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Immunohistochemical, Quantitative Immunoelectron Microscopic, and DNA Cytofluorometric Characterization of Chemically Induced Rat Malignant Fibrous Histiocytoma

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Malignant fibrous histiocytoma (MFH) was induced in rats by injection of 9,10-dimethyl-1,2-benzanthracene. Using cell suspensions prepared from the heterotransplanted nude mouse tumor as immunogen, a monoclonal antibody, (MAb), MEP-1, against fibroblastlike MFH tumor cells was generated. In the primary rat tumors and transplanted rat or nude mouse tumors, MEP-1 reacted specifically with the fibroblastlike cells but not with the histiocytelike cells or xanthoma cells. Anti-rat macrophage MAbs RM-1 and TRPM-3 did not stain the fibroblastlike cells, but both were reactive with the histiocytelike cells. Double stainings with both MEP-1 and RM-1 or TRPM-3 did not detect any double positive cells. Immunoelectron microscopy using these MAbs showed that the fibroblastlike cells were the major cell component of the primary and transplanted rat tumors and that their cell membrane was stained positively with MEP-1, but not for RM-1 or TRPM-3. By the double staining method using a MAb against prolyl 4-hydroxylase β and MEP-1 or TRPM-3, this enzyme was demonstrated in MEP-1-positive cells but not in TRPM-3-positive cells. Results obtained by DNA cytofluorometry with 4,6-diamidino-2-phenylindole dihydrochloride staining or by the combined method of DNA cytofluorometry and indirect immunofluorescence, using MEP-1, RM-1, and TRPM-3, indicate that MEP-1-positive cells are neoplastic cells of rat MFH having proliferation activity. In the transplanted nude mouse tumors, no differentiation of MEP-1-positive rat tumor cells into histiocytelike cells was detected, and all histiocytelike cells were immunostained by F4/80 and most of them were positive for M5/114. These results suggest that fibroblastlike cells and intermediate cells are tumor cells of 9,10-dimethyl-1,2-benzanthracene-induced rat MFH showing differentiation toward fibroblasts and that histiocytelike cells are infiltrated macrophages. (Am J Pathol 1993, 143:431–445)

Malignant fibrous histiocytoma (MFH) is the most common soft part sarcoma of human adults[1] and is composed mainly of fibroblastlike cells and histiocytelike cells, variably intermixed with intermediate cells, bizarre giant cells, immature cells, xanthoma cells, and/or inflammatory cells.[2] These variable types of cells of MFH are arranged in a storiform pattern. The cell components have led to three theories of its histogenesis; 1) histiocytic cell origin,[3–7] 2) undifferentiated mesenchymal cell origin,[8–14] and 3) fibroblast or its related cell origin.[9,12,15] To elucidate the nature of the tumor cells, experimental MFH has been produced in rats,[16–18] mice,[19] and rabbits.[7] In rats, the experimental tumor is known to be produced by injection of various chemicals such as 9,10-dimethyl-1,2-benzanthracene (DMBA)[16,17] or methylcholanthrene.[18] In previous experimental studies, however, conflicting results concerning its histogenesis were obtained, which have led to differing proposals for human cases.[7,17,18]

In our previous study, we produced MFH in rats by intra-articular injections of DMBA and found that none of the cell lines established from the tumor reacted

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with anti-rat macrophage monoclonal antibodies (MAbs), possessed any Fc receptors, or conducted immune phagocytosis or Latex particle phagocytosis.[17] Inoculation of cloned tumor cells of the established cell lines into syngeneic rats and nude mice produced tumors with the histology of MFH and induced marked macrophage infiltration.[17] In our further approach by [^{3}H]thymidine flash labeling, we demonstrated that the histiocytelike cells in the transplanted nude mouse tumors were non-neoplastic and infiltrated macrophages derived from the bone marrow of the host animals.[17] More recently, we substantiated the production of monocyte chemotactic activity in tumor cell lines of human and rat MFH and the expression of messenger RNA of monocyte chemoattractant protein-1 in all of the tumor cell lines examined.[20,21] However, the nature of fibroblastlike cells in MFH remains unclear.

To elucidate the nature of tumor cells in DMBA-induced rat MFH, we generated an anti-rat MAb, MEP-1, against chemically induced rat MFH tumor cells. Besides MEP-1, we used three anti-murine macrophage MAbs to identify the histiocytelike cells and to examine the relationship between them and the fibroblastlike cells. By inoculating cloned rat MFH tumor cells into rats or nude mice, we produced transplanted tumors to examine whether or not differentiation of the tumor cells into histiocytelike cells occurs in the tumors.

Materials and Methods

Animals

Wistar white rats, athymic BALB/c nu/nu mice, and BALB/c mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan) and kept under routine laboratory conditions.

Production of Experimental Tumors

Specific pathogen-free 7-week-old Wistar white rats, weighing approximately 200 g, were injected in the right knee joint cavity with 0.1 ml of 2% paraffin solution of DMBA (Nakarai Chemicals, Kyoto, Japan) and injected once again 4 weeks later, as described previously.[16,17] The animals were sacrificed with ethyl ether anesthesia at 10 to 20 weeks after the initial injection, and their tumors were removed and examined.

Transplantation

MFR11, a tumor cell line established previously from a chemically induced rat MFH by single cell cloning,[17] was used. Subcutaneous inoculation of MFR11 (1×10^7 cells) was performed into the back of Wistar white rats and nude mice. The animals were sacrificed with ether anesthesia 7, 9, 11, and 14 days after inoculation; tumors were removed and examined.

Generation of Monoclonal Antibody

Tumor tissues were excised from transplanted nude mouse tumors and then mashed in phosphate-buffered saline (PBS) with a steel grater to make a free cell suspension. BALB/c mice were immunized three times by intraperitoneal injection of 1 ml of the tumor cell suspension (approximately 1×10^7 cells) at 2-week intervals. Fusion and selection of hybrids were performed as described previously.[22,23] Screening of the MAb-producing hybrids was performed by the two-step immunoperoxidase method on cryostat sections of the primary rat MFH tumor tissues and normal rat spleen. A hybrid producing an antibody reactive with MFH tumor cells but not reactive with splenic macrophages was selected, cloned twice by the limiting dilution procedure and designated as MEP-1. Ascites was obtained by injection of 5×10^6 MEP-1 cells into pristane-primed BALB/c mice. The isotype of MEP-1 was determined with a mouse MAb isotyping kit (Amersham Int., Amersham, UK).

Estimation of Molecular Weight

To estimate the molecular weight (MW) of MEP-1-recognized antigen, proteins were extracted from the primary rat MFH tumor tissues with an extraction buffer (5% NP40 in PBS, 2 mmol/L phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitory units of aprotinin), dissolved in sample buffer (0.5 mol/L Tris-HCl buffer 25 ml, 10% sodium dodecyl sulfate 4 ml, glycerin 2 ml, 2-mercaptoethanol 1 ml, 0.05% bronophenol blue 0.54 ml, and distilled water 10 ml) and boiled for 4 minutes. The proteins were run on an 8% sodium dodecyl sulfate-polyacrylamide gel and were blotted on an Immobilon PVDF transfer membrane (Millipore, Bedford, MA) as described previously.[24] After blocking with a 10% dried milk solution, the membranes were immunostained with MEP-1.

