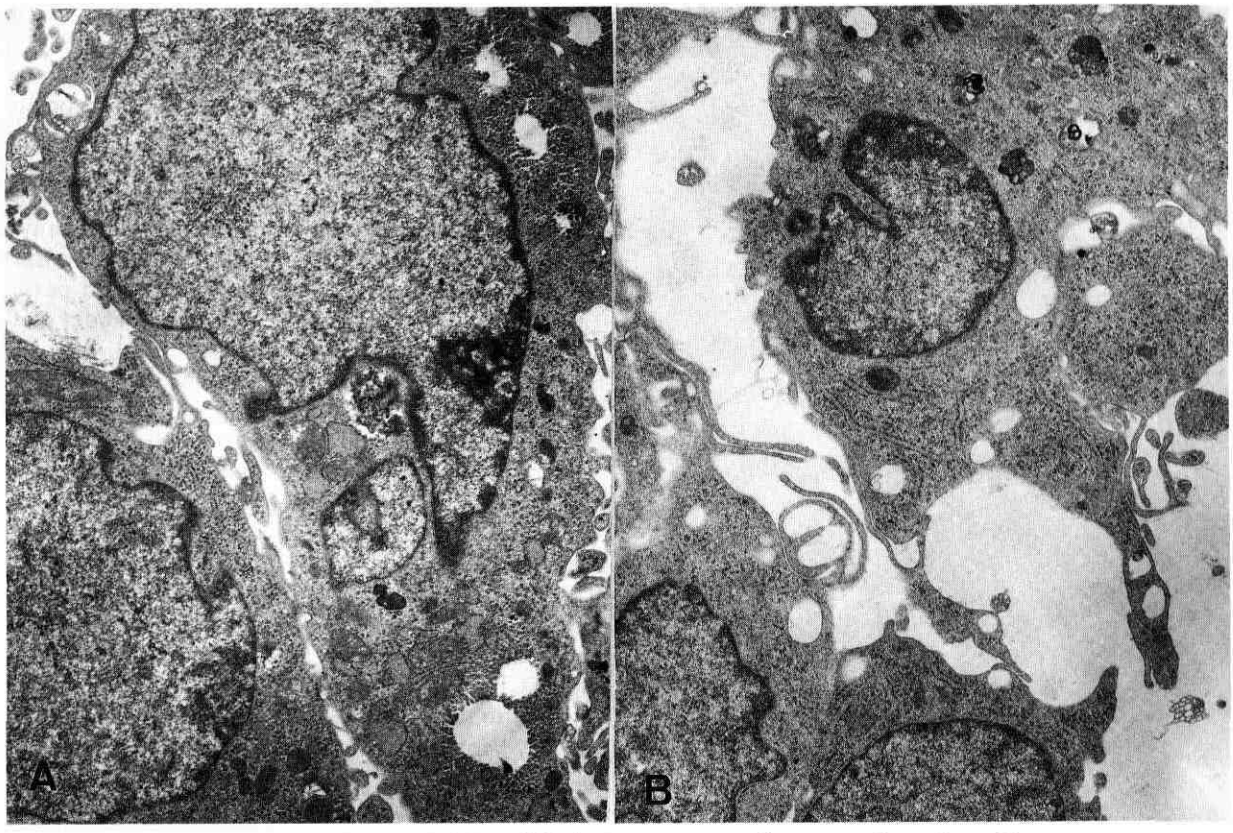

Fig. 3. Electron micrographs of MT-8 (A and B). Cells have scant organelles, a small number of lysosomes, numerous free ribosomes and glycogen granules in their cytoplasm (×6,000).

Table I. Results of Enzyme/Immunocytochemical and Functional Examinations on Cloned Cells Established from a Transplantable Rat Malignant Fibrous Histiocytoma

