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Establishment and Characterization of a Murine Cell-Line Derived from Malignant Fibrous Histiocytoma of A/Jackson Mouse

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Watanabe, I., Kurosawa, N. and Nishihira, T. *Establishment and Characterization of a Murine Cell-Line Derived from Malignant Fibrous Histiocytoma of A/Jackson Mouse.* Tohoku J. Exp. Med., 1998, **184** (3), 173-187 —— A hard mass incidentally found in the right buttock of an elderly male A/J mouse was morphologically consistent with malignant fibrous histiocytoma; storiform-pleomorphic subtype. Diced pieces or suspensions of the tumor tissue were readily transplantable subcutaneously in the flank of mice of the strain. The tumor-bearing mice died mostly of rupture of the tumor into the thorax or abdomen within 4 weeks. Pulmonary and splenic metastasis were recognized only in a mouse which survived more than 10 weeks. A new cell-line was established from the original tumor without necessary cloning and was maintained in MEM (minimum essential medium) supplemented with 10%FCS for 6 months under 22 passages. Frozen stocks were then made, from which the recovery of the cells was successful. The cell growth, requiring about 20 hours for doubling of population, was a mixture of plump or stellate histiocytic cells and fibroblastic ones, both of which contained lipid droplets and retained phagocytic activity. The cell-line was designated as murine sarcoma Sendai (MuSS). Morphological versatility of the cultured cells suggested the origin of the neoplasm from multipotential, primitive mesenchymal cells. ———— malignant fibrous histiocytoma (MFH); A/Jackson (A/J) mouse; continuous cell-line; phagocytosis; murine sarcoma Sendai (MuSS)

Malignant fibrous histiocytoma (MFH) is a neoplasm which is histologically composed of neoplastic cells in several figures such as histiocytic, fibroblastic and bizarre-giant cells. Even myoblastic cells are often intermingled. In order to elucidate the origin of these cells, not only human MFH cells in short-term culture (Ozzello et al. 1963; Fu et al. 1975; Enjoji et al. 1980; Oku et al. 1984) or

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continuous culture (Shirasuna et al. 1985; Iwasaki et al. 1987, 1992; Roholl et al. 1991; Takeya et al. 1991; Yokota et al. 1995) but also chemically induced (Tsuchiya et al. 1993) or spontaneously developed (Takahashi et al. 1990; Yamamoto and Yamamoto 1996; Yamate et al. 1996) animal MFH cell-lines have so far been investigated. The results are, however, not always conformable with each other and the histogenesis of this neoplasm is still being debated.

An additional murine cell line derived from spontaneously developed MFH in an A/Jackson (A/J) mouse is herein reported, because of its easy homotransplantability and stable growth in culture. A/J mice are renowned for their bearing of C 1300 neuroblastoma and also for developing lung cancer in the elderly. The newly established cell-line of MFH of this murine strain is expected to provide further information on various aspects of this neoplasm, which is the most frequent soft tissue sarcoma in human adults.

Materials and Methods

A hard mass was incidentally found in the buttock of an elderly male A/J mouse at 60 weeks of age. The tumor was expanding into the lower abdomen and up to 27 mm in the largest diameter. Fracture of the right femur was recognized. Autopsy specimens after extirpation of the tumor showed no other neoplastic changes in the mouse. Tumor tissue, minced and diluted with culture medium (MEM [minimum essential medium] 1 : 1) was first inoculated subcutaneously in the flank of four A/J mice. The tumors appeared at the site of the four mice 2 weeks after the inoculation and grew steadily. These were subjected to successive homo transplantation and to continuous cell culture, as the source of MuSS (murine sarcoma Sendai).

A/J mice had been purchased from the Jackson Laboratory, Bar Harber, ME, USA.

Homo transplantation

Diced pieces or suspensions of the tumor tissue obtained from the tumors at the flank of the mice were transplanted in the same way into other mice, every 3–4 weeks for succession. Suspension of the tumor tissue consisted of $3\text{–}5 \times 10^6$ tumor cells in 0.3–0.5 ml MEM per mouse. Small lumps appeared in every mouse at the site of inoculation after 2 weeks. About 3 weeks after the inoculation, the tops of the growing lumps (ca 10 mm in diameter) began to ulcerate and within 4 weeks the tumor-bearing mice died, mostly of rupture of the tumor into the thorax or abdomen. Behaviors in vivo were almost the same when the inoculation of the tumor was similarly done, and doubling time of the largest diameter of the lumps was about 2 weeks. A few mice survived more than 4 weeks but died of cachexia. No signs of fatal infection nor metastasis were histologically recognized among them, even in the rare mice which survived more than 8 weeks. Only one mouse which survived 10 weeks showed pulmonary and splenic metastasis. Fifteen

passages in vivo have been thus carried out for 17 months including 4 months' frozen interval.

Specimens of the tumor were fixed in 10% formalin. From paraffin blocks the sections for H-E and special stains were made. The original tumor cells for flow cytometric DNA analysis were also obtained from the blocks. Ultrastructural observations were performed on the specimens which were fixed in 2% glutarformal solution and ultimately embedded in epoxy resin.

Cell culture

Original tumor tissue was minced into fine fragments with a sharp blade and explanted on the bottom of T25 (3109) plastic culture bottles (Nippon Becton Dickinson Co., Ltd., Tokyo). After attachment of the fragments became firm, growth medium (Dulbecco's modified Eagle's medium [DMEM] supplemented with 10% fetal calf serum [FCS] and antibiotics) was added and the bottles were kept in the humidilfied 5% CO_2 incubator at 37°C. After 2 weeks, when tumor cells began to grow out of the fragments and some colonies became visible, the growth medium was first replaced. One week thereafter when abundant outgrowth of the cells was seen, the fragments and the cells were removed with 0.05% trypsin and 0.02% EDTA (ethylene-diamine-tetraacetic acid) solution, and only single cells were subjected to subcultivation. After these procedures, the growth of the tumor cells accelerated overcoming that of native fibroblasts and, without the necessity of special cloning, a clean and monotonous cell-layer of the tumor cells was obtained. Later than 40 days after the beginning of this cell culture, the growth medium (MEM or DMEM, supplemented) was usually replaced twice weekly. When the cell layer became confluent, the cells were detached with the trypsin solution for subcultivations. Thus, for 6 months the cells were maintained in vitro under 22 passages and then the cells in stock solution with 10% DMSO (dimethyl-sulpho-oxide) were reserved in fluid nitrogen. After a 4-months' period of interruption of the culture, recovery of the cells from the stock solution was successful both in vitro and in vivo.