Tissue or Cell Preparation and Fixation

Tumor tissues were excised from the primary rat MFH tumors and the transplanted rat or nude mouse tumors. Some of the tumor tissues were cut by a knife, mashed with a grater, and filtrated through steel meshes to prepare tumor cell suspensions. After sacrificing the Wistar rats with anesthesia, tissues were obtained from the brain, lungs, liver, spleen, kidneys, intestines, thymus, lymph nodes, synovia, skin, and bone marrow. These tissues were fixed in 10% buffered neutral formalin or in a periodate-lysine-paraformaldehyde fixative.[25] Subepidermal and other mesenchymal tissues were excised from the rat fetuses at fetal day 14, 16, 18, and 20, and subcutaneous tissues from the knee joints of the rat neonates at 2 days after birth. To obtain a mononuclear cell fraction, peripheral blood was sampled by a cardiac puncture with a heparinized syringe and centrifuged for 10 minutes after the addition of a Hypercol pack. Peritoneal cells were collected by washing the peritoneal cavity with PBS, and alveolar cells were obtained by bronchioalveolar lavage. Their cytospin preparations or cloned tumor cells were fixed in acetone and dried in air.

Light and Electron Microscopy

Formalin-fixed tissue specimens were embedded in paraffin, cut into 3-μ-thick paraffin sections, and stained with hematoxylin and eosin for histological examination. For electron microscopy, fresh specimens were cut into small pieces, fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and processed as described previously.[17]

Monoclonal Antibodies

For immunohistochemistry and immunoelectron microscopy, two anti-rat macrophage MAbs, RM-1 and TRPM-3, an anti-mouse macrophage MAb, F4/80, and an anti-mouse MAb against Ia antigens, M5/114, were utilized. RM-1 and TRPM-3 were produced in our laboratory,[22,23] F4/80 was kindly supplied by Dr. Siamon Gordon, the Sir William Dunn School of Pathology, University of Oxford,[26] and M5/114 by Dr. G. Kraal, Department of Histology, Free University, Amsterdam.[27] MEP-1 generated above was used for detection of fibroblastlike cells of rat MFH. Anti-human prolyl 4-hydroxylase β MAb was purchased from Fuji Pharmaceutical Co. (Takaoka, Japan) and employed for immunohistochemistry to detect prolyl 4-hydroxylase β, as this MAb cross-reacts with rat enzyme.[28]

Immunohistochemistry and Immunoelectron Microscopy

For immunohistochemistry, periodate-lysine-paraformaldehyde-fixed tissue specimens were washed with PBS containing a graded series of sucrose (10%, 15%, and 20%), rinsed in PBS containing 20% sucrose and 10% glycerol for 60 minutes, and embedded in OCT compound (Miles, Elkhart, IN). They were frozen in dry ice-acetone, cut 6 μ thick by a cryostat, and dried in air. These sections were stained by the indirect immunoperoxidase method with MEP-1, RM-1, or TRPM-3 for rats and with biotinylated MEP-1, biotinylated RM-1, biotinylated TRPM-3, F4/80, or M5/114 for mice as described previously.[17,22] Cytospin preparations and cultured cells were stained in the same way as mentioned above.

For immunoelectron microscopy, immunostaining was performed as described previously.[22] After demonstration of the enzyme activity by 3,3′-diaminobenzidine tetrahydrochloride, tissue specimens were postfixed in 1% osmium tetroxide and embedded in epoxy resin. They were sectioned and observed in the same manner as described elsewhere.[17]

Immunohistochemical Double Staining

Double staining was performed according to the method described previously.[29] In brief, cryostat sections or tumor cell suspensions were treated by the indirect immunoperoxidase method using RM-1 or TRPM-3 and stained brown with 3,3′-diaminobenzidine tetrahydrochloride. After removing the first and second antibodies by incubating in 0.1 mol/L glycine-HCl buffer (pH 2.2), the same sections were stained with MEP-1 by the alkaline phosphatase anti-alkaline phosphatase method using a kit from DAKO Corporation (Carpinteria, CA). This reaction was stained blue in a substrate solution consisting of naphthol AS-MX phosphate, fast blue BB salt, and levamisole. To confirm the reaction specificities of each step, the first antibodies in the first or second step were omitted in control experiments. According to the same procedures, we performed double staining using MEP-1 or TRPM-3 and anti-prolyl 4-hydroxylase β MAb.

Cell Enumeration

On immunoelectron microscopy, cells positive for each MAb were photographed and counted according to the ultrastructural classification of the constituent cells in the primary or transplanted rat tumors and in the transplanted nude mouse tumors. Cells were judged positive when they showed a positive reaction on their entire cell surface; cells showing a positive reaction on only one side of their surface were excluded. Percentages of positive cells were calculated.

DNA Cytofluorometry

Isolated cell suspensions were prepared according to the method previously described,[30] with a minor modification. The cells isolated from the unfixed primary rat tumor tissues were smeared on micro slide glasses (Iwaki Co., Osaka, Japan) using Autosmear CF-12D (Sakura Seiki, Tokyo, Japan) at 800 rpm for 5 minutes. The smear slides were air-dried and fixed with 4% paraformaldehyde in PBS (pH 7.4) at 4 C. Then, these slides were rinsed with running water for one minute and with PBS three times for 3 minutes each time. The slides were incubated in 5% fetal calf serum in 0.05 mol/L Tris buffer containing 0.015 mol/L NaN_3 at room temperature for 10 minutes and then incubated with primary antibody (MEP-1, RM-1, or TRPM-3) for 90 minutes. After three washes with PBS, the slides were incubated with fluorescein isothiocyanate-conjugated rabbit immunoglobulins to mouse IgG (DAKO F232, Lot N. 088; DAKO, Denmark) in PBS as the secondary antibody for 60 minutes at 4 C. After rinsing in PBS at 4 C, the smears were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI)[31] diluted in 10 mmol/L Tris buffer (pH 7.4) containing 10 mmol/L ethylene diamine tetraacetic acid disodium salt-2Na, 100 mmol/L sodium chloride (NaCl), and 100 mmol/L 2-mercaptoethylamine for 2 hours. A few of the smear slides were stained with DAPI solution alone. These were mounted with the staining solution, covered by a glass slip, and sealed with nail polish. The fluorescent intensity of the cell nuclei was measured with an epi-illumination cytofluorometer, Nikon P1 (Nikon, Tokyo, Japan) equipped with two sets of filters: 1) a 365-nm interference excitation filter, 450-nm dichroic mirror, and 470-nm interference band pass filter for DAPI and 2) a 490-nm interference filter, 510-nm dichroic mirror, and 520-nm interference band pass filter for FITC. The lens was a 40× objective Nikon Fluor 40/1.30 oil (Nikon, Tokyo, Japan).

To determine the DNA ploidy pattern, we measured the nuclear DAPI fluorescence intensity of 300 to 400 cells. By observing the membranous fluorescence of FITC, these cells were discriminated into positive or negative cells for each monoclonal antibody, MEP-1, RM-1, or TRPM-3. For cell enumeration, multinuclear giant cells were excluded because it was difficult to measure their fluorescence intensity and compare it with uninuclear cells. As a control, the fluorescence intensity of 20 neutrophils included in the same smear specimens was measured to obtain the internal standard of diploid DNA content. The nuclear DNA content of the G_2/M stage was defined as 4C, twice the 2C control mean values. The term "aneuploidy" was used for cells showing a peak outside 2C, 4C, or 8C. The measured data were demonstrated as a histogram of DNA content of cell nuclei after calculation by an EPSON 286 personal computer (Seiko Co., Nagano, Japan) connected to the cytofluorometer.

Results

Isotype and Molecular Weight of MEP-1

The isotype of MEP-1 was IgG1 κ. MEP-1 immunostained a single protein band of 35 kd MW in comparison with the MW standard stained with Amido black (Figure 1).