Clone	*In vitro* passage level	Staining[a]				Cytological function (%)[b]			
		ACP	NSE	ALP	MAB	EA	EAC	EA-P	L-P
MT-7	1–4	3+	3+	3+	2+	7	2	5	8
	12	3+	2+	2+	NE[c]	NE	NE	NE	NE
	16–18	3+	2+	2+	NE	6	2	13	5
MT-8	1–4	1+	1+	2+	1+	23	7	19	3
	12	2+	1+	2+	NE	NE	NE	NE	NE
	16–18	1+	1+	2+	NE	10	2	11	7
MT-9	1–4	3+	2+	2+	2+	8	3	6	3
	12	3+	2+	3+	NE	NE	NE	NE	NE
	16–18	3+	3+	2+	NE	7	4	3	6
MT-10	1–4	3+	2+	—	3+	11	7	15	5
	12	3+	3+	—	NE	NE	NE	NE	NE
	16–18	3+	2+	—	NE	21	3	5	6

a) ACP, acid phosphatase; NSE, nonspecific esterase; ALP, alkaline phosphatase; MAB, anti-rat monocytes/macrophages monoclonal antibody; −, negative; 1+, faint; 2+, moderate; 3+, strong.
b) EA, rosette formation for the Fc-receptor; EAC, rosette formation for the C3-receptor; EA-P, phagocytosis of SRBC used for the Fc-receptor assay; L-P, phagocytosis of latex particles.
c) NE, not examined.

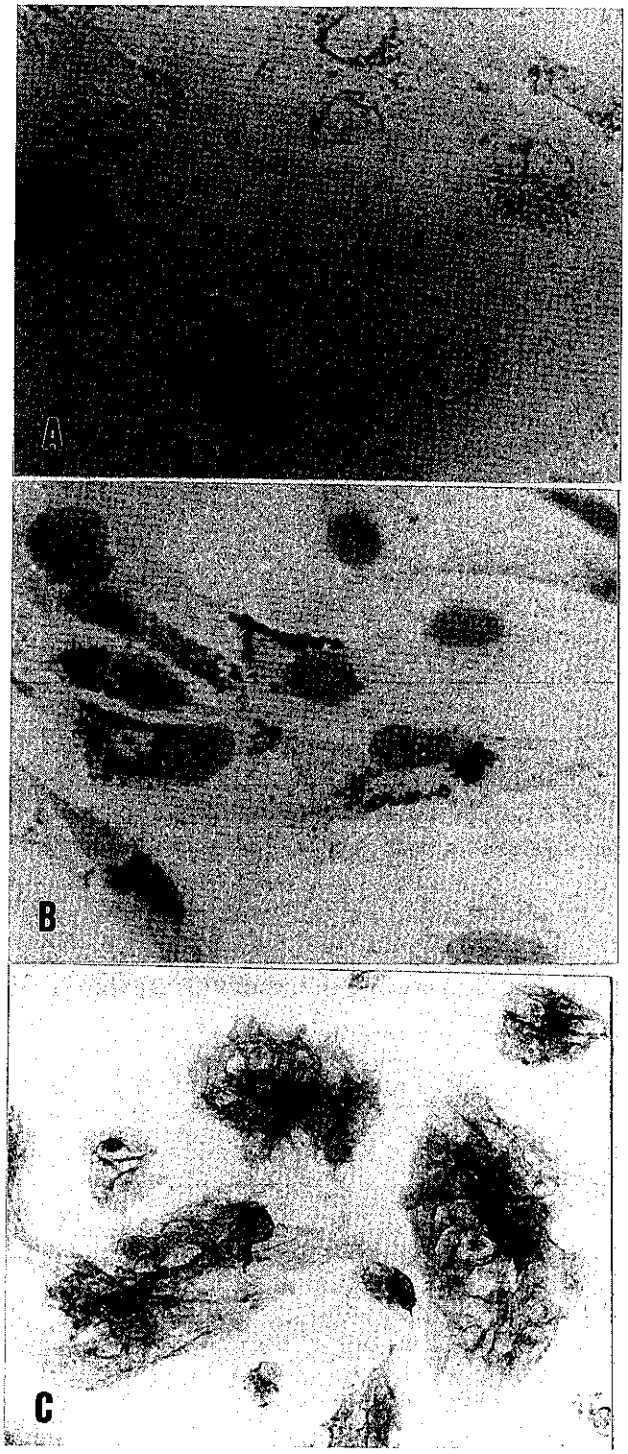

Fig. 4. Cytologic markers of *in vitro*-passaged cloned cells. A, MT-7 reacts strongly for ACP (Gomori's method, ×700). B, MT-10 contains coarse intracytoplasmic granules positive to MAB1435 (indirect immunoperoxidase staining, counterstained with hematoxylin, ×700). C, MT-9 reacts positively for ALP (naphthol AS method, ×600).