Analysis of biological characteristics of the cells was performed on these cells, at the 24th or 25th subcultivation. Population doubling time was estimated during the exponential growth of the cells in MEM (10% FCS). The number of tumor cells was counted daily after staining with trypan-blue. For chromosome preparation of the tumor cells, those growing in the culture bottles were treated with Colcemide (0.05 μg/ml) for 3 days. After being rinsed several times with prewarmed phosphate buffered saline or balanced salt solutions, the cells were subjected to hypotonic conditions and then fixed in methanol: acetic acid (3 : 1) solution. These were smeared on slide-glasses and air-dried. The preparations were stained with the Giemsa-banding Technique using trypsin, and 20 metaphase cells were analyzed. For DNA histogram, nuclei of the tumor cells were stained with Propidium iodide and their fluorescences were assayed with a flow-cytometer

(Nippon Becton Dickinson Co., Ltd.).

Observations

Histopathological findings

The original tumor tissue was gray-white to light yellow, and was microscopically mainly occupied by an aggressive growth of undifferentiated sarcoma cells which showed round to spindle shapes with prominent pleomorphism. Round to oval nuclei contained sparse chromatin and conspicuous nucleoli, and frequently showed typical to atypical mitotic figures. Cytoplasm with ill-defined cell borders was thin-eosinophilic or amphophilic and sometimes contained lipid droplets (Fig. 1). Solitary, enlarged and highly acidophilic cells with nuclear atypism were often found. Their cytoplasm resembling those of myoblasts was periodic acid Schiff (PAS) positive and not rarely contained non-lipid, hyalinous substance. These cells were occasionally multinucleated with bizarre nuclei which were not like those of osteoclast-type giant-cells (Fig. 2). Intermingling of plump histiocyte-like cells other than the eosinophilic cells was also recognized. They were scattered haphazardly or connected with spindle cells and occasionally showed engulfing of hemosiderin particles. Clusters of foam cells were not found.

These tumor cells, in small area of the tumor, showed gradual transition into fibrous bundles of elongated fibroblastic cells which were manifested as clusters in a storiform or cartwheel pattern (Fig. 3). Their nuclei were also elongated but still oval in shape and mitosis was rare in this area. Apart from ample distribution of capillary branches throughout the tumor, an angiomatoid pattern in another small area was formed by tumor cells, endothelial cells and collagenous matrix, where dilated vessels were often flanked by tumor cells (Fig. 4).

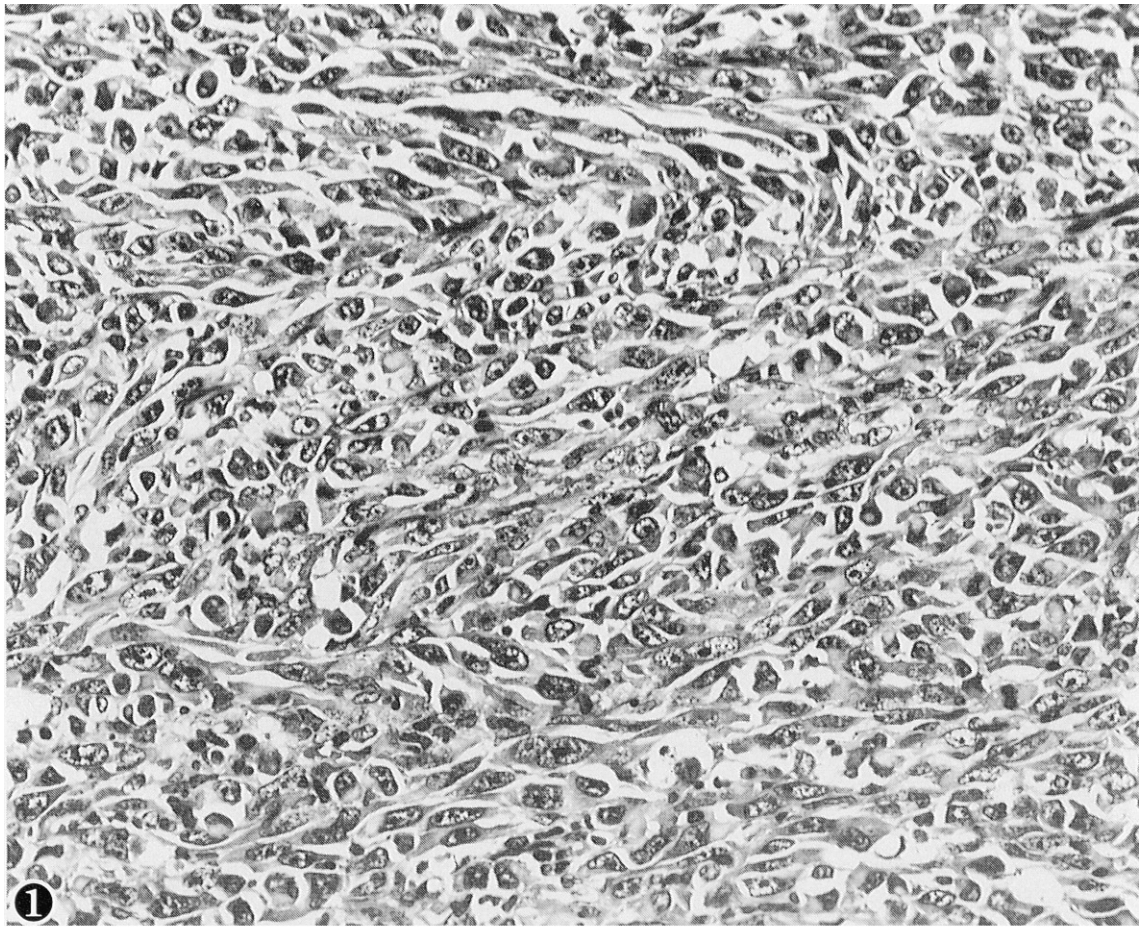
This histologic picture of the whole tumor was modified by common hemorrhage and patchy necrosis, where several inflammatory cells had accumulated. Invasive growth of the tumor into surrounding tissue was prominent and tumor tissue occasionally contained entrapped bundles of normal muscle fibers.

