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Metamorphosed fibroblasts and their relation to the histogenesis of malignant fibrous histiocytoma in experimental murine model

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Summary. Malignant fibrous histiocytoma (MFH) is a clinicopathologically established entity, but its histogenesis remains to be clarified. We have reported the existence of a specific cell type, the "fibrohistiocytoid (FH) cells", in various chronic inflammatory tissues. The FH cells are the metamorphosed fibroblasts and we have revealed the morphological resemblance between FH cells and MFH cells. In the present study we carried out some experiments to ascertain whether the FH cells have a possibility of neoplastic potential for the development of MFH in mice. A total of 50 female Balb/c mice treated with a chemical carcinogen, 9,10-dimethyl-1,2-benzanthracene (DMBA), were examined histopathologically from 8 to 22 weeks after the initial treatment. It was found that 1) the chemically induced tumors in the mice resembled human pleomorphic/storiform variant of MFH and cells from the tumor were transplantable subcutaneously in the back of another mouse, 2) the tumors were composed mainly of malignant FH cells, and there were many benign FH cells and fibroblasts in granulation tissues obtained at the initial stage of the experiment, 3) all DNA histograms obtained from MFHs were aneuploid and granulation tissues were diploid, and 4) benign FH cells in the granulation tissue appeared to have higher DNA synthesis activity than typical fibroblasts on the basis of bromodeoxyuridine (BrdU) labeling and cytofluorometric studies. From these findings, we suggest that the FH cells are not only a merely morphologically changed fibroblast, but also a biologically ominous cell which may contribute to develop MFH in mice.

Key words: 9,10-dimethyl-1,2-benzanthracene (DMBA), Malignant Fibrous Histiocytoma, Fibrohistiocytoid cell, Fibroblast, Chemically induced tumor

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Introduction

Malignant fibrous histiocytoma (MFH) is a clinicopathologically established entity and it has been recognized as the most common soft tissue sarcoma of late adult life. More recent studies have produced controversial results with regard to its histogenesis, and the progenitor cells of MFH have been proposed to be fibroblasts (Tsuchiya et al., 1993; Takeya et al., 1995), histiocytes (Binder et al., 1992) or primitive mesenchymal cells (Iwasaki et al., 1992; Yamate et al., 1996). MFH should be diagnosed using strict criteria (Meister, 1996; Stadler et al.,1998), and it is important to clarify the histogenesis of MFH.

We have previously reported morphological changes of fibroblasts in granulation tissues, and the existence of a specific cell type, the "fibrohistiocytoid (FH) cells" (Imai et al., 1989). The FH cells were found in a variety of chronic inflammatory tissues, and we demonstrated morphological, enzyme histochemical and immunohistochemical similarities between FH cells and human MFH cells. Under in vitro conditions, we also found that long-term-cultured human dermal fibroblasts were transformed into FH cells (Takagi et al., 1988).

There are a few reports of chemically induced MFH in the animal. The chemical carcinogen, 4-hydroxyaminoquinoline l-oxide (4-HAQO) can produce MFH in the rat (Mii et al., 1982). The substance 9,10-dimethyl-1,2-benzanthracene (DMBA) has been known as a potent carcinogen (Safer et al., 1998) and can also induce MFH in the rat (De Santis et al., 1981; Sakamoto, 1986) and the rabbit (Homma and Wiinsch, 1983).

The objective of the present study was to ascertain whether DMBA could produce MFH in the mouse and FH cells could have neoplastic potential for development of MFH. We carried out light and electron microscopic observations, BrdU labeling method, and fluorocytometric analysis of DNA content in a series of tissues obtained from this animal model of MFH.

Materials and methods

Production of experimental tumors

A total of 50 female Balb/c mice (Nippon Charles River Animal Farm, Tokyo, Japan) each weighing approximately 30g were used. The mice were housed in wire cages and fed commercial food pellets (CE-2: CLEA Japan Inc., Tokyo, Japan) and water ad libitum in an air-conditioned room at 24 °C. The animals were treated by injection of 0.05 ml of a 1% paraffin solution of DMBA (Nakarai Chemical Co., Kyoto, Japan) into the left knee joint, which was repeated twice at an interval of 4 weeks. As controls, several mice were treated with 0.05 ml paraffin solution only. The animals were sacrificed under ether anesthesia between 8 and 22 weeks after the first injection of DMBA and tissue specimens were obtained.

Serial transplantation

Tumor tissues obtained from a tumor were aseptically minced into small pieces in Dulbecco's minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and cultured in a humidified 5% CO_2 incubator at 37 °C. Confluent cell sheets were subcultured by dispersing the cells with a mixture of 0.1% trypsin in phosphate-buffered saline (PBS). The cell suspension (0.5 ml) was transplanted subcutaneously with a 20 gauge injection needle into the back of conventional Balb/c mouse.