rough-surfaced endoplasmic reticulum (RER), prominent Golgi complex and some mitochondria in their cytoplasm (Figs. 2A, 2B). The cytoplasm of occasional cells contained glycogen granules, lipid droplets and phagosomes including cellular debris. MT-9 cells had many cytoplasmic extensions and indented nuclei. Their abundant cytoplasm possessed well-developed RER with dilated cisterna, prominent Golgi complex, many small lysosomes and a moderate number of mitochondria (Fig. 2C). Actin-like microfilament bundles with partly filamentous condensations were observed in about one-third of MT-9 cells examined (Figs. 2C, 2D). MT-8 had a slightly irregular cell surface with some long cytoplasmic processes and a small oval nucleus. The cytoplasmic organelles were poorly developed, although there were numerous free ribosomes, a small number of lysosomes, some mitochondria and glycogen granules (Figs. 3A, 3B).

The results of enzyme/immunocytochemical and functional examinations on cloned cells are shown in Table I. MT-7, MT-9 and MT-10 reacted moderately or strongly for ACP (Fig. 4A) and NSE. Positive reaction to MAB-1435 in these clones appeared to correspond to the results for ACP and NSE (Fig. 4B). In contrast, MT-8 reacted weakly to these stainings. MT-7, MT-8 and MT-9 gave a positive reaction for ALP (Fig. 4C), while MT-10 did not. These staining properties were almost the same in all the passage levels examined. All the functional markers examined showed small positive percentages in all the cloned cell lines. No significant differences were observed in positive rates between the clones and between passage levels.

Histology of tumors induced in rats by inoculating cloned cells All the cloned cell lines were tumorigenic. Tumors became palpable two to three weeks after inoculation and during the following three to four weeks they developed into nodules ranging in diameter from 15 to 25 mm and in weight from 10 to 25 g. All tumors were well-circumscribed, multilobulated and grayish in color. The tumors induced by MT-9 were firm masses with a fascicular structure on the cut surface, whereas those induced by the remaining clones were soft masses with several small cysts containing myxomatous liquid.

Tumors induced in syngeneic rats by the four cloned cell lines were classified into four types according to the criteria of human MFH.[2] The pleomorphic type was composed of round and pleomorphic cells and a small amount of collagenic fibers (Fig. 5A). These cells had abundant cytoplasm and a pale nucleus with a prominent nucleolus. The myxoid type was composed of loosely arranged polygonal and round cells supported by alcian blue-positive material, and occasionally contained giant cells with bizarre nuclei and hyaline globules (Fig. 5B). The globules stained red with PAS and were diastase-resistant. In myxoid areas cells containing oil red O-