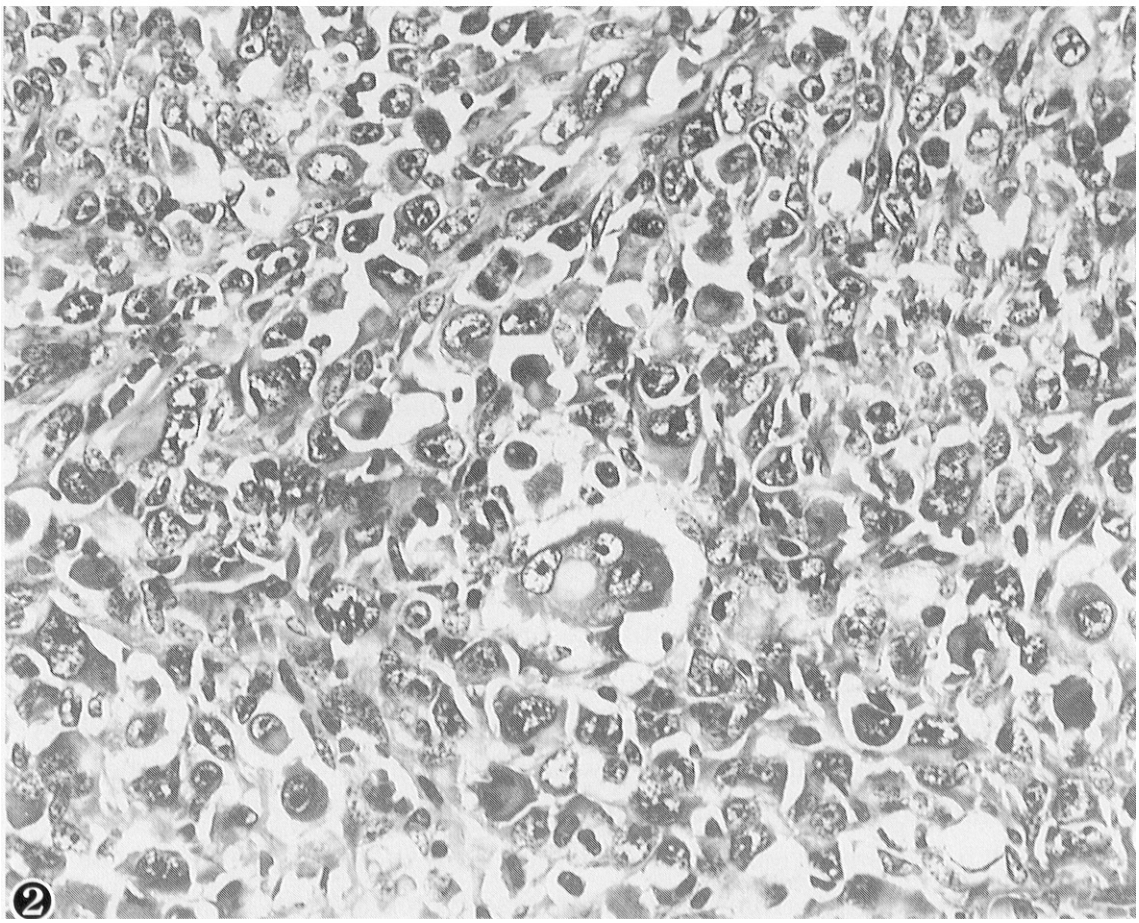
Tumor tissue which had grown at the flank of the mice after the inoculation showed essentially the same histological findings as those of the original tumor. Ulcers at the tops of the lumps, however, showed bacterial colonies in the surface and collagenous layers at the bottom adjacent to the neoplastic growth. Rupture of the tumor due to invasion into the thorax or abdomen had caused fatal massive hemorrhage in each mouse.

Ultrastructural observations of the original tumor supported the histological findings of several kinds of tumor cells. Spindle or polygonal tumor cells had round to oval nuclei with single to three nucleoli. Their abundant cytoplasm

Fig. 1. Undifferentiated tumor cells occupying most of the primary tumor. A mixture of atypical polygonal cells and spindle cells. H–E, ×200

Fig. 2. An area of the primary tumor where eosinophilic round cells are accumulated. H-E, ×250