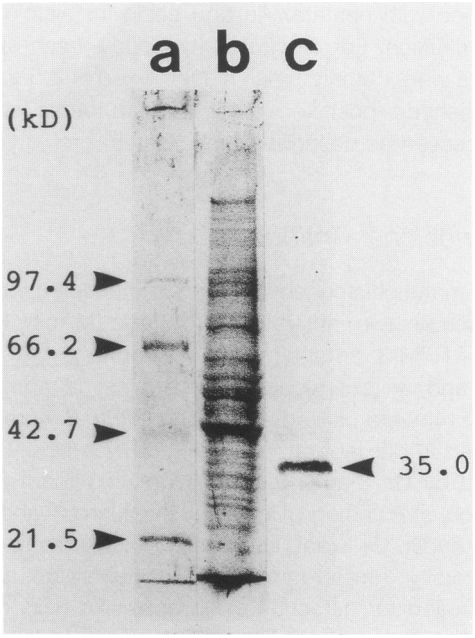

Figure 1. *Molecular weight of the antigen recognized by MEP-1.* **a**: *Molecular weight standard.* **b**: *Whole protein pattern of rat MFH.* **c**: *MEP-1 immunostaining.*

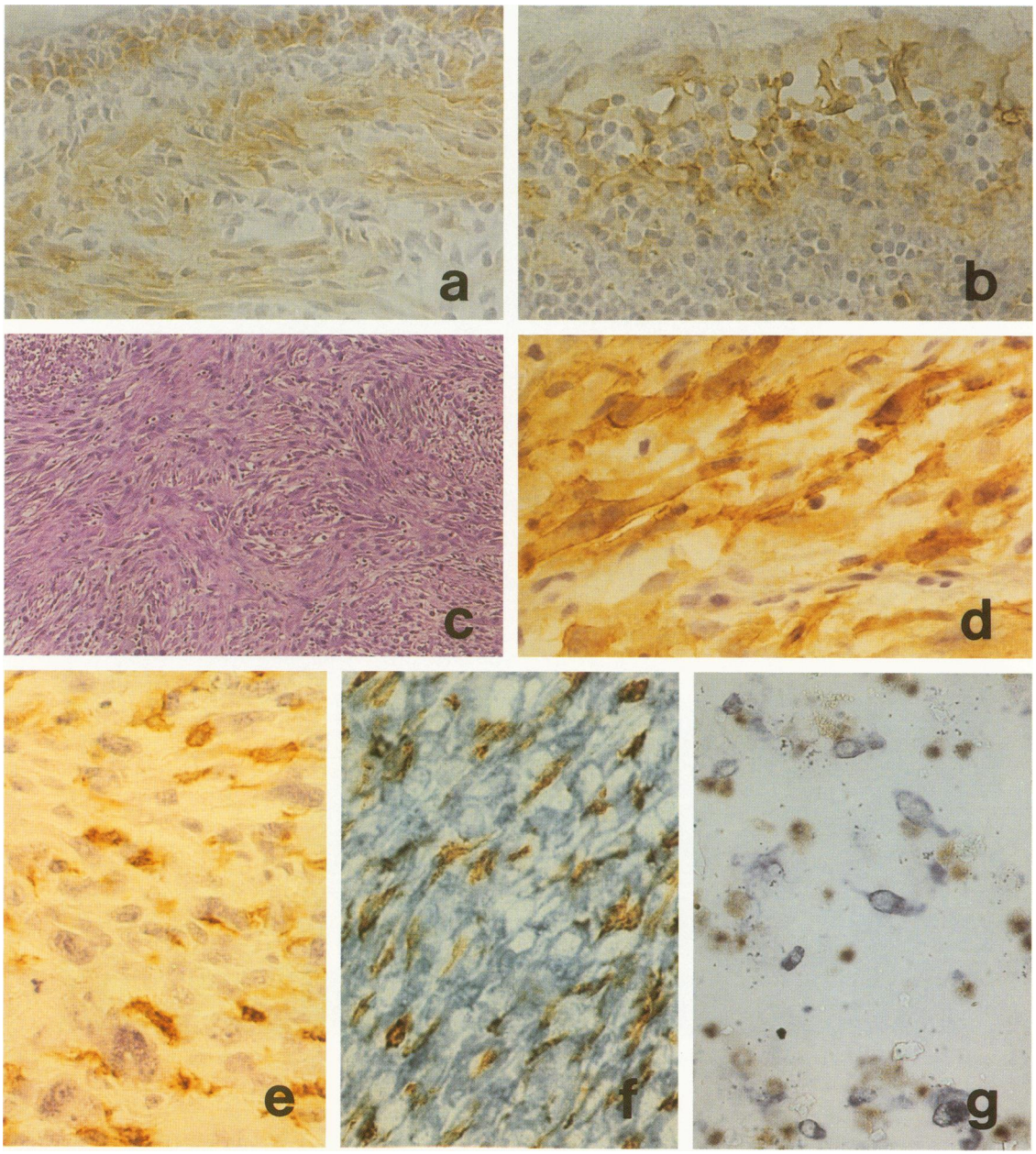

Figure 2. *Immunoreactivity of MEP-1* (a,b *and histology and immunohistochemical staining of chemically induced primary rat tumors* (c *to* g). a: *Mesenchymal cells or fibroblasts are reactive for MEP-1 in the fetal rat tissue* (*410×*). b: *Sinus lining cells and reticulum cells of the lymphatic sinuses are reactive in an adult rat lymph node* (*410×*). c: *The tumors show the pleomorphic-storiform pattern of MFH* (*hematoxylin and eosin stain, 120×*). d: *Large spindle fibroblastlike cells are immunostained with MEP-1, but histiocytelike cells are negative* (*410×*). e: *TRPM-3 immunostains the histiocytelike cells, but not the fibroblastlike cells* (*410×*). f: *By double staining of tissue sections using MEP-1 and TRPM-3, the large spindle cells are stained blue with MEP-1 and the polygonal cells are stained brown with TRPM-3* (*410×*). g: *In the tumor cell suspension, the double staining clearly demonstrates no doubly positive cells* (*320×*).

Immunoreactivity of MEP-1 in Normal Rat Tissues

In the mesenchymal tissues of rat fetuses, mesenchymal cells and fibroblasts were reactive to MEP-1 from fetal day 14 on (Figure 2a). In adult rats, MEP-1 was reactive with sinus lining cells and reticulum cells of the lymphatic sinuses in the lymph nodes (Figure 2b), with mesothelial cells of body cavities, with endothelial cells of the lymphatic vessels in the dermis and Glisson's sheath of the liver, with the innermost periosteal cells or osteoblasts, and with some epithelial cells such as alveolar epithelia, renal glomerular epithelia, and epidermal basal cells. However, MEP-1 did not show any reactivity to fibrocytes in the connective tissues,