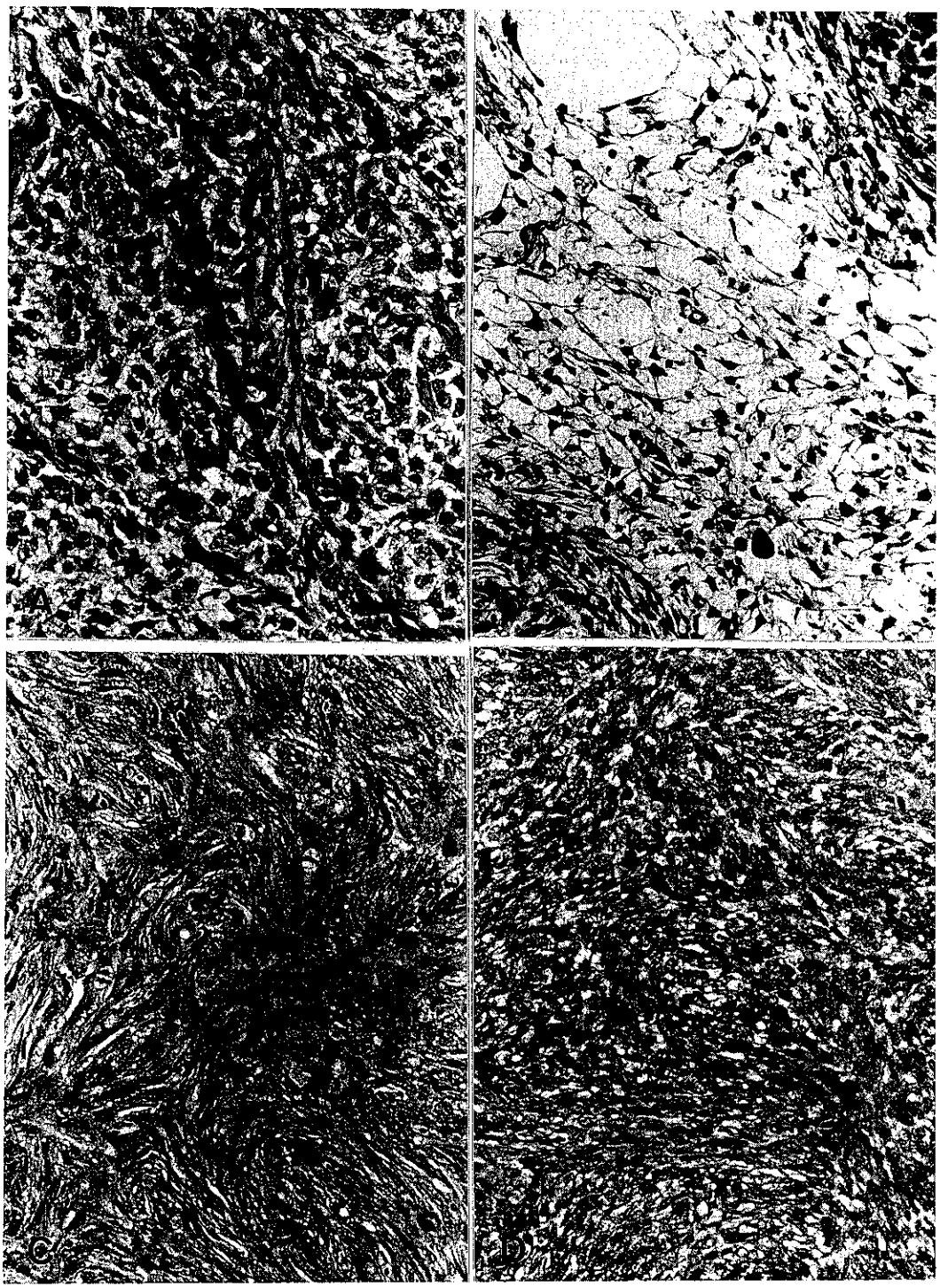

Fig. 5. Histology of tumors induced in rats by cloned cell lines. A, pleomorphic-type tumor induced by MT-7 consists of pleomorphic, round cells and a small amount of collagenic fibers (HE, ×250). B, myxoid-type tumor induced by MT-10 consists of loosely arranged neoplastic cells (HE, ×180). C, storiform-type tumor induced by MT-9 consists of round and elongated cells and a large amount of collagenic fibers (HE, ×200). D, spindle-cell-type tumor induced by MT-8 consists of spindle or fusiform cells arranged in an interlocking pattern (HE, ×200).

Table II. Enzyme/Immunocytochemical Findings of Tumors Induced in Syngeneic Rats by Four Cloned Cell Lines

Clone	Tumor type[a]	Staining[b]			
		ACP	NSE	ALP	MAB
MT-7	TC-1, TC-7, TC-21; pleomorphic, myxoid and storiform types	3+	2+	3+	2+
	#3, #5; spindle-cell type	—	—	3+	NE
MT-8	TC-1, TC-7, TC-21; spindle-cell type	—	—	3+	—
	#3, #5; spindle-cell type	—	—	3+	NE
MT-9	TC-1, TC-7, TC-21; storiform type	2+	2+	3+	3+
	#3, #5; storiform type	3+	3+	3+	NE
MT-10	TC-1, TC-7, TC-21; pleomorphic, myxoid and storiform types	2+	2+	1+	2+
	#3, #5; spindle-cell type	—	—	3+	NE

a) TC-1, TC-7 and TC-21 are tumors induced by cloned cells at 1, 7, 21 passage levels, respectively. #3 and #5 are serial transplantation numbers of tumors induced by cloned cells at *in vitro* passage level 7.
b) See footnotes of Table I.

positive lipid droplets in their cytoplasm were frequently seen. The storiform type consisted predominantly of round histiocytic cells and elongated fibroblastic cells often arranged in a storiform or cartwheel pattern (Fig. 5C). This type had a considerable amount of collagenic fibers among neoplastic cells. Tumors consisting mainly of spindle or fusiform cells arranged in an interlocking pattern or a compact sheet and containing no demonstrable collagenic fibers are referred to as spindle-cell type in this paper (Fig. 5D). In all the types neoplastic cells contained PAS-positive and diastase-digestible material, probably glycogen granules, and reticulin fibers were around neoplastic cells.