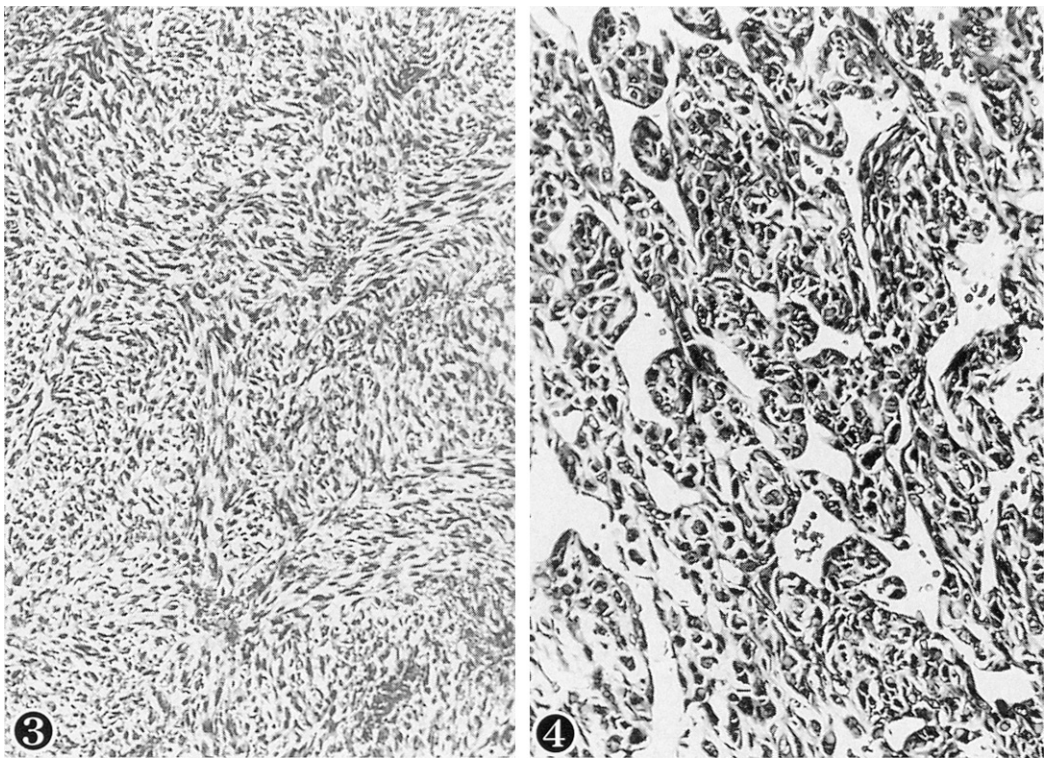


Fig. 3. Storiform pattern found in a small area of the original tumor. H-E, ×100

Fig. 4. Angiomatoid pattern found in another small area of the original tumor. H-E, ×120

containing many dilated rough endoplasmic reticula was elongated and showed intersection each other, while they showed intermediate to tight junctions in other areas. Round tumor cells also appeared showing nuclear pleomorphism and contained lysosome-like dense bodies (Fig. 5A). Fine lipid droplets were seen in both fibroblastic and histocytic cells, of which nuclei were not displaced or indented by them (Fig. 5B). Numerous ribosomes were occasionally found in the cytoplasm of these cells of various figures. Other than well-differentiated sarcomeres in entrapped muscle fibers, no Z-band material was recognized in any myoblast-like tumor cells. In some locations of the original tumor, large amounts of intracytoplasmic virus particles were noticed electron microscopically.

Characteristics of cultured cells

At the beginning of primary culture or subcultivation, the scattered growth of stellate cells was remarkable. The stellate cells stretched a few processes, and were sometimes binucleated with prominent nucleoli (Fig. 6). They otherwise showed an easily dissociating cobble-stone arrangement. Among the growing colonies, round cells and elongated bipolar cells were only sparsely intermingled. Until day 3–5, before a confluent monolayer of the cells was seen, elongated fibroblastic cells distinct from native fibroblasts increased in number among the population and compressed the stellate and round cells. Polygonal or stellate

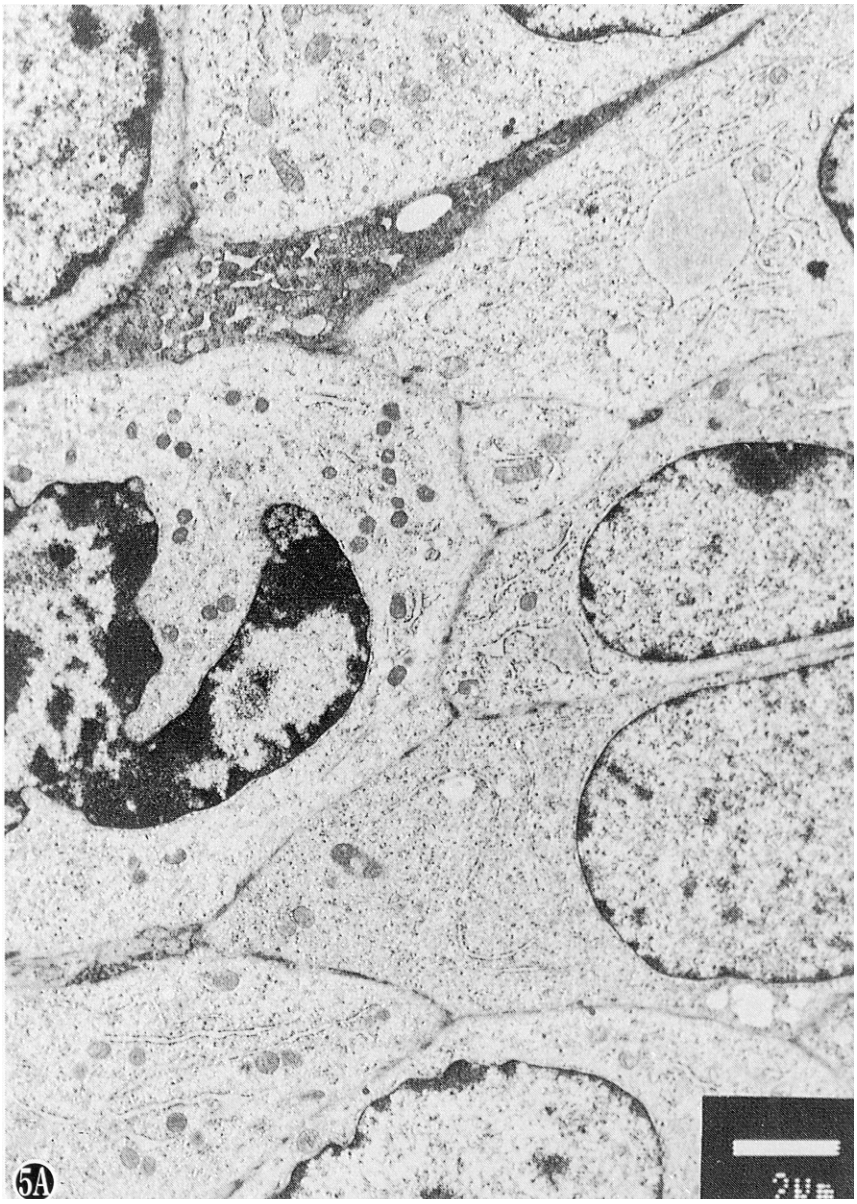


Fig. 5-A. Ultrastructure of tumor cells showing tight junctions. Intracytoplasmic well-developed RER in fibroblastic cells, and lysosomes in a histiocytic cell with lobate nucleus are seen.

cells often showed gradual transition of the figures into spindle or fibrous ones. At the terminal stage of subcultivation, day 5–7, multinucleated large cells became visible and piling of accumulated fibroblastic cells was recognized (Fig. 7). When the culturing was protracted to day 7–9, fibroblastic cells constituted the whole population forming a storiform-like pattern, where hardly any stellate or round cells were seen. The process of these morphological changes in vitro was repeated in every subcultivation until at least 26 passages.