In vivo BrdU labeling

One hour before sacrifice, each mouse received an intraperitoneal injection of 0.5 mg of BrdU (Sigma Chemical Co., St. Louis, MO) dissolved in 2ml of distilled water.

Morphological examinations

Tissue specimens obtained from the series of model animals were fixed in B5 fixative containing 10ml of formalin, 90ml of distilled water, 6 g of $HgCl_2$, and 2.047 g of CH_3COONa, embedded in paraffin and cut into 4-μm-thick sections. The sections were stained with hematoxylin-eosin, elastica-Masson, alcian blue, phosphotungstic acid-hematoxylin, and periodic acid-Schiff.

Fresh specimens were fixed in ice-cold 1.25% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4 for 2 h, post-fixed in osmium tetroxide for 1 h, then dehydrated and embedded in Epon 812. Sections, 1-μm-thick, were stained with toluidine blue, and ultrathin sections were then cut from selected areas, stained with uranyl acetate and lead citrate, and examined with a Hitachi HS-9 transmission electron microscope (Hitachi Co., Tokyo, Japan).

Detection of proliferating cells

The avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981) was used for detection of S-phase cells in the cell cycle. B5-fixed paraffin-embedded sections, 4-μm-thick, were used for immunohistochemical staining. The sections were treated with 2N HCl for 30 min at room temperature and then neutralized with 0.1M $Na_2B_4O_7$. After inhibition of endogenous peroxidase activity, the sections were incubated with mouse monoclonal antibody against BrdU (Becton Dickinson, Mountain View, CA) at 4 °C overnight. Subsequent staining procedures were performed by the usual ABC method (DAKOpatts, Glostrup, Denmark). After counterstaining with methyl green, the percentage of BrdU-positive cells among the 400 objective cells was obtained and referred to as the labeling index (LI).

Fluorocytometric analysis of DNA content

For the isolation of single cells from B5-fixed paraffin-embedded materials, several sections, 100-μm-thick, from the selected area were dewaxed in xylol and rehydrated in a graded ethanol series. The tissue fragments were incubated in culture bottles containing 10 ml of Ringer-Locke solution supplemented with 0.05% collagenase type IV (Sigma) for 60 min at 37 °C. The specimen was resuspended in 2% Na-EDTA (Sigma) in 0.2M phosphate buffer, pH 7.3, for 120 min at 37 °C. Further mechanical cell isolation was performed by an ultrasonicator (UR-20P: TOMY SEIKO Co., Tokyo, Japan) for 2 min at room temperature. The free cell suspension was smeared on non-fluorescent glass slides using an auto-smear (Cytospin: Shandon Co., Pittsburgh, PA), followed by DNA staining with 4',6-diamidino-2-phenylindole (DAPI) (Nakarai Chemical Co., Kyoto, Japan). The cell nuclear DNA content of 200 cells was measured in each specimen using a fluorescence cytophotometer (OSP-1: Olympus Co., Tokyo, Japan). We defined 2C as the mean value of 20 small lymphocytes.

Results

Development of tumors

Macroscopic and microscopic examinations of mice revealed tumors in the series of animals sacrificed 12 weeks after the first treatment with DMBA (Table 1). The tumors developed around the knee joint as intramuscular and subcutaneous lesions, most of which were unrelated to the joint cavity. Once the tumor became palpable, it grew rapidly and logarithmically. Tumor size varied, and the largest measured approximately 3 cm in maximum diameter. Animals merely observed and not killed subsequently died of massive tumor growth. In section, each tumor appeared pale yellow with a thin fibrous capsule. Large tumors occasionally contained necrotic foci. No tumors were

Table 1. Pathological diagnosis and cell kinetic findings on the animal model of MFH.

TIME KILLED (weeks)	TUMOR INCIDENCE	MOUSE no. EXAMINED	DIAGNOSIS	F/H RATIO[a]	L.I. (%)	PLOIDY[b]
8	0/3					
9	0/3					
10	0/3					
11	0/3					
12	1/3	1	MFH	2.9	19.9	A
13	0/3					
14	3/4	2	Granulation	2.3	7.1	D
		3	MFH	2.0	4.5	A
		4	MFH	1.4	4.5	A
15	2/4	5	Granulation	8.0	7.7	D
		6	MFH	2.1	7.1	A
16	2/5	7	MFH	-	-	-
		8	MFH	2.1	18.3	A
17	2/4	9	MFH	-	-	-
		10	MFH	-	-	-
18	1/3	11	MFH	6.5	8.9	A
19	2/4	12	MFH	6.4	17.0	A
		13	MFH	-	-	-
20	1/4	14	MFH	1.3	4.3	A
21	2/2	15	MFH	6.2	14.5	A
		16	MFH	5.6	9.4	A
22	2/2	17	MFH	7.0	12.0	A
		18	MFH	4.3	8.5	A

[a]F/H ratio: fibroblast-like cell/histiocyte-like cell. [b]Ploidy: A, aneuploid; D, diploid.