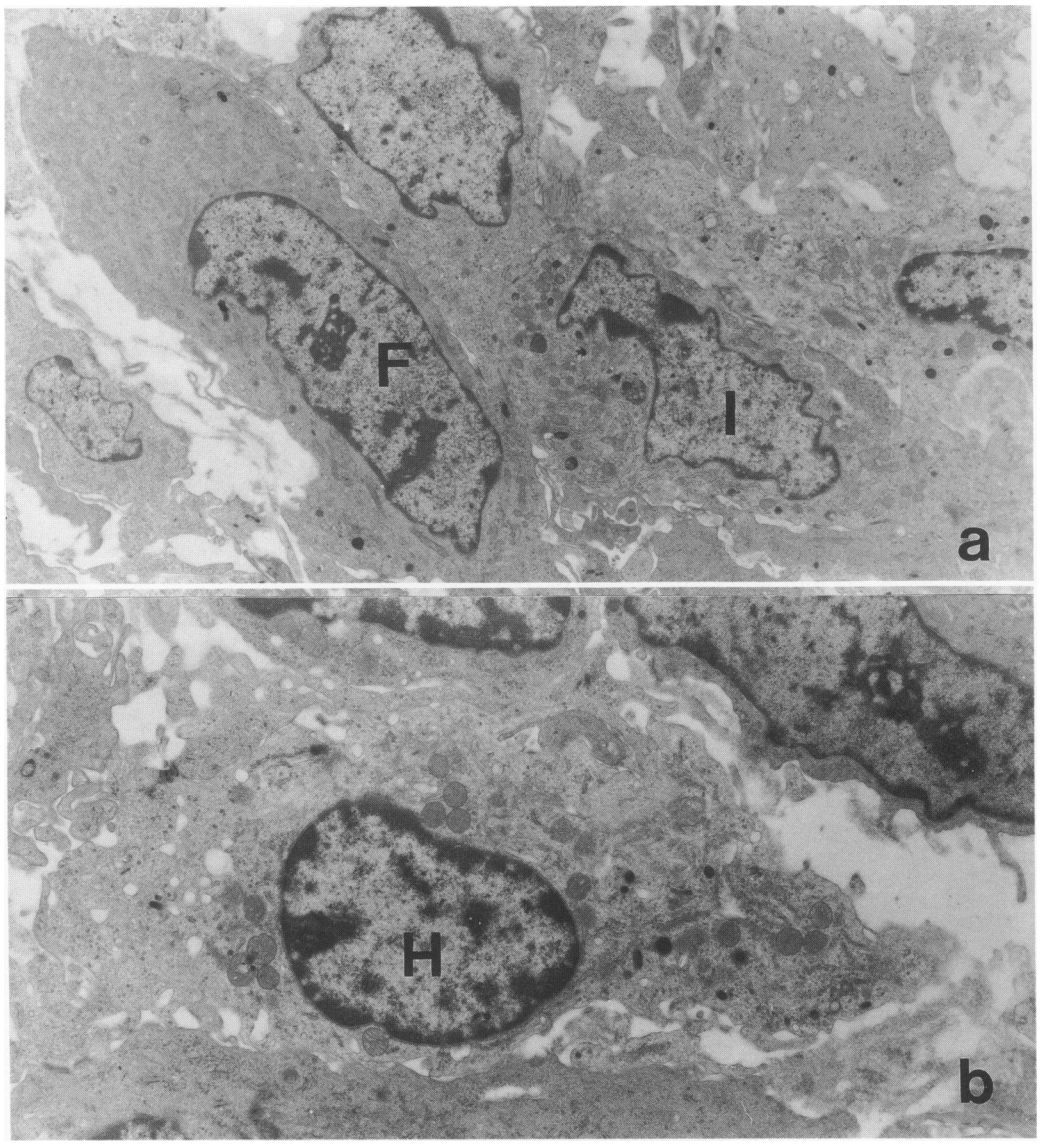

Figure 3. *Ultrastructure of the primary rat tumors.* a: *Fibroblastlike cell (F) and intermediate cell (I) (4,000×).* b: *Histiocytelike cell (H) (7,700×).*

macrophages residing in tissues and body cavities, monocytes, lymphocytes, granulocytes in peripheral blood, or parenchymal and neuronal cells.

Cellular Components of Chemically Induced Rat Malignant Fibrous Histiocytoma and Their Characterization by Immunohistochemistry Using Various MAbs

Consistent with the results of our previous study,[17] primary tumors developed around the knee joints from 16 weeks on. These tumors showed a histology typical of MFH consisting of fibroblastlike cells and histiocytelike cells as major cell constituents, intermixed with bizarre giant cells, immature round cells, xanthoma cells, and inflammatory cells (Figure 2c). On immunohistochemistry, MEP-1 immunostained the fibroblastlike cells (Figure 2d) and the cloned tumor cells but not the histiocytelike cells. In contrast, anti-rat macrophage MAbs, RM-1 and TRPM-3, reacted to the histiocytelike cells but not to the fibroblastlike cells (Figure 2e) or the cloned tumor cells. Upon double staining using TRPM-3 and MEP-1, the fibroblastlike cells stained with MEP-1, whereas the histiocytelike cells and xanthoma cells reacted with TRPM-3 (Figure 2f). Similar findings were obtained by double staining with MEP-1 and anti-rat macrophage MAb, RM-1, instead of

TRPM-3. In the tumor cell suspensions, the double staining demonstrated no doubly positive cells (Figure 2g).

On electron microscopy, six cell types were distinguished in the tumors: fibroblastlike cells, histiocytelike cells, intermediate cells, undifferentiated cells, xanthoma cells, and multi- or uninuclear giant cells. Fibroblastlike cells possessed an elongated or spindle nucleus and had a well-developed rough endoplasmic reticulum (Figure 3a). Their cell surface was smooth and often attached to collagen fibers. Histiocytelike cells had a round or indented, heterochromatic nucleus, developed lysosomal granules, endocytic vesicles, or phagocytic vacuoles, and projected many cytoplasmic processes (Figure 3b). Intermediate cells showed ultramicroscopic features similar to those of the fibroblastlike cells, and they also developed lysosomal granules, vesicles, or vacuoles (Figure 3a). Immature round cells observed light microscopically had a large euchromatic nucleus with prominent nucleoli, scant cytoplasm, and poor development of intracellular organelles except for abundant polyribosomes. Table 1 shows percentages of constituent cells of the primary rat tumors.

Immunoelectron microscopically, RM-1 and TRPM-3 were localized on the cell surface of the histiocytelike cells (Figure 4a) and xanthoma cells, whereas reaction products for MEP-1 were detected on the surface membrane of the fibroblastlike cells and intermediate cells (Figure 4b). The numbers and percentages of positive cells for each MAb are summarized in Table 2. Almost all the fibroblastlike cells and intermediate cells were positive for MEP-1, but none reacted with RM-1 or TRPM-3. Almost all the histiocytelike cells and all the xanthoma cells reacted to RM-1 and TRPM-3. Most immature undifferentiated cells reacted to MEP-1 (Figure 4c), but some that did not were positive for either RM-1 or TRPM-3. Judging from their ultrastructural features, the former seemed to be undifferentiated mesenchymal cells; the latter, immature macrophages. As for multinuclear giant cells, half were immunoreactive with MEP-1 (Figure 4d), the others with RM-1. In the MEP-1-positive multinuclear giant cells, the rough endoplasmic reticulum developed markedly, occasionally accompanied by many small vesicles (Figure 4d). By contrast, the RM-1-positive giant cells possessed numerous lysosomal granules, endocytic vesicles, and some phagocytic vacuoles, and their cell-surface projections were prominent. These results suggest that some multinuclear giant cells are related to fibroblastlike cells and their related cell types whereas the others belong to a macrophage subpopulation.

Table 1. *Quantitative Electron Microscopic Analysis of the Cells in Primary Rat MFH*

Cell type	Number (%)
Fibroblastlike cells	95 (45.2)
Intermediate cells	18 (8.6)
Histiocytelike cells	77 (36.7)
Undifferentiated cells	10 (4.8)
Xanthomatous cells	3 (1.4)
Multinucleated giant cells	7 (3.3)
Total	210 (100)

To examine collagen production of the constituent cells in rat MFH, double staining with MEP-1 or TRPM-3 and anti-prolyl 4-hydroxylase β MAb was performed: MEP-1-positive cells showed a positive reaction for prolyl 4-hydroxylase β (Figure 5a) demonstrating their ability to produce collagen, but all TRPM-3-positive cells were negative for this enzyme (Figure 5b).