In these cells of several shapes, various amounts of lipid droplets were always recognized. When they were exposed to the lithium carmine solution (Ozzello et al. 1963) at day 3 of subcultivation, the stellate cells in any arrangement rapidly engulfed the dye-particles (Fig. 8). Occasional cytoplasmic vacuoles rimmed

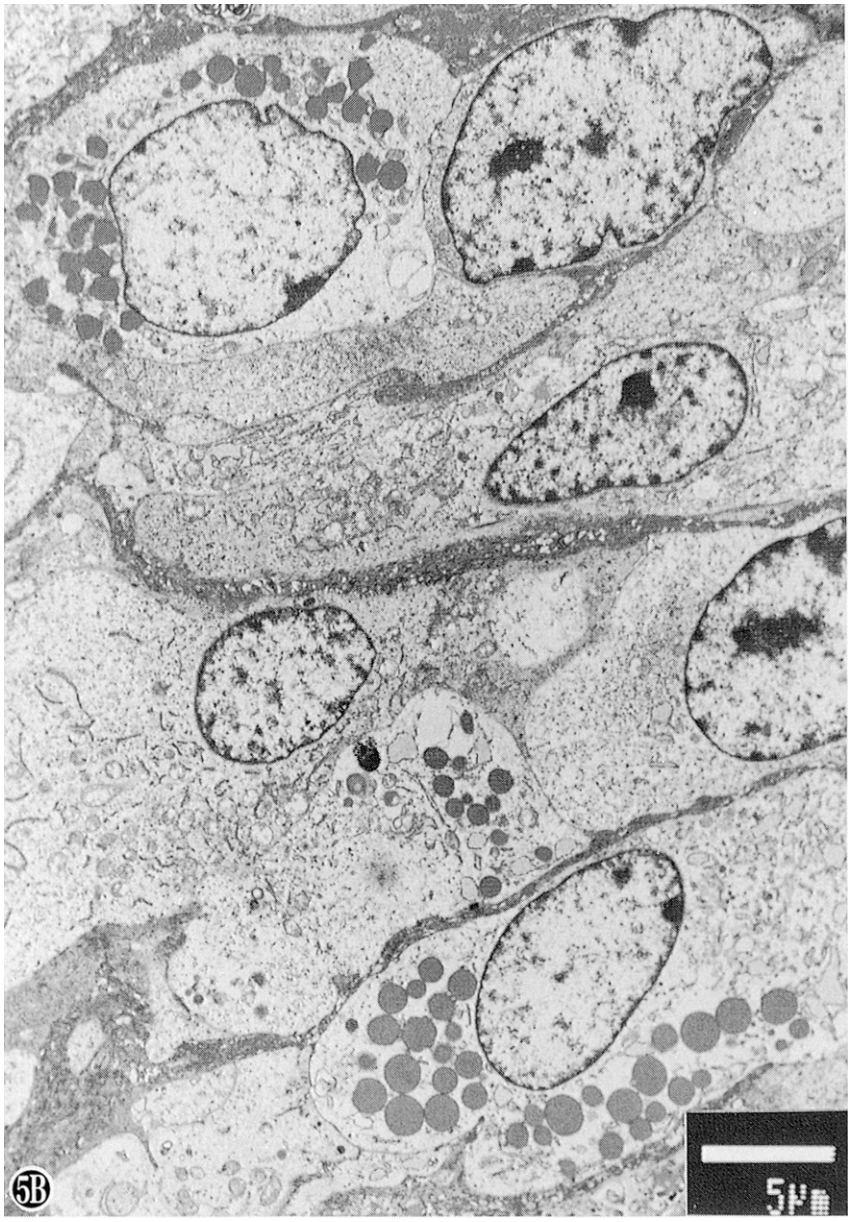


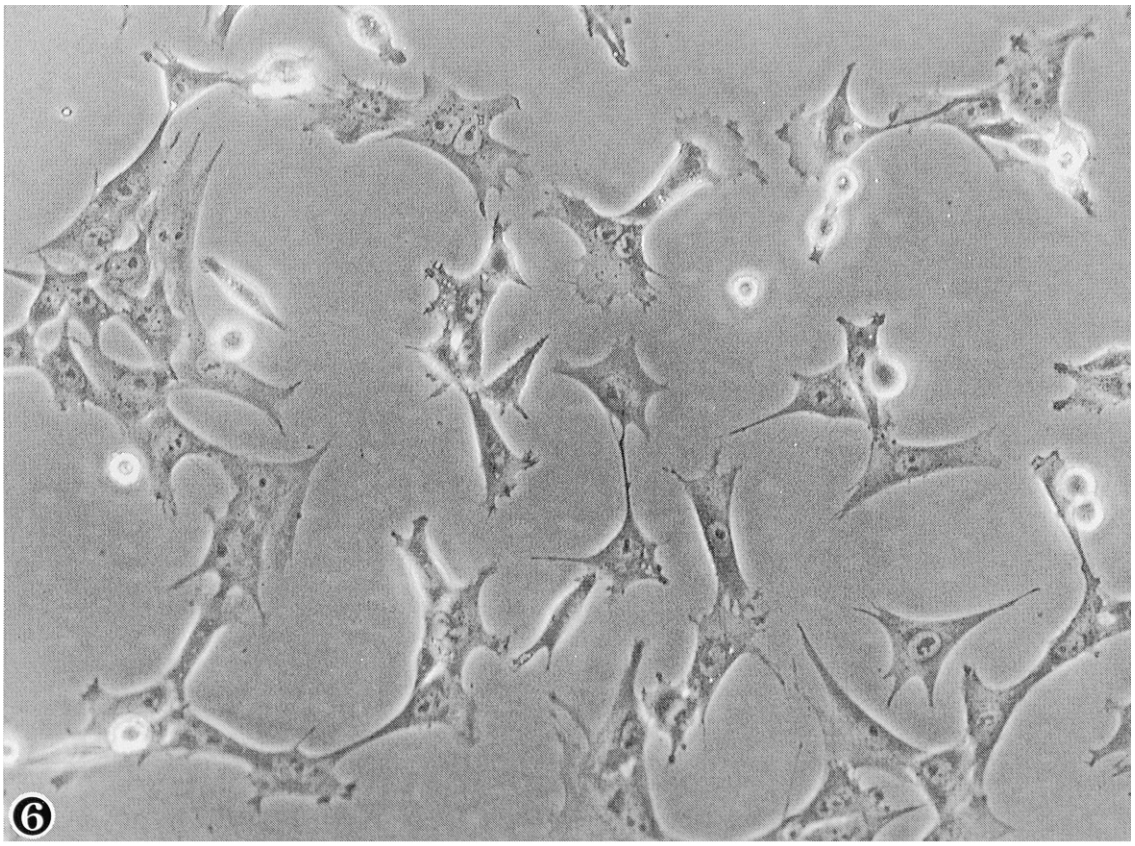
Fig. 5-B. Ultrastructure of tumor cells separated by delicate collagenous matrix. Fine lipid-droplets are seen in cytoplasm.