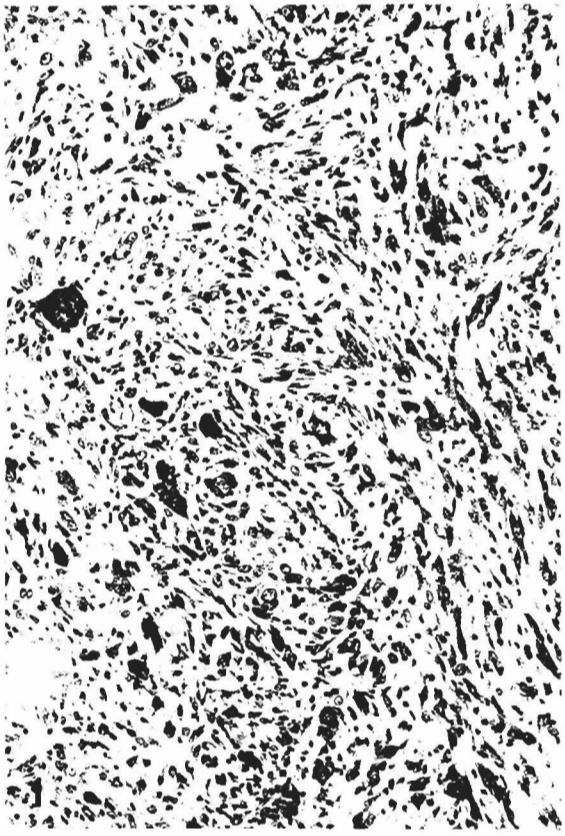


Fig. 1. Chemically induced tumor of a mouse, which morphologically resembles human pleomorphic-type MFH. H&E stain, x 160

Fig. 2. Tumor of a mouse is composed of fibroblast-like cells, histiocyte-like cells, multinucleated giant cells and foamy xanthomatous cells. H&E stain, x 500

observed in the control mice.

Serial transplantation

The cell line was transplantable and developed a tumor growing logarithmically. The tumor microscopically resembled the original tumor, but almost all cells were fibroblastic. Multinucleated giant cells and foamy xanthomatous cells were not observed. The cell line had been maintained for 40 passages.

Light microscopic findings

Light microscopy revealed that all the developed

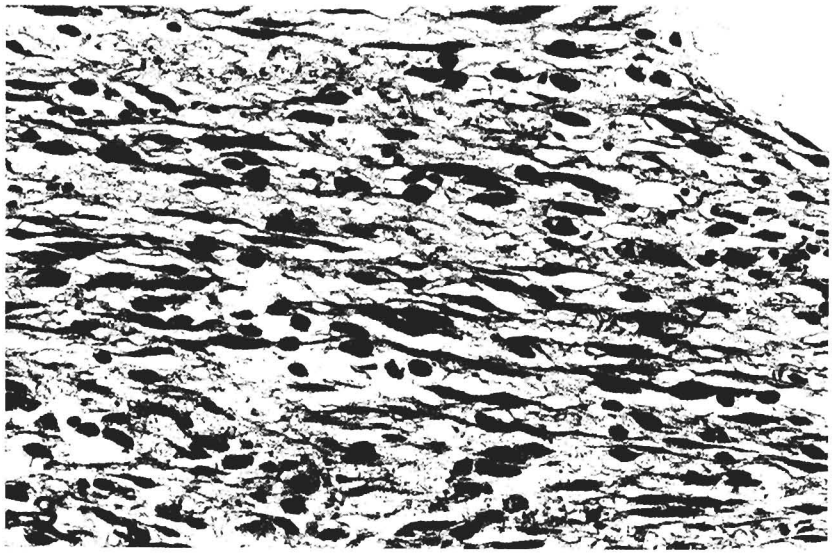
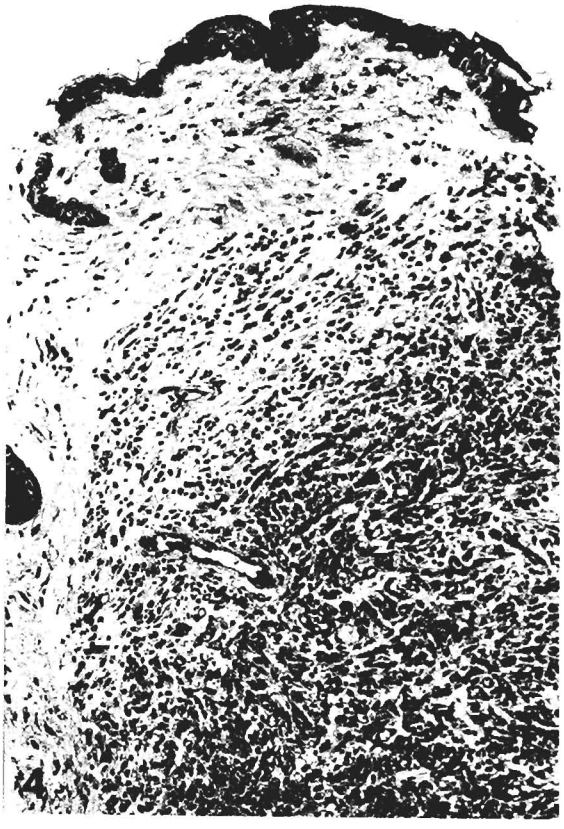