All four cloned cell lines produced tumors with a similar histologic pattern irrespective of their passage level (Table II). Tumors induced by MT-7 and MT-10 were composed of a mixture of the pleomorphic, myxoid and storiform types, and there was no distinct border between these types. Tumors induced by MT-9 consisted uniformly of the storiform type. Rats inoculated with MT-8 developed spindle-cell type tumors. Some parts of these tumors contained the pleomorphic and myxoid types.

Enzyme/immunocytochemically, neoplastic cells constituting the pleomorphic, myxoid and storiform types gave moderately or strongly positive reactions for ACP and NSE and reacted to MAB1435 (Fig. 6A). In contrast, the spindle-cell type did not react to these stainings (Fig. 6B). Tumors induced by MT-7, MT-8 and MT-9 reacted strongly for ALP, while those induced by MT-10 gave a faint reaction for ALP (Table II). Positive reactions for α_1-antitrypsin, S-100 protein and lysozyme were sporadically observed in all induced tumors. On the other hand, no positive reactions were demonstrated for factor VIII-related antigen, keratin and desmin in any of the tumors induced.

To investigate possible histologic variations that may occur during serial transplantation of tumors, the tumors induced by cloned cells were serially transplanted in syngeneic rats up to the 5th generation. The transplanted tumors from MT-9 retained histologic features of the storiform type throughout the generations examined. Transplants at the 2nd passage from MT-7 and MT-10 bore a close resemblance histologically to those of the 1st passage. In subsequent transplantations, however, spindle-cell areas consisting of undifferentiated cells made an appearance. In serial transplantation of the tumor induced by MT-8, the spindle-cell type became gradually predominant and it was accompanied with organoid structures in which small, round or fusiform cells with scanty cytoplasm and hyperchromatic nuclei proliferated around vascular channels (Fig. 7A). Neoplastic cells constituting storiform-type transplants from MT-9 gave positive reactions for ACP and NSE,, whereas those of spindle-cell-type transplants from MT-7, MT-8 and MT-10 were negative for both enzymatic markers (Table II). ALP was positive in transplants from all four cloned cell lines (Table II). Osseous tissues consisting of osteoids, osteoblasts and calcifying areas were sporadi-