with the particles appeared to be phagosomes.

The growth-curve of the cultured cells was obtained through daily counts of the stained cells. They were 10×10^4 at day 1; 60×10^4 at day 2; 340×10^4 at day 3; 780×10^4 at day 4; 1400×10^4 at day 5; 1600×10^4 at day 6 and 1600×10^4 at day 7. Population doubling time was estimated to be about 20 hours, which seemed to be in accord with the in vivo doubling time of the inoculated tumors.

Fig. 6. Day 3 cultured cells at passage 20 in the MEM. Stellate or polygonal cells grow dispersedly. Occasional binuclear cells are seen. Phase contrast $\times200$

Fig. 7. Day 7 cultured cells at passage 20, in the same bottle as that in Fig. 6. Abundant growth of fibroblastic cells and a few giant cells are compressing stellate cells. Phase contrast $\times100$



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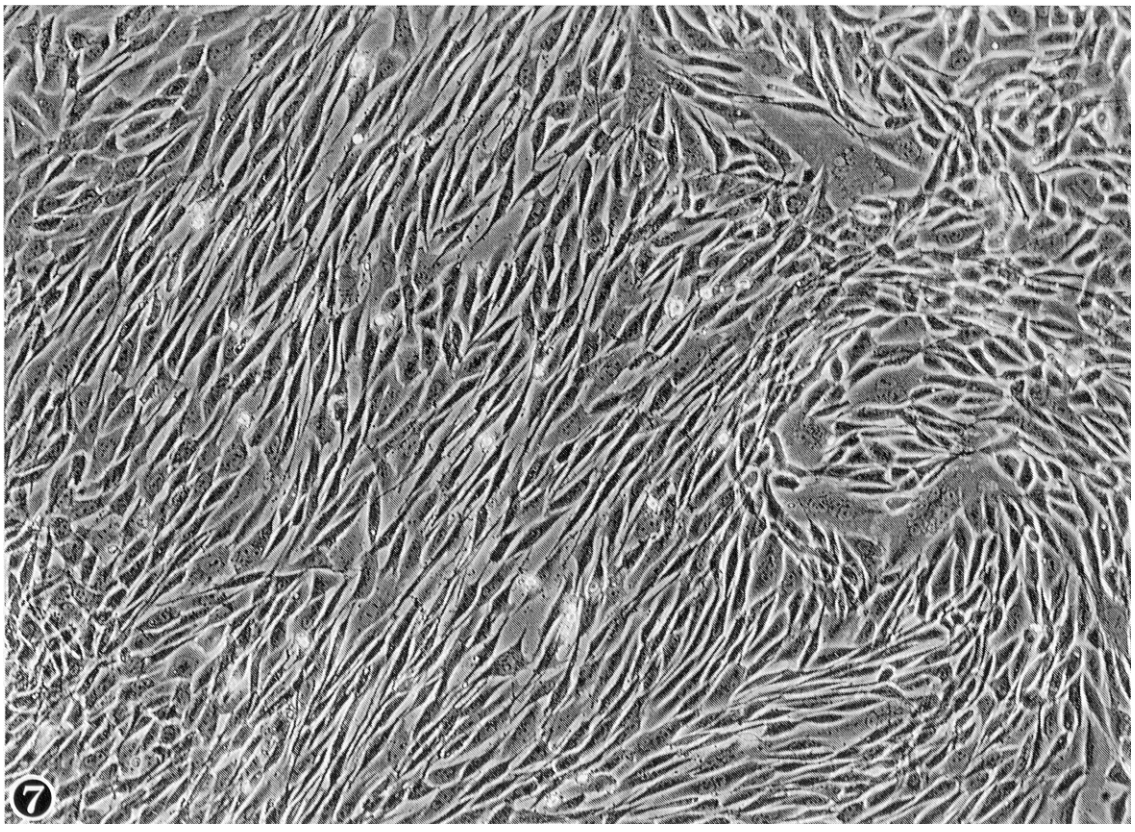

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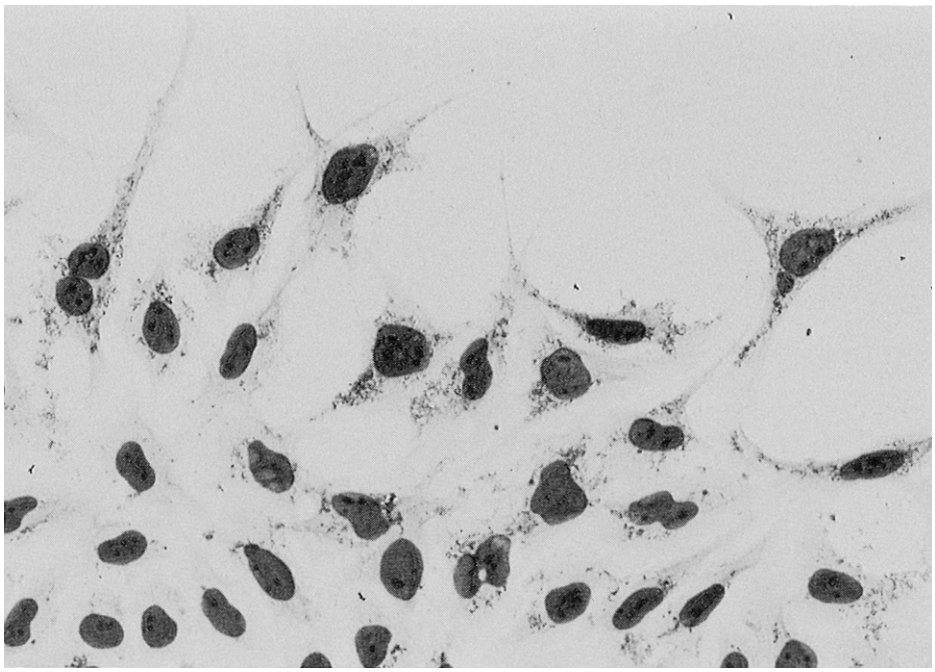