Differentiation of Cloned Tumor Cells Transplanted into Nude Mice and Syngeneic Rats

About 2 weeks after subcutaneous inoculation of the cloned tumor cells into nude mice, tumors developed *in situ* and showed histology typical of the pleomorphic storiform type of MFH. In these transplanted tumors, we observed light and electron microscopically all the cell types seen in the original tumors. Immunohistochemically, almost all the fibroblastlike cells, immature round cells, and bizarre giant cells exhibited a positive reaction for biotinylated MEP-1 (Figure 5c) but not for F4/80 (Figure 5d), biotinylated RM-1, or biotinylated TRPM-3 (Figure 5e). Among tumor cells, numerous F4/80-positive cells were demonstrated (Figure 5d), most of which showed Ia expression (Figure 5f). Within a week after inoculation, F4/80-positive cells were found in the periphery of the developing tumors, and thereafter distributed throughout the tumors. Immunoelectron microscopically, we classified biotinylated MEP-1- or F4/80-positive cells into the above mentioned six cell types, counted the number of positive cells, and calculated their percentages (Table 3). The results were that most of the fibroblastlike cells, intermediate cells, undifferentiated cells, and multinuclear giant cells showed immunoreactivity with biotinylated MEP-1 on the cell-surface membrane, but all these cell types were negative for F4/80 and did not show the ultrastructural features of macrophages. In contrast, F4/80-positive cells were mature macrophages. These

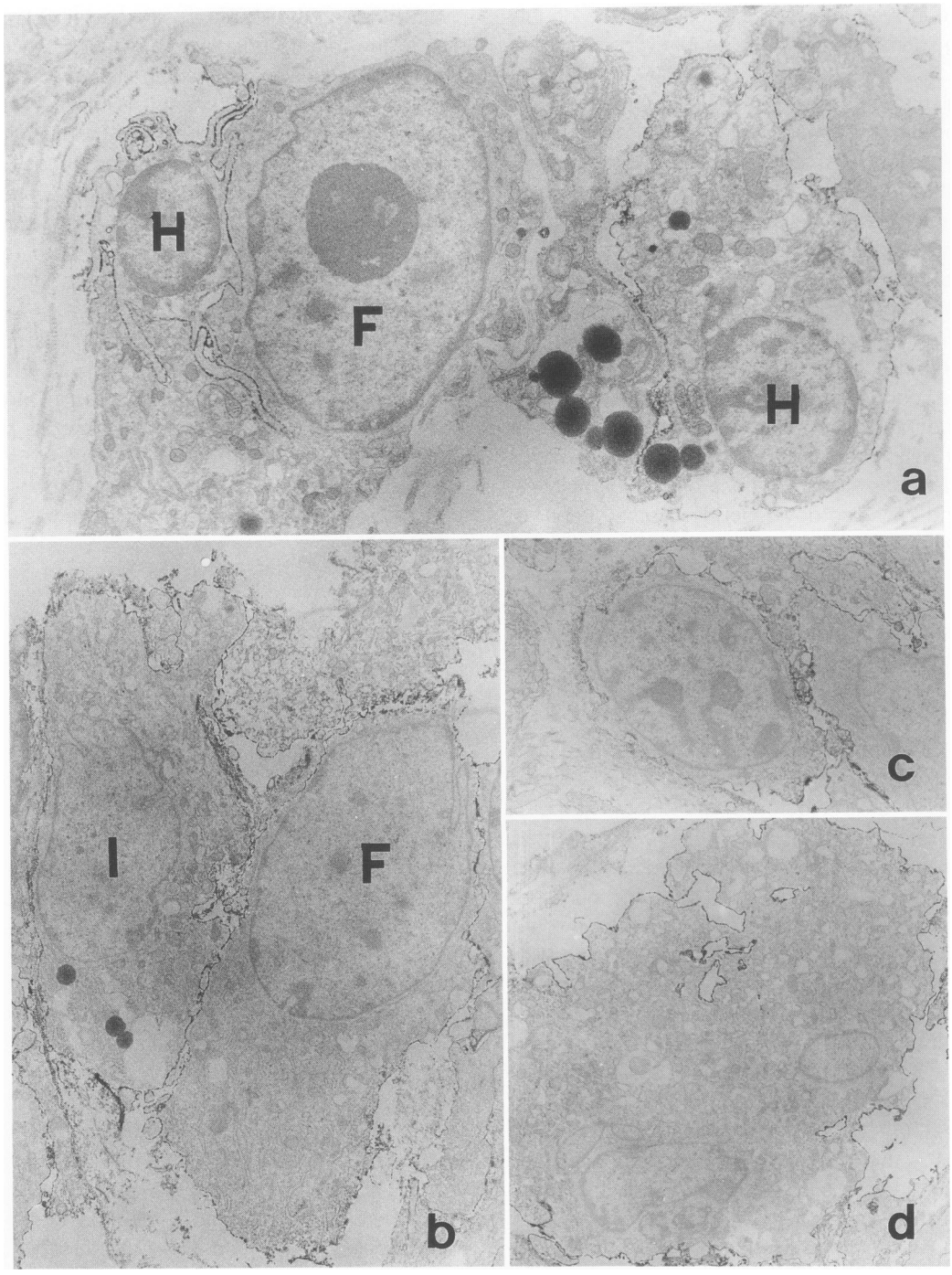

Figure 4. *Immunoelectron microscopy of the primary rat tumors.* a: *Positive reaction products for RM-1 are demonstrated on the cell membrane of the histiocytelike cells (H) but not on the fibroblastlike cells (F) (5,400×).* b: *Positive reaction products for MEP-1 are demonstrated on the cell membrane of fibroblastlike cell (F) and an intermediate cell (I) (3,700×).* c: *An immature undifferentiated cell shows a positive reaction for MEP-1 (3,600×).* d: *A MEP-1-positive giant cell (3,000×).*

results indicate that tumor cells transplanted into the nude mouse show no differentiation into histiocytelike cells and that the histiocytelike cells are infiltrating reactive macrophages of host origin.

Two weeks after subcutaneous inoculation of the cloned tumor cells into Wistar white rats, tumors histologically similar to the primary tumors developed. In the transplanted rat tumors, we examined differentiation of the cloned tumor cells both immunohistochemically and immunoelectron microscopically. Transplanted tumor cells reacting to MEP-1 were large and pleomorphic (Figure 5g). On the other

Table 2. *Quantitative Immunoelectron Microscopic Analysis of Positive Cells for Monoclonal Antibodies in the Primary Rat Tumors*

Cell type	Number (%)		
	MEP-1	RM-1	TRPM-3
Fibroblastlike cells	416 (76.4)	0 (0)	0 (0)
Intermediate cells	82 (15.0)	0 (0)	0 (0)
Histiocytelike cells	0 (0)	159 (93.0)	107 (94.6)
Undifferentiated cells	41 (7.5)	7 (4.1)	3 (2.7)
Xanthomatous cells	0 (0)	2 (1.2)	3 (2.7)
Multinucleated giant cells	6 (1.1)	3 (1.7)	0 (0)
Total	545 (100)	171 (100)	113 (100)

hand, the MEP-1-negative cells in the tumors were small and mostly round (Figure 5g), corresponding to RM-1- or TRPM-3-positive cells (Figure 5h). Double staining with MEP-1 and RM-1 or TRPM-3 did not demonstrate any doubly positive cells; all large and pleomorphic tumor cells stained blue with MEP-1, and round or polygonal cells stained brown with RM-1 or TRPM-3 (Figure 5i). In the early stage of transplanted tumors, RM-1- or TRPM-3-positive cells were found in the periphery, but they distributed diffusely in the tumors with the advance of tumor development. Thus, RM-1- or TRPM-3-positive cells were regarded as macrophages infiltrating from outside the transplanted tumors. Two weeks after transplantation, MEP-1-positive cells exhibited the ultrastructural features of the fibroblastlike cells, intermediate cells, undifferentiated cells, and giant cells, but not those of the histiocytelike cells, which were negative for MEP-1.