Fig. 3. In addition to the typical fibroblasts, metamorphosed fibroblasts are seen in the granulation tissue adjacent to the tumor. Short spindle-shaped cells and elliptical cells are categorized in "fibrohistiocytoid cell". H&E stain, x 320



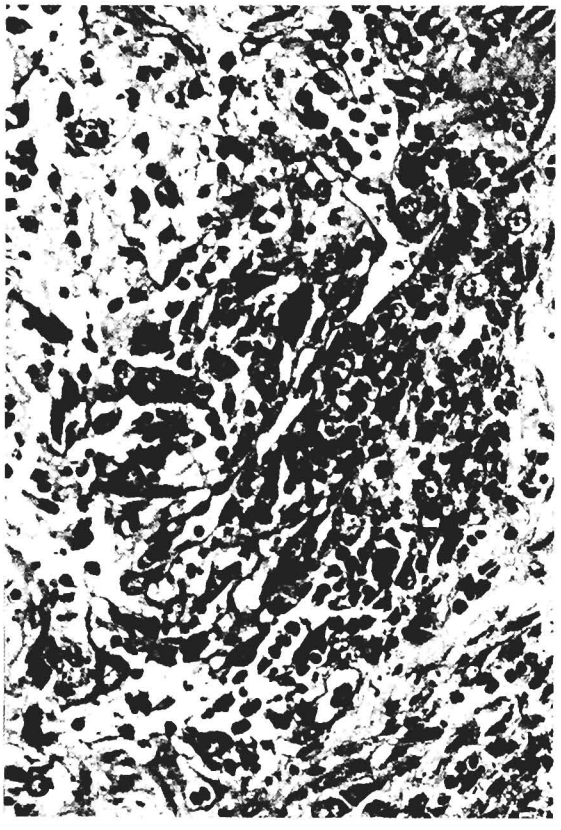


Fig. 4. The early-stage MFH as a very small lesion in the subcutaneous tissues. No invasive proliferation is observed. H&E stain, x 80

Fig. 5. High-power view of the very small tumor mass shown in Fig. 4. The tumor is composed of fibroblast-like cells and histiocyte-like cells, which resemble the tumor cells shown in Fig. 2, with infiltrating small lymphocytes. H&E stain, x 320

tumors were comprised of two major cell types, fibroblast-like cells and histiocyte-like cells. In addition, a few other cell types, i.e. multinucleated giant cells and foamy xanthomatous cells, were also observed (Figs. 1, 2) (Table 1). The fibroblast-like cells were spindle-shaped with an elliptical or irregularly shaped nucleus containing one or two large nucleoli. The storiform pattern, one of the pathognomonic features of MFH, was composed mainly of this cell type, and occasionally a herring-bone pattern was also evident. The histiocyte-like cells were larger, with polygonal nucleus surrounded by a vacuolated, drab, eosinophilic cytoplasm. By light microscopy, the proportions of the two major cell types in the tumor varied, but the fibroblast-like cells tended to be dominant. Multinucleated giant cells had two or three large nuclei with a thickened membrane and strongly eosinophilic cytoplasm. Foamy xanthomatous cells had a small, round nucleus without an obvious nucleolus. These findings were compatible with the pleomorphic/storiform type of human MFH (Enzinger and Weiss, 1995).

Macroscopically, most tumors had a thin fibrous capsule, but microscopic examination revealed that some tumors had partly infiltrated into the surrounding tissues. In the connective tissues adjacent to the tumor, FH cells (short spindle-shaped, or elliptical cells) were found in addition to the typical fibroblasts. Morphologically, the FH cells resembled fibroblast-like tumor cells (Fig. 3).