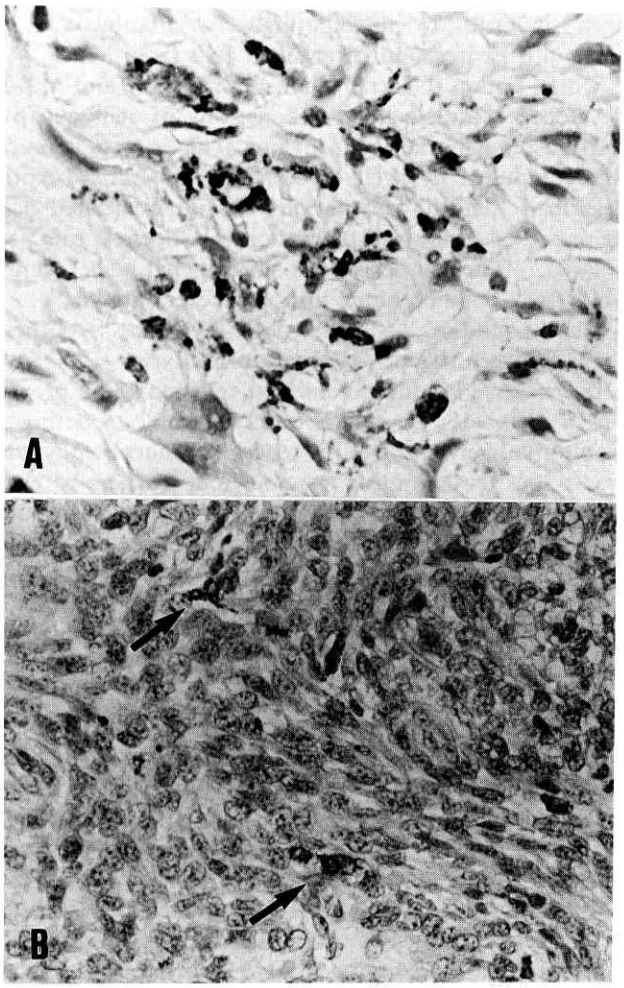

Fig. 6. Cells in a storiform-type tumor induced by MT-9 are positive to MAB1435 (A, ×400), whereas neoplastic cells in a spindle-cell-type tumor induced by MT-8 are negative to MAB-1435, although infiltrating macrophages are positive to the antibody (arrows) (indirect immunoperoxidase staining, counterstained with hematoxylin, B, ×300).

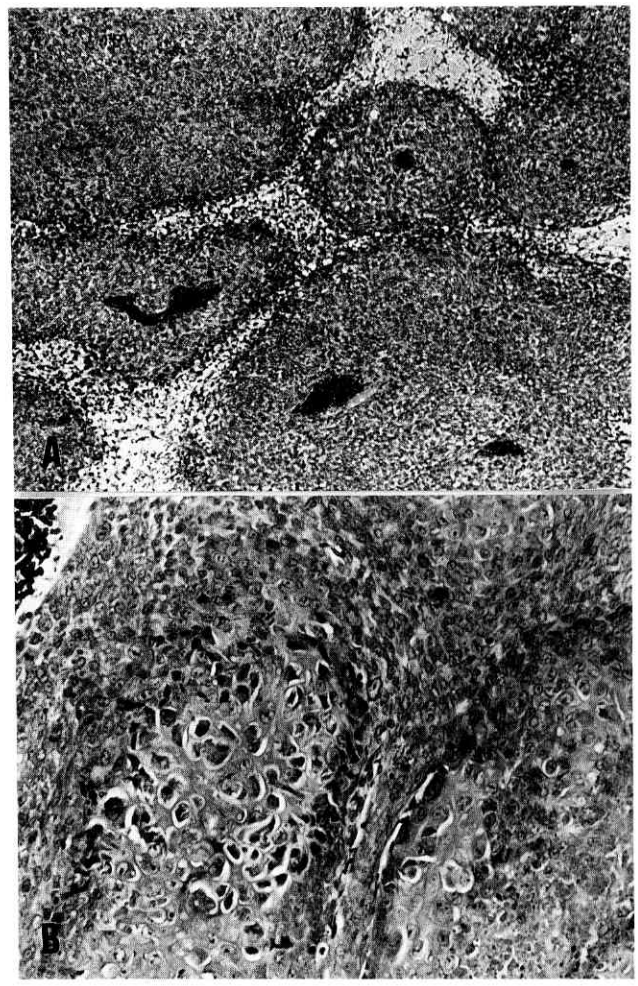

Fig. 7. A, spindle-cell area in a transplant from MT-8 shows an organoid structure (HE, ×80). B, a portion of osseous tissue in a spindle-cell area of a transplant from MT-8 (HE, ×280).

cally observed in spindle-cell areas of transplants from MT-8 and MT-10 (Fig. 7B).

DISCUSSION

In the present study we established four cloned neoplastic cell lines from a transplantable rat MFH. The results obtained by examining the cloned cells and their transplants in syngeneic rats confirmed the diversity in cellular constituents of MFH, as described in human and animal MFH by many previous workers.[2, 3, 13]

Electron microscopy of cells from MT-7 and MT-10 revealed several features characteristic of histiocytes such as numerous cytoplasmic extensions, many lysosomes and well-developed cytoplasmic organelles. The histiocytic nature of these cells was further supported by positive reactions for ACP and NSE and by a positive reaction with MAB1435. Cells of MT-9 had similar ultrastructural and enzyme/immunocytochemical features to those of MT-7 and MT-10, but they contained microfilaments and well-developed dilated RER in their cytoplasm. Neoplastic cells forming human fibrosarcomas were characterized by smooth cytoplasmic membrane, elongated nuclei and well-developed dilated RER, but lysosomes were not observed in their cytoplasm.[24] Fibroblasts having intracytoplasmic actin-like thin filaments were often observed in dermatogenic contracture and are known as myofibroblasts.[25] Fine structures of