DNA Cytofluorometric Analyses

In smear preparations stained with DAPI,[23] the cells could be divided into two cell types: large atypical cells with large nuclei and prominent nucleoli and small round cells with small nuclei showing smooth nuclear margins (Figure 6a). The DNA histograms of these two cell types are shown in Figure 7. The mode of DNA content of the large atypical cells was 4.5C (range: 3C to 5C) with polyploid cells (Figure 7 I-a). Whereas cells with a 4.5C ploidy class are considered to be in the G_0/G_1 phase, cells with a 9C ploidy class (12.9%) are thought to be in the G_2/M phase. Some large atypical cells (10.0%) had an intermediate nuclear DNA content between the 5C and 9C class; these were judged to be S-phase fraction. In other words, the large atypical cells showed aneuploidy with polyploidy over the 8C class. In contrast, the mode of DNA content of small round cells was diploid without any S-phase fraction or 4C class (Figure 7 I-b).

With the combined method of DNA cytofluorometry and the indirect immunofluorescence staining using monoclonal antibodies MEP-1, RM-1, and TRPM-3, the mode of DNA content of the MEP-1-positive, RM-1-negative, and TRPM-3-negative cells was shown to be 4.6C (range: 4C to 5C) containing aneuploid and polyploid cells (Figure 7 II-a, III-b, IV-b). The proportion of G_2/M cells (in the peak around 9C) and S-phase cells (between 5C and 9C) to MEP-1-positive cells was 24.4% and 21.5%, respectively. Large atypical cells positive for MEP-1 (Figure 6b) and negative for RM-1 or TRPM-3 were considered to be fibroblastlike cells and showed a higher proliferative activity than the MEP-1-negative, RM-1-positive, or TRPM-3-positive cells. Most MEP-1-negative cells showed 2C ploidy classes with a few tetraploid cells and S-phase cells (6%) (Figure 7 II-b), though RM-1-positive cells (Figure 6c) and TRPM-3-positive cells revealed 2C ploidy classes without tetraploid or S-phase cells (Figure 7 III-a, IV-a).

Discussion

As mentioned briefly in the introduction, divergent views have been presented on the histogenesis of MFH. Based on the results obtained in tissue cultures of human MFH, Stout and his associates speculated that tumor cells derive from histiocytes that may act as facultative fibroblasts capable of producing collagen fibers.[3,4] According to this theory, tumor cells of MFH are considered to share the cytological features of both histiocytes (macrophages) and fibroblasts and to show transition between them. Against this view, Fu et al proposed their monistic idea that the tumor originates from undifferentiated mesenchymal cells showing a dual differentiation toward fibroblasts and histiocytes (macrophages).[10] The presence of cells judged as intermediate at the ultrastructural level has been considered as evidence to support this theory. Against these views, other investigators maintained that histiocytelike cells are not neoplastic and that true MFH tumor cells are fibroblastlike cells and their related cells.[17] To make clear whether such transitional or intermediate cells are virtually present in chemically induced rat MFH, we generated a MAb against rat MFH tumor cells and designated it MEP-1. By immunohistochemical and immunoelectron microscopic analysis, MEP-1 reacted with fibroblastlike cells, but not with histiocytelike cells. In addition, it reacts with cloned tumor cells and with immature fibroblasts or mesenchymal cells in the fetal or neonatal rat tissues, but not with fibrocytes in

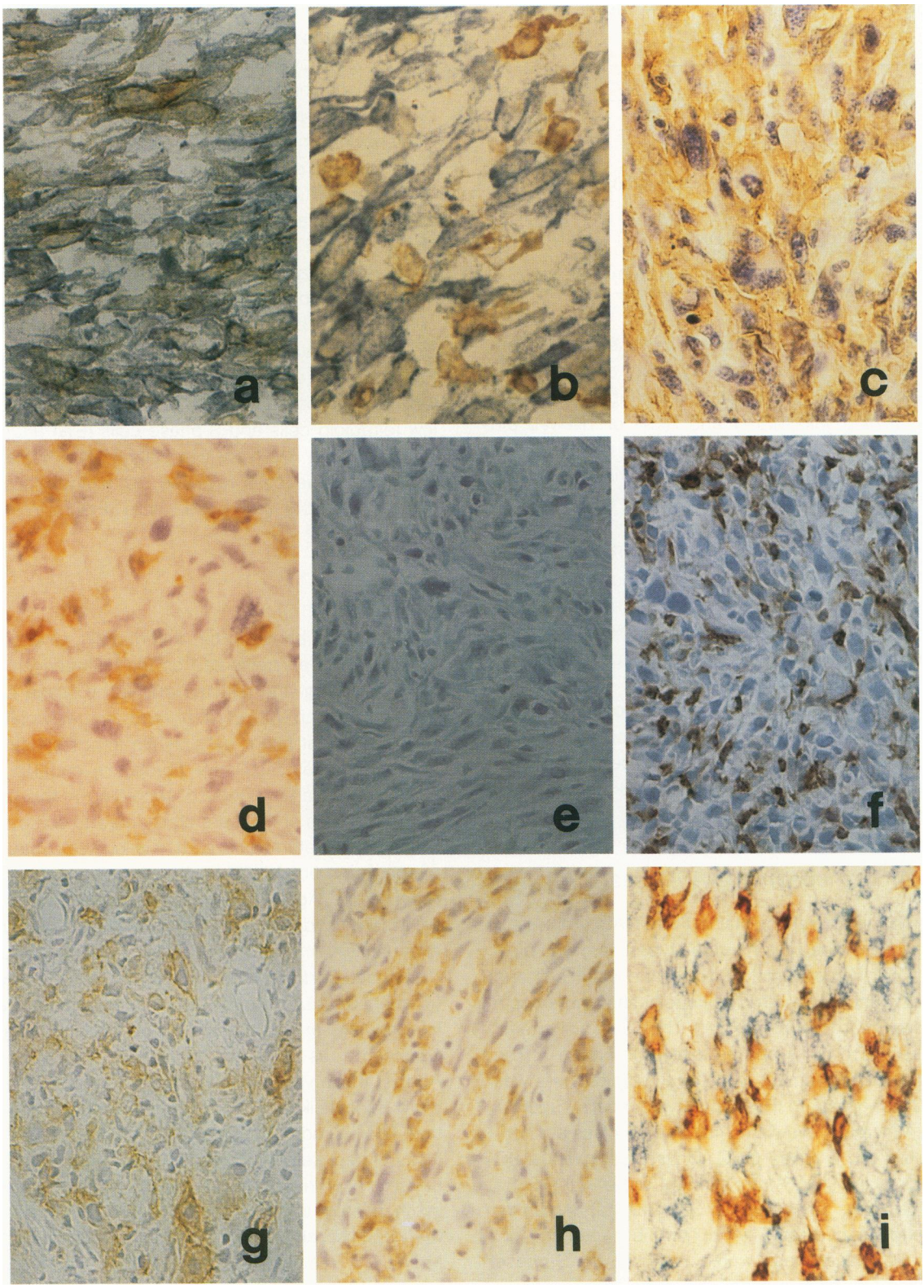

Figure 5. *Immunohistochemical double staining of primary rat tumors using MEP-1 or TRPM-3 and anti-prolyl 4-hydroxylase β (a,b) and immunohistochemical staining of transplanted nude mouse tumors (c to f) and syngeneically transplanted rat tumors (g to i).* a: *Large spindle cells are stained blue with anti-prolyl 4-hydroxylase β and brown with MEP-1 (410×).* b: *TRPM-3 positive cells stained brown are not stained with anti-prolyl 4-hydroxylase β (540×).* c: *Transplanted tumor cells show a positive reaction for biotinylated MEP-1 (410×).* d: *Between the rat tumor cells, numerous F4/80-positive cells are seen (410×).* e: *No positive cells for biotinylated TRPM-3 are detectable in the tumor (320×).* f: *Between the rat tumor cells, numerous M5/114-positive cells are infiltrated (320×).* g: *Large fibroblastlike tumor cells show a positive reaction with MEP-1, whereas MEP-1 negative cells are small and round or polygonal (410×).* h: *Small round or polygonal cells are positive for TRPM-3, but large spindle cells are negative (340×).* i: *By double staining using MEP-1 and TRPM-3, large spindle cells stain blue with MEP-1 and polygonal cells brown with TRPM-3 (410×).*