Some tumors were detectable only by microscopic examination as small foci. These foci were composed of

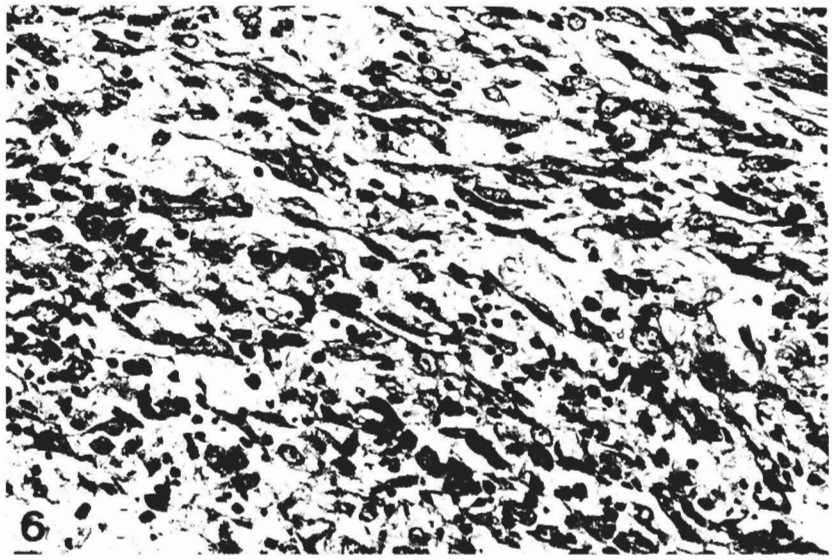


Fig. 6. Atypical fibroblasts are observed in granulation tissues. They have short spindle and elliptical morphology, but nuclear atypism and mitotic figures are not observed. H&E stain, x 320

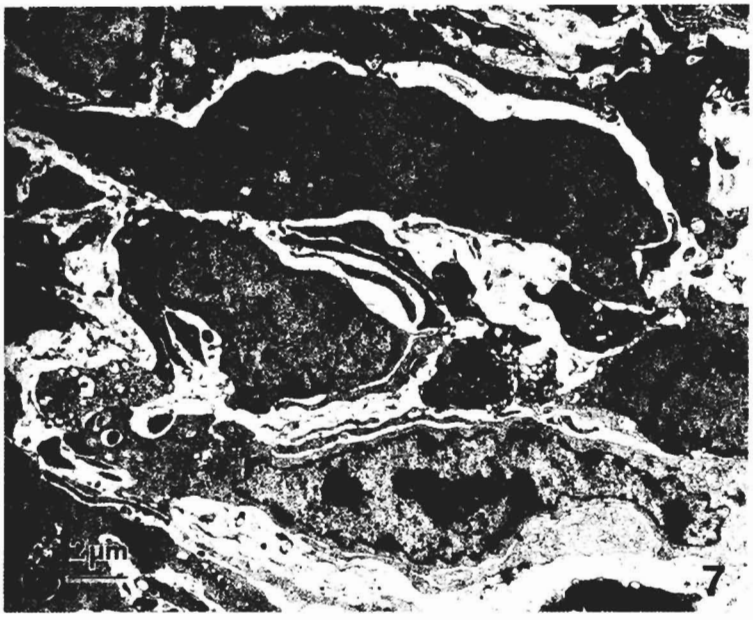
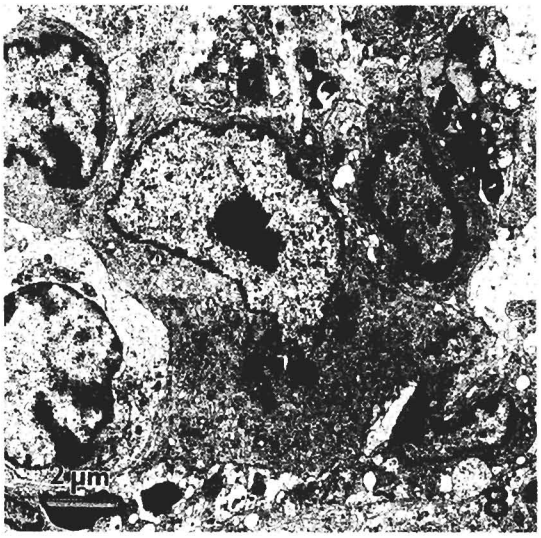


Fig. 7. Electron micrograph shows fibroblastic FH cells in MFH. They have a large elliptical shaped nucleus with prominent nucleolus. Note their well developed rough endoplasmic reticulum (rER). x 4,000

the same cell types as those of the developed tumors, and were assumed to be early-stage of the tumorigenesis (Figs. 4, 5). In the granulation tissues diagnosed by light microscopy, in addition to the typically elongated fibroblasts, several FH cells were observed with lymphocytic inflammatory cells (Fig. 6).