MT-9 cells suggested that they may be intermediate forms between histiocytes and fibroblasts. Such cells have occasionally been detected in human and animal MFH.[3, 13, 20] Cells of MT-8 stained faintly positive for ACP and NSE and with MAB1435. These cells had a rather smooth cytoplasmic membrane and poorly developed cytoplasmic organelles and were interpreted as undifferentiated mesenchymal cells that have occasionally been seen in MFH.[3, 11, 13] The incidences of rosette formation and phagocytic activities were low in all the clones. This may be due to a lower differentiation stage of the cloned cells examined. Cloned cells established from human MFH were polygonal in shape and exhibited histiocytic differentiation with positive reactions for lysosomal enzymes and high incidences of functional markers.[7, 8] Roholl *et al.*[11] isolated two cell lines from human MFH. *In vitro* neoplastic cells from the first MFH ultrastructurally resembled primitive mesenchymal cells and those from the second MFH resembled fibroblastic cells. The two lines did not exhibit immunorosetting or phagocytosis. The authors suggested heterogeneity of the MFH group.

All the clones induced subcutaneous MFH with various histologic patterns. Tumors induced by MT-7 and MT-10 cells having a histiocytic appearance were of the pleomorphic, myxoid or storiform type. Cells of MT-9, a possible intermediate form between histiocyte and fibroblast, produced storiform-type tumors containing a large amount of collagenic fibers, suggesting that the cells have the capability to produce collagen fibers during the tumor development. Fibrosarcomas have abundant collagen fibers. However, the histology of the storiform type differed from fibrosarcoma in that the latter consisted predominantly of neoplastic fibroblasts negative for lysosomal markers arranged in a herring-bone pattern.[24] It has been reported that some histiocytic cells underwent morphologic alteration into fibroblastic cells during long-term cultivation[8, 20] and that neoplastic histiocytes in MFH may be able to behave as facultative fibroblasts under appropriate conditions.[6, 9]

Tumors induced by MT-8 were of the spindle-cell type consisting of undifferentiated cells negative for lysosomal markers, and were accompanied with organoid structures. Such structures have never been described in human MFH. These tumors appeared to be undifferentiated sarcomas. Undifferentiated sarcomas have also been induced by inoculating *cis*-diamminedichloroplatinum-selected undifferentiated cells from MT-P.[23] It is worth noting that transplants from MT-7 and MT-10 developed spindle-cell areas consisting of cells negative for lysosomal markers. Maruyama *et al.*, in a study on rat MFH induced by 4-(hydroxyamino)quinoline 1-oxide, described that the population of undifferentiated cells increased as serial transplantation in syngeneic rats progressed.[13] These observations suggest that cells forming MFH may dedifferentiate into undifferentiated cells.

Recent studies[3, 12, 13, 26] seem to favor the hypothesis that the precursor of MFH is a primitive mesenchymal cell. Presumably, undifferentiated cells may be a precursor of cells with histiocytic nature capable of acting as facultative fibroblasts. Differences in morphology and reactivities to stainings for cytological markers between the clones may depend on different stages of cellular differentiation. However, it is still uncertain why inoculation of each clone, having a homogeneous cell population, into rats resulted in the development of an MFH with a pleomorphic pattern. This may be explained by assuming that each clone has the capability to differentiate in various directions.

Osseous tissue has occasionally been found in human and experimental rat MFH,[1, 12] as observed in spindle-cell areas of transplants from MT-8 and MT-10. It has recently been reported in a study using monoclonal anti-MFH antibodies that MFH and liposarcoma have a common origin from the perivascular mesenchymal cells.[15] Moreover, xenografts of human MFH in nude mice have been shown to express leiomyogenic or schwannian differentiation.[27] MFH has been reported to contain a pluripotential progenitor for mesenchymal differentiation.[1] The cloned cell lines we established and transplantable MFH induced by the clones may provide useful experimental systems for studying the histogenesis and growth behavior of this tumor.

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