Table 3. *Quantitative Immunoelectron Microscopic Analysis of Positive Cells for Biotinylated MEP-1 and F4/80 in the Transplanted Nude Mouse Tumors*

	Number (%)	
	Biotinylated MEP-1	F4/80
Fibroblastlike cells	130 (68.0)	0 (0)
Intermediate cells	33 (17.3)	0 (0)
Histiocytelike cells	0 (0)	102 (100)
Undifferentiated cells	24 (12.6)	0 (0)
Xanthomatous cells	0 (0)	0 (0)
Multinucleated giant cells	4 (2.1)	0 (0)
Total	191 (100)	102 (100)

the normal connective tissue of adult rats. The recognition site of MEP-1 was demonstrated by immunoelectron microscopy to be the cell membrane of the immunoreactive cells. This MAb was shown to recognize an epitope of an antigen of 35 kd MW present on the cell membrane of fibroblastlike tumor cells of rat MFH. Although several MAbs have been produced against human MFH,[13,32–35] MEP-1 is the first MAb generated for rat MFH.

Consistent with several authentic reports on human MFH[8,11,12] and animal models,[16,17] we also found six cell types comprising rat MFH: fibroblastlike cells, histiocytelike cells, intermediate cells, undifferentiated cells, giant cells, and xanthoma cells. Compared to those in human MFH cases,[11] percentages of these cell types in the primary rat tumor were mostly similar. By immunoelectron microscopy using MEP-1, we found that all the fibroblastlike cells and intermediate cells were positive for MEP-1 but that all the histiocytelike cells and xanthoma cells were negative. In contrast, the fibroblastlike cells and intermediate cells were not reactive for anti-rat macrophage MAbs, RM-1 and TRPM-3, whereas the histiocytelike cells and xanthoma cells stained positively with both MAbs. By immunohistochemical double staining, we could not detect any double positive cells in not only tissue sections but also tumor cell suspensions. These findings indicate that the fibroblastlike cells are clearly distinguished from the histiocytelike cells and xanthoma cells and that the intermediate cells belong to the family of fibroblastlike cells. Although the intermediate cells develop endocytic vesicles or vacuoles to a certain extent, almost all of them have a well-developed rough endoplasmic reticulum typical of fibroblastlike cells. These data suggest that the intermediate cells are a form of fibroblastlike cells.

As for the other cell types, immature or undifferentiated cells were divided into two groups: those

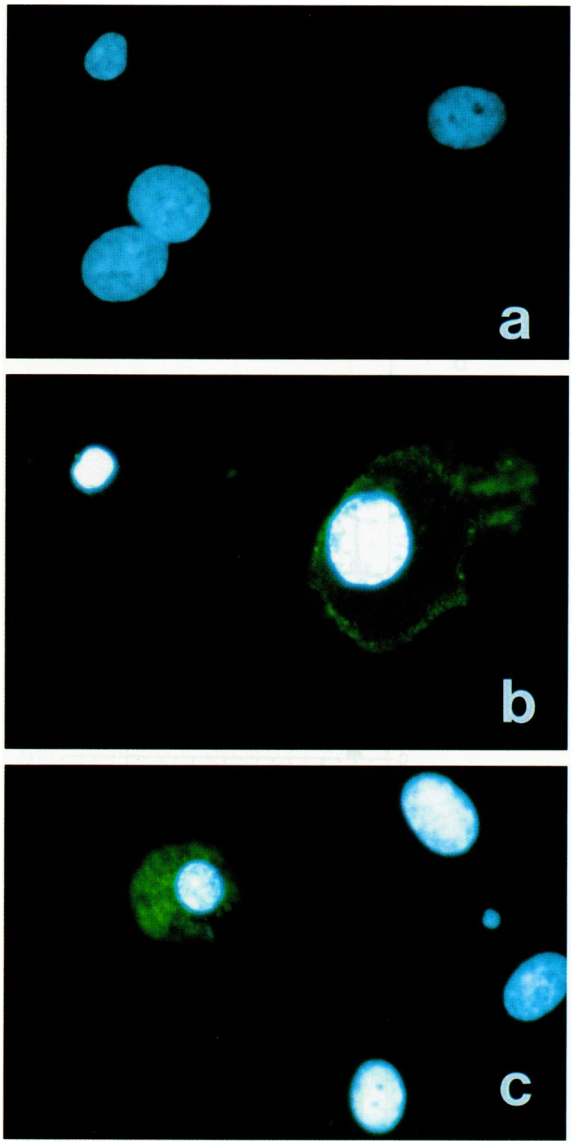

Figure 6. *DAPI staining with or without FITC immunofluorescence using MEP-1 or RM-1* (**a** *to* **c**). **a:** *Large atypical cells with large nucleus (lower and right) and small round cells (upper) are found in DAPI staining (600×).* **b:** *By the combined DAPI and FITC immunofluorescence method using MEP-1, the MEP-1-positive large atypical cell (right) shows green fluorescence, whereas the small cell (left) does not (600×).* **c:** *Using RM-1, small cells emit green fluorescence but large atypical cells with large nuclei do not (600×).*

positive for MEP-1 and those that stained with RM-1 or TRPM-3. Among these cells, also, no double positive cells were detected. MEP-1-positive immature cells are considered to be undifferentiated mesenchymal cells or immature fibroblastlike cells, whereas most RM-1- or TRPM-3-positive immature cells are small and round, suggesting that they are immature macrophages known to exist in ontogeny[36,37] and adult life.[38] As for giant cells, half were reactive for MEP-1 and markedly developed a rough endoplasmic reticulum, suggesting that they