Electron microscopic findings

Electron microscopy revealed a clearly identifiable variety of cell types in the tumors, including fibroblast-like cells, histiocyte-like cells, small undifferentiated cells, xanthomatous cells and multinucleated giant cells. However, most of the cells composing the tumors were fibroblast-like cells and histiocyte-like cells. The fibroblast-like cells had a large, elliptical or somewhat irregularly shaped hyperchromatic nucleus with one or two prominent nucleoli. The nuclear envelope had complicated folds. The cytoplasm contained well developed, fragmented and often dilated rough endoplasmic reticulum (rER) (Fig. 7). The histiocyte-like cells had an oval or elliptical nucleus, one or two



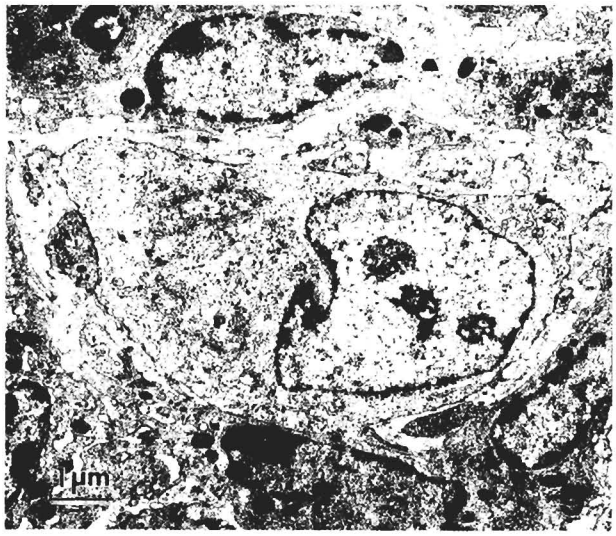
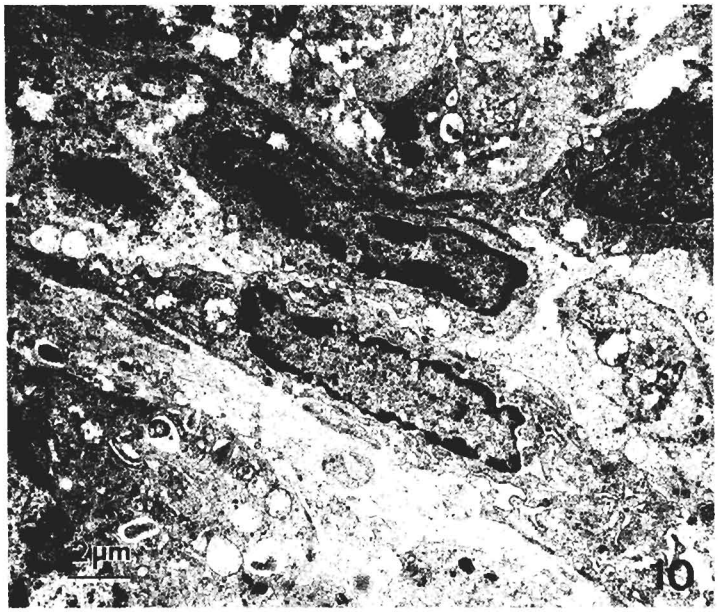


Fig. 8. A histiocytic FH cell in MFH has well developed, fragmented rER with free ribosomes and lipid droplets. No identifiable cytoplasmic projection or phagocytotic vacuoles are found. x 4,500

Fig. 9. An undifferentiated cell in MFH. It shows scanty cytoplasm and smooth cell surface. x 8,200



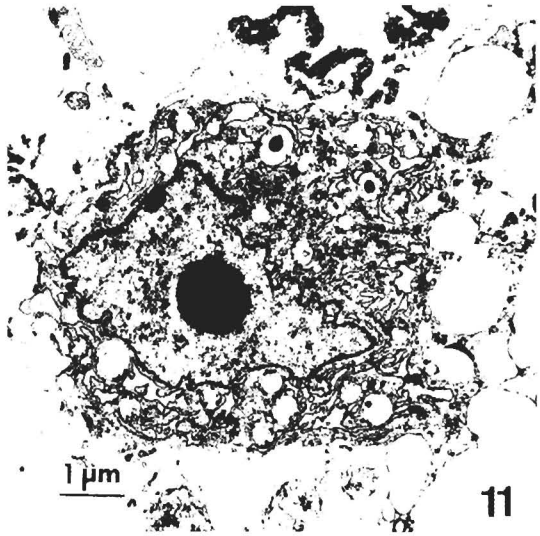


Fig. 10. Short, spindle-shaped cells in granulation tissue. x 4,000

Fig. 11. An elliptical cell in granulation tissue. x 8,200. Those cells shown in Figs.10 and 11 differ from malignant FH cells shown in Figs.7 and 8 in the amount of nuclear chromatin, nuclear atypism and so on.

small, and often large nucleoli, and a cytoplasm which contained fragmented, branching rER, many free ribosomes and a few dense bodies which resembled lysosomes. They had no typical characteristics of histiocytes, i.e. phagocytotic vacuoles and pseudopodial cell processes (Fig. 8). A few small undifferentiated cells were observed, with a smooth cytoplasmic membrane and few organelles (Fig. 9).