Fig. 8. Day 3 cultured cells at passage 24 showing easily dissociating cobble-stone arrangement. Each cell contains lithion-carmine particles. Giemsa ×250

The cells at passage 25 revealed a mixed population of various karyotypes showing either one of the following:

1) As shown in Fig. 9
2) 81, −X, −X, −Y, −Y, −1, +3, +4, +6, +6, +6, +7, +7, +7, +7, +8, +9, +9, +10, +10, +12, +12, +12, +der(12)t(12; 13)?, +14, 15+, +15, +15, +16, +16, +17, +17, +17, +18, +19, +19, +mar12
3) 81, −X, −X, −Y, −Y, −1, −1, −4, +der(2)t(2; ?), +5, +5, +6, +6, +6, +6, +7, +7, +8, +8, +8, +8, +9, +9, +10, +10, +10, +11, +11, +12, +12, +der(12)t(12; 13)?, +13, +13, +13, +14, +14, +14, +15, +16, +17, +17, +18, +18, +mar10
4) 80, −X, −X, −Y, −Y, −1, −1, +der(2)t(2; ?), +3, +3, +4, +4, +4, +6, +7, +7, +7, +9, +9, +12, +12, +12, +12, +13, +13, +13, +14, +14, +14, +16, +16, +16, +17, +17, +18, +18, +mar12
5) 83, −X, −X, −Y, −Y, −1, −1, +der(2)t(2; ?), +3, +3, +4, +6, +7, +7, +7, +8, +8, +9, +9, +10, +10, +10, +11, +12, +12, +der(12)t(12; 13)?, +der(12)t(12; 13)? +13, +13, +13, +14, +14, +14, +15, +16, +16, +16, +17, +17, +18, +18, +19, +mar12

Details of the chromosomal analysis will be described elsewhere.

Flow-cytometric analysis of the original tumor cells showed a DNA histogram of diploid pattern having G1, 74.2%; S, 14.0%; and G2+M, 11.8%. The G2+M/G1 ratio was 2.01. However, that of the cultured cells at passage 25, subcultured for 8 days, displayed an aneuploid pattern (Fig. 10). Dual G_0G_1 peaks and supposedly an accumulation of the cells in S-phase fraction were suggestive of the highly malignant character of the cells (Radio et al. 1988) and possibly a mixed proliferation of at least more than two constituent neoplastic cells.

No virus was isolated from the cultured cells at the 24th subcultivation.

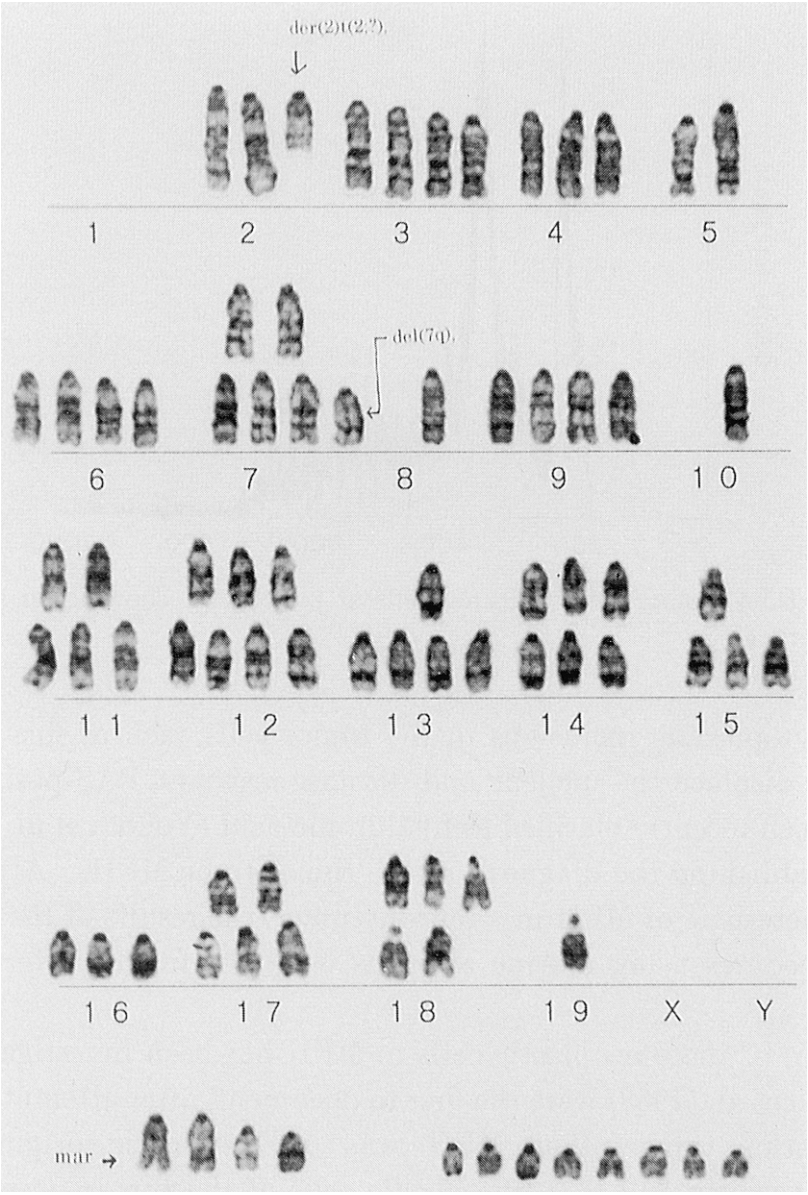

Fig. 9. A representative chromosomal pattern of cultured cells at passage 25 showing karyatype of 79, −X, −X, −Y, −Y, −1, −1, −8, −10, −19, +der(2)t(2; ?), +3, +3, +4, +6, +6, +der(7)del(7q), +7, +7, +7, +9, +9, +11, +11, +11, +12, +12, +12, +12, +12, +13, +13, +13, +14, +14, +14, +14, 15+, +15, +16, +17, +17, +17, +18, +18, +18, +mar 12