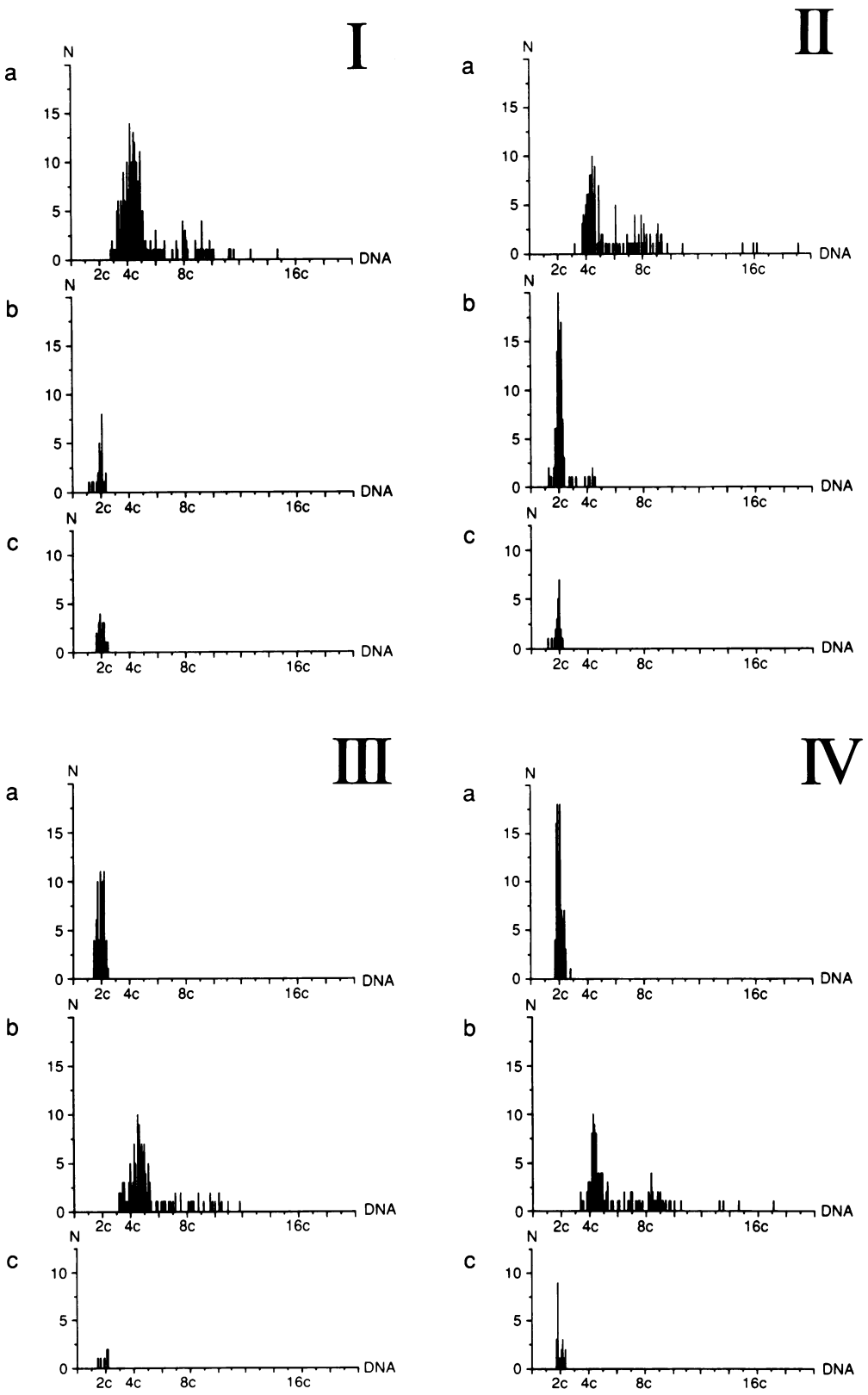

Figure 7. *DNA cytofluorometric histograms of various types of cell in the primary rat tumors.* I-a: *Large atypical cells with large nuclei.* I-b: *Small round cells.* I-c: *Neutrophils.* II-a: *MEP-1-positive cells.* II-b: *MEP-1-negative cells.* II-c: *Neutrophils.* III-a: *RM-1-positive cells.* III-b: *RM-1-negative cells.* III-c: *Neutrophils.* IV-a: *TRPM-3-positive cells.* IV-b: *TRPM-3-negative cells.* IV-c: *Neutrophils.*

are related to the fibroblastlike cells. In contrast, RM-1-positive giant cells possessed numerous lysosomal granules, endocytic vesicles, and some phagocytic vacuoles, and projected numerous cytoplasmic processes. These data suggest that RM-1-positive giant cells are derived from macrophages. In this manner, the constituent cells of rat MFH may be classified into two groups: MEP-1-positive cells and RM-1- or TRPM-3-positive cells. The former includes the fibroblastlike cells, intermediate cells, bizarre giant cells, and immature mesenchymal cells, whereas the latter comprises histiocytelike cells (macrophages), xanthoma cells, giant cells, and round immature macrophages.

In previous *in vitro* studies of human MFH or animal models, transformation of histiocytelike cells into fibroblastlike cells or dual differentiation of undifferentiated mesenchymal cells toward fibroblastlike cells and histiocytelike cells were reported.[3–14] However, it remains unclear whether these phenomena occur within the tumor. To elucidate this problem, we examined the differentiation of cloned rat MFH tumor cells in transplanted rat and nude mouse tumors both immunohistochemically and immunoelectron microscopically. In adult rats and nude mice, tumors showing the typical histology of MFH developed at about 2 weeks after subcutaneous inoculation of cloned rat tumor cells, and MEP-1-positive cloned tumor cells showed the ultrastructural features of fibroblastlike cells, intermediate cells, immature mesenchymal cells, or bizarre giant cells. However, the transplanted rat tumor cells showed no differentiation into histiocytelike cells or xanthoma cells and no immunoreactivity to RM-1 or TRPM-3 in the syngeneic rat or nude mouse tumors. In the nude mouse tumors, transplanted rat tumor cells also exhibited immunoreactivity to MEP-1 and the ultrastructural features of fibroblastlike cells or intermediate cells. However, the histiocytelike cells reacted with anti-mouse macrophage MAb F4/80 or anti-mouse MAb against Ia antigens M5/114 but not with biotinylated RM-1 or TRPM-3. In addition, their distribution pattern into the tumors provides evidence that the histiocytelike cells are macrophages of host origin infiltrating from outside the tumors, as we maintained previously.[17] The similar results were observed in our preliminary experiment of human MFH heterotransplanted into nude mice.[39]

Other investigators of tumor cells in MFH proposed a fibroblast cell family origin[9,12,15] or a perivascular mesenchymal cell origin,[13] or they considered them a specific cell type (fibrohistiocytoid cells).[40] Based on our data, MEP-1 does not react with fibrocytes in the normal connective tissue of adult rats. However, it does show immunoreactivity to mesenchymal cells or fibroblasts in rat fetuses and neonates. In adult rats, although MEP-1 recognizes epitopes present on the cell surface membrane of various epithelial cells, it also reacts with certain mesenchymal cells related to fibroblasts, including mesothelial cells, periosteal or osteoblastic cells, reticulum cells or sinus lining cells of the lymph nodes, and endothelial cells of the lymphatic vessels. The present study revealed that MEP-1-positive cells were also positive for the MAb against prolyl 4-hydroxylase-β, which catalyzes the 4-hydroxylation of proline residues in procollagen, whereas all of the TRPM-3-positive cells were negative. This indicates that MFH tumor cells have the ability to produce collagen.

Because abnormalities in the chromosomal structure and cellular DNA content are well-recognized characteristics of human soft tissue malignancies and because aneuploidy in human MFH is closely correlated with malignancy of the tumor,[41] we used DNA cytofluorometry to test for proliferative activity of the constituent cells of the chemically induced rat MFH. Because of difficulty in comparing the fluorescence intensity of bizarre (mostly multinuclear) giant cells and uninuclear cells, we excluded giant cells from the present DNA cytofluorometric analysis. The constituent cells could be divided into large atypical cells and small round cells. The large atypical cells showed aneuploidy with polyploidy of more than 8C class, whereas the mode of DNA content in the small round cells was diploid without S-phase fractions and 4C class. Employing the combined DNA cytofluorometry and indirect immunofluorescence method using MEP-1, RM-1, and TRPM-3, we examined the immunoreactivity of the large atypical cells to these MAbs. The results demonstrated that the large atypical cells are MEP-1-positive (fibroblastlike) cells showing a high proliferative activity. In contrast, most RM-1- or TRPM-3-positive cells showed 2C ploidy class but no tetraploidy or S-phase fractions, indicating that these cells are macrophages having no proliferative potential. Although most MEP-1-negative cells showed a 2C ploidy class, there were also a few tetraploid and S-phase cells. The MEP-1-negative cells with intermediate or tetraploid classes may be macrophages reactively proliferating *in situ* or MEP-1-false negative fibroblastlike tumor cells. However, they are a very minor population.

From the data presented, it seems reasonable that MEP-1-positive cells are neoplastic cells includ-

ing a variety of mesenchymal cells differentiating toward fibroblasts, whereas RM-1- or TRPM-3-positive cells are macrophages infiltrated from the outside of the tumor.

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