In the granulation tissue, the electron microscopic features of the long, spindle-shaped fibroblasts were similar to those of typical fibroblasts. In contrast, FH cells (the short, spindle-shaped cells and elliptical cells) resembled MFH cells in many aspects except that those in the granulation tissue displayed no atypical mitotic figures, and had hypochromatic nuclei with lower nucleus/cytoplasm ratios (Figs. 10, 11).

Comparison between the BrdU LI and cell types

The LI for MFHs in mice ranged from 4.3 to 19.9 (average, 10.7) (Table 1). Positive staining with anti-BrdU monoclonal antibody was shown in short spindle-shaped cells and elliptical cells, but multinucleated giant cells and foamy xanthomatous cells were stained negatively with the antibody (Fig. 12). The LI for FH cells in the granulation tissue was approximately 7.0, and typical elongated fibroblasts did not show immunoperoxidase localization of labeled BrdU.

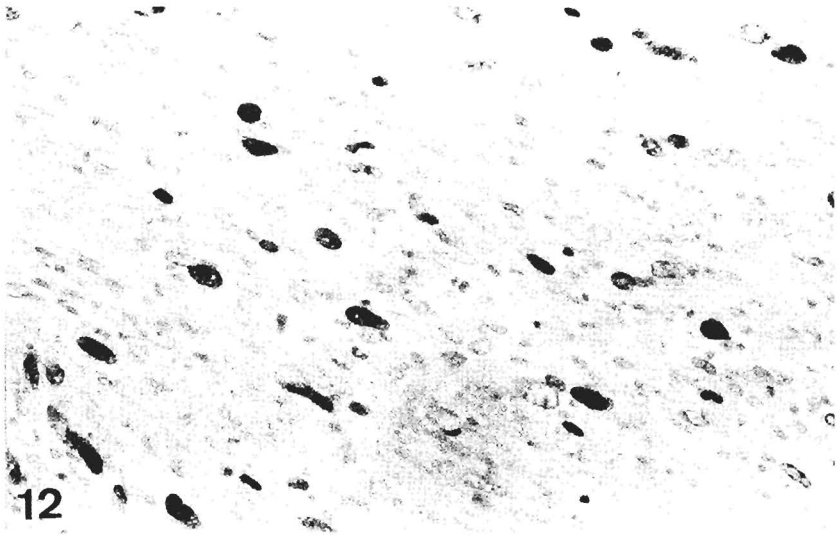


Fig. 12. Immunoperoxidase localization of labeled BrdU in MFH of a mouse. Positive stains are seen on the nuclei of short, spindle-shaped cells and elliptical cells. Multinucleated giant cells are stained negatively. Counterstained with methyl green, x 320

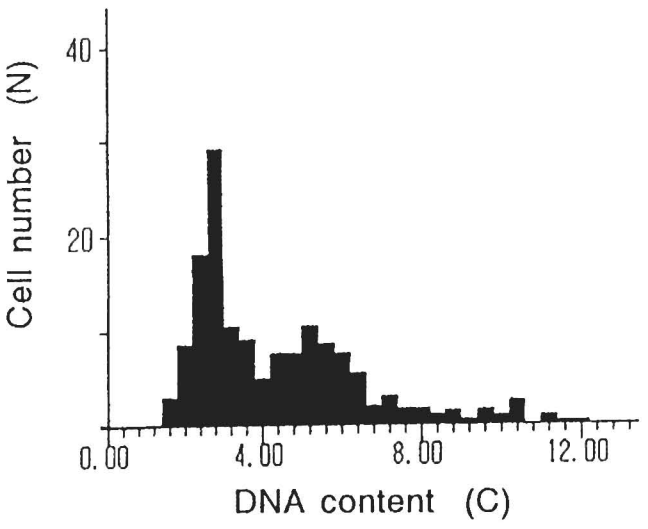


Fig. 13. DNA histogram of MFH of a mouse. Vertical axis is the number of cells and the horizontal one is for relative DNA contents. Note the abnormally positioned stem line peak corresponding to triploid cells and abundant hyperploid cell populations.

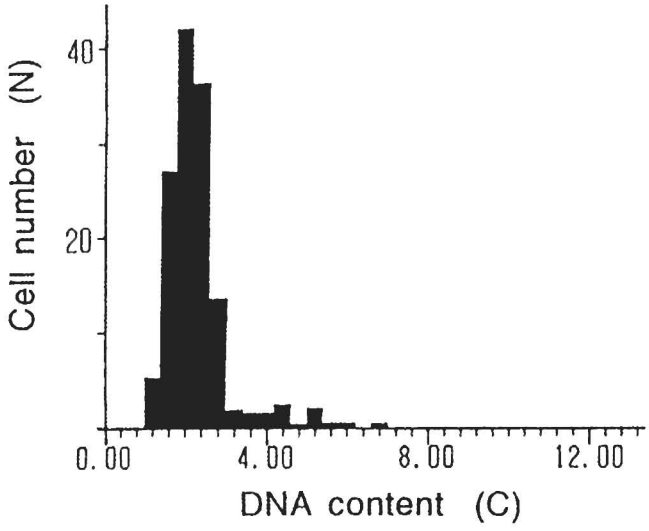


Fig. 14. Most of the cells in granulation tissue have 2C DNA content. There are several replicating cells and aneuploid cells which are not shown in the histogram of normal connective tissues.