Discussion

Human malignant fibrous histiocytoma (MFH) usually manifests a broad range of histological appearances and so divided into subtypes. The subtype, which the murine tumor imitated was the storiform-pleomorphic one, which was an admixture of several neoplastic cells including plump histiocytic and elongated fibroblastic cells. The differential diagnosis of these non-epithelial pleomorphic tumors is generally confusing unless the histological component displaying storiform and/or angiomatoid pattern is found, even in small amounts (Enjoji et

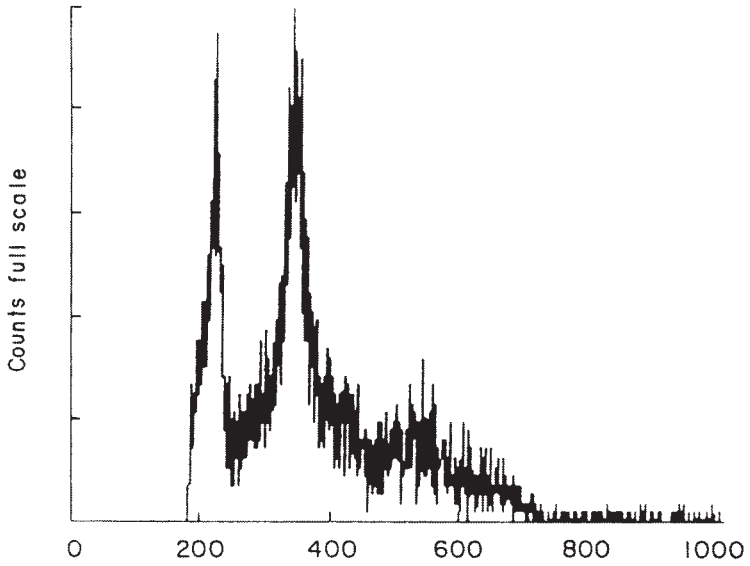

Fig. 10. DNA histogram of cultured cells at passage 25, showing an aneuploid pattern.

al. 1980). Cytoplasmic inclusions in the tumor cells such as fine lipid-droplets which do not displace the nucleus and diastase-resistant PAS-positive materials which have been recently clarified as hyaluronic-acid (Yokota et al. 1995) are also helpful in establishing the diagnosis of the tumor to be MFH. After finding the features characteristic of MFH in a murine tumor, the results of the immunohistochemical procedures using murine antigens were not further referred to in this study.

The origin of these neoplastic cells in MFH has been investigated for a long time. Ozzello et al. (1963) were the first to document, after attempting supravital stainings of this tumor, that MFH was of histiocytic origin manifesting phagocytotic activity and that fibroblastic cells of the tumor were also derived from histiocytes but were acting as facultative fibroblasts. Thereafter, alpha-1-antitrypsin and -antichemotrysin and lysozyme were stained immunohistochemically and both tumor cells were also described to be of common origin from tissue histiocyte (de Bouley 1982). MFH as a whole tumor reacted with the antibodies directed to monocytes and macrophages in another experiment (Strauchen and Dimitriu-Bona 1986), and monocyte - macrophage lineage was generally suspected to be the origin of this tumor.

Against the view, recent immunoelectron microscopic observations of chemically induced rat MFH, with the use of anti-rat MFH monoclonal antibodies, suggested that the histiocyte-like cells in it were merely infiltrated macrophages and that fibroblast-like cells and intermediate cells were virtual tumor cells showing differentiation toward fibroblasts (Tsuchiya et al. 1993; Takeya et al. 1995). Those histiocytic cells, also in the human MFH cell-line, had been defined as being attracted by tumor-derived MCP-1; macrophage chemoattractant protein-1 (Takeya et al. 1991). Furthermore, heterogeneity in the origin and

immunophenotypes of the histiocytic cells gathering in a transplantable rat MFH was indicated (Yamate et al. 1996). Non-fibroblastic tumor cells, which were histiocytic in figures and function but not induced by MCP-1 were, therefore, facultative histiocytes. On the other hand, in major constituent fibroblastic cells of MFH, immunophenotypic characteristics very similar to those of fibrosarcoma and fibroblastic cells were detected by Takaya et al. (1995) and the tumor cells were strongly suggested to belong to fibroblastic lineage differentiated from mesenchymal cells. Multipotential mesenchymal cells, not yet known but arising during normal embryonic development (Roholl et al. 1991), were thus deemed as the origin of MFH. To determine precursor cells of MFH in actual human tissue, Iwasaki et al. (1987, 1992) generated monoclonal antibodies FU_3, FU_4 by a mouse hybridoma technique using established human MFH cell lines. It was concluded immunohistochemically that MFH and liposarcoma might have a common origin from the perivascular mesenchymal cells having potential for multidirectional differentiation.

The stellate cells which always appeared at the beginning of subcultivation in this study might be estimated to be the multipotential mesenchymal cells in vitro, since gradual transition of stellate figures into fibroblastic ones were usual findings. When subcultivations were repeated from the cells in fibroblastic full-sheet population, the behaviors of the cells were the same as seen in the preceding subcultivation; the stellate cells were predominant at the beginning but then were replaced by the subsequent growth of fibroblastic cells and giant cells. The stellate cells might differentiate into several types of MFH-tumor cells in vitro, reflecting potential versatility of original cells of MFH also in the mouse. DNA cytoflowmetric analysis and the chromosomal pattern analyzed in cultured cells also suggested that a few morphologically distinct cell types had originated from the same cell clone rather than from separate clones. Further elaborative studies of the stellate cells are mandatory to verify whether they differentiate, as primitive mesenchymal cells, not only into MFH but also into several mesenchymal tumors including liposarcoma.

Acknowledgments

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