Fluorocytometric analysis of DNA content

The DNA histogram patterns of MFH in mice varied, but all showed a non-diploid pattern (Table 1). Most of the patterns were aneuploid, defined by the presence of a G0/G1 peak (stem line) outside the diploid (2C) range (Fig. 13). The cells composing the granulation tissue were distributed in the diploid range with several cell populations located at S-phase (2C to 4C) and a few aneuploid cells (Fig. 14).

Discussion

The number of cases of MFH treated clinically at a single institute is not always sufficient for investigation of the histogenesis, and in recent years several experimental animal models of MFH have been established and made available for basic researches. Yumoto and Morimoto (1980), who were able to induce MFH in mice by injection of SV-40-transformed macrophages, suggested that the progenitor cell of MFH was histiocytic. Katenkamp and Neupert (1982) showed that MFH developed in nude mice after implantation of rat fibrosarcoma cell line and emphasized the relation between MFH and fibroblasts. Kato et al. (1990), using rat MFH induced by DMBA, revealed that infiltrating macrophages, which did not have neoplastic characteristics, contributed to the typical morphology of MFH.

The substance DMBA, one of the aromatic carbohydrates, is a potent chemical carcinogen and available for oncological researches. Sakamoto (1986) produced soft tissue MFH in the rat by intra-articular injection of DMBA and reported the development of storiform-pleomorphic type in 67.2%, myxoid type in 15.5% and giant cell type in 13.8%. In our experiment, which is the first report of DMBA-induced MFH in the mouse, all tumors were storiform-pleomorphic type. In the report of rat MFH of the bone induced by 4-HAQO, tumors were histologically divided into fibrous, giant cell, myxoid, and inflammatory type (Mii et al., 1982). It is uncertain that the variety of histological subtype depends on the kind of chemical carcinogens, animals, and organs (Schneider et al., 1999).

Recently, the role of electron microscopy in the diagnosis of soft tissue tumors has been re-evaluated (Woodruff, 1998). In this study, electron microscopic examinations were performed to clarify the morphological resemblance between FH cells and MFH cells. It was shown that the histiocytic cells in light microscopy actually had some characteristics of fibroblast in electron microscopy.

Some markers of cellular proliferation were reported in the normal and neoplastic tissues (Heatley, 1998). To compare the proliferation activity between typical fibroblasts, FH cells and MFH cells, we also accomplished cell kinetic analysis, using in vivo BrdU labeling technique and cytofluorometric measurement of DNA content.

BrdU, an analogue of thymidine, is taken up specifically by cells in the DNA synthetic phase (S-phase) of the cell cycle (Gratzner, 1982). BrdU immunostaining revealed that some FH cells in granulation tissues were able to synthesize DNA. In contrast, typical elongated fibroblasts of connective tissue in the control animals did not clearly show positive staining for BrdU. We considered that the difference between FH cells and typical fibroblasts was not only morphological but also biological. Fibroblast-like cells and histiocyte-like cells of the developed tumors were also positive for BrdU staining, but foamy xanthomatous cells and multinucleated giant cells had no reactivity with the antibody. This may suggest that xanthomatous cells and giant cells are reactive ones or already lack the potential for DNA synthesis.

With regard to soft tissue tumors, there are several reports on analysis of DNA content (Oshiro et al., 1995; Collin et al., 1997; Dunn and Everett, 1997). Although the criteria for analysis of DNA histograms vary, in general, benign lesions show a diploid pattern whereas malignant entities may be both diploid and hyperploid (including polyploid and aneuploid). All the DNA histograms obtained from our experimental tumors were categorized as aneuploid. Most of the cells composing granulation tissue were located in the diploid range with an increase of S-phase cells and a few hyperploid cells. It is accepted that some benign lesions contributed to the development of carcinomas, but in soft tissue tumors, malignant change of a benign tumor is thought to be uncommon, except for neurofibromatosis, which often develops into malignant schwannoma. It was difficult to define granulation tissue as a precancerous lesion of human MFH, but an experimental tumor induced by a chemical carcinogen might have such a preneoplastic lesion.

We consider that MFH has fibroblasts and benign FH cells as stromal cells in addition to the major neoplastic cells, namely malignant FH cells (including fibroblast-like and histiocyte-like cells). FH cells observed in granulation tissue had higher proliferation activity than typical fibroblasts, suggesting that the FH cells, and ultimately fibroblasts, may be the progenitor cells of MFH.

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