Heterogeneity in the Origin and Immunophenotypes of "Histiocytic" Cells in Transplantable Rat Malignant Fibrous Histiocytoma

Jyoji YAMATE, Masanori TAJIMA¹⁾, Kazumoto SHIBUYA¹⁾, Mitsuru KUWAMURA, Takao KOTANI, Sadashige SAKUMA, and Motohiro TAKEYA²⁾

Department of Veterinary Pathology, College of Agriculture, Osaka Prefecture University, Gakuencho 1-1, Sakai, Osaka 593, ¹⁾Nippon Institute for Biological Science, Shinmachi 2221-1, Ome, Tokyo 198, and ²⁾Second Department of Pathology, Kumamoto University School of Medicine, Kumamoto 860, Japan

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ABSTRACT. To clarify the origin and nature of "histiocytic" cells in malignant fibrous histiocytoma (MFH), immunoreactivities to rat macrophage/histiocyte-specific monoclonal antibodies (ED1 and ED2) and monocyte chemoattractant protein-1 (MCP-1) production were investigated using rat transplantable MFH-derived cloned cells (undifferentiated MT-8 and fibrohistiocytic MT-9). In different cultures, the positive rates for ED1 and ED2 ranged from 2.5% to 26.0% in MT-8, and from 6.0% to 40.0% in MT-9. In homotransplants, ED1-positive cells were frequently observed in MT-9 tumors, but barely seen in MT-8 tumors. In both MT-8 and MT-9 tumors, ED2-positive cells were not detected within tumors. Immunophenotypic characteristics of MT-8 and MT-9 to these antibodies seemed to be easily altered depending on *in vitro* and *in vivo* conditions. By ELISA, MCP-1 was hardly detected in culture supernatants of MT-8 and MT-9, but its level was very high in sera of MT-8- and MT-9-tumor-bearing rats. In rats with these tumors, the number of circulating monocytes was significantly increased and the presence of factors stimulating macrophage proliferation was demonstrated by the colony formation assay using rat bone marrow cells. Histiocytic cells in MT-8 and MT-9 tumors that were produced in nude mice reacted only with antibody to mouse macrophage-associated antigen. This suggests the existence of non-neoplastic, infiltrated macrophages of host origin that were induced probably by MCP-1 and factors stimulating macrophage proliferation. The present studies indicate the presence of heterogeneities in the origin and immunophenotypes of "histiocytic" cells in rat MFH. — **KEY WORDS:** heterogeneity, histiocytic cell, immunophenotype, monocyte chemoattractant protein-1, rat MFH cell.

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Malignant fibrous histiocytoma (MFH) is a soft tissue sarcoma consisting of various cell types such as fibroblastic, histiocytic, giant, xanthomatous and undifferentiated cells [5, 17, 22]. The histogenesis of MFH is still debatable, although fibroblasts [11, 15, 21], histiocytes [3, 19, 20] or undifferentiated cells [4, 7, 10, 16, 17] have been proposed as a possible progenitor. Recently, it has been reported that histiocytic cells in MFH might be fibroblastic cells acting as facultative histiocytes [11, 13, 15, 21] or non-neoplastic cells induced by monocyte chemoattractants produced by MFH cells [15, 21]. Thus, a concept has been drawn that MFH is not a tumor derived from cells belonging to the mononuclear phagocyte system (MPS) [11, 13, 16, 21]. On the other hand, it has been demonstrated that human MFH-constituting cells reacted with macrophage-related antibodies [3, 20]. Hence it appears that so-called "histiocytic" cells in MFH may be heterogeneous in their nature and origin [13].

Two rat MFH-derived cloned cell lines (named MT-8 and MT-9), which had been established by the present authors, showed characters differing from each other and seemed to be useful experimental models for studying the histogenesis of MFH [25–27]. To clarify the nature of "histiocytic" cells, the authors examined immunoreactivities of these cells to rat macrophage/histiocyte-specific antibodies (ED1 and ED2) *in vitro* and in homo- and heterotransplants. Monocyte chemoattractant protein-1 (MCP-1) has been considered to be a possible factor causing macrophage influx into tumor tissues [21, 23]. Therefore,

productions of MCP-1 and other bioactive molecules by these cloned cells and macrophage kinetics in rats with MT-8 and MT-9 tumors were also examined to pursue a possible existence of infiltrating macrophages.

MATERIALS AND METHODS

MT-8 and MT-9 cells: MT-8 and MT-9 cells are regarded as undifferentiated mesenchymal cells and fibrohistiocytic cells having both fibroblastic and histiocytic characters, respectively [25, 26]. The derivation of these cells from parent cells (MT-P) and their features have been described [26, 27]. The cloned cells have been passaged in a humidified 5% CO₂ incubator at 37°C by the methods described [26]. The growth medium used was Dulbecco's minimum essential medium (MEM; GIBCO) supplemented with 10% fetal bovine serum, streptomycin (100 µg/ml) and penicillin (100 U/ml).

***In vitro* observations:** Cells grown in chambers of tissue culture slides (LAB-TEK, IL, U.S.A.) were fixed in cold acetone and stained immunocytochemically with monoclonal antibodies (ED1 and ED2). ED1 (Chemicon International Inc., CA, U.S.A.) recognizes a cytoplasmic antigen present in all types of macrophages, and ED2 (Serotech, Tokyo) recognizes antigens correlated with different stages of differentiation of macrophages/histiocytes in the rat [2].

The indirect immunoperoxidase stainings were performed as described [24–26]; after treatment with 3% H₂O₂ for 15 min, the fixed cells were incubated for 14 hr at 4°C with

400-fold-diluted ED1 or ED2. Subsequently, the cells were incubated for 1.5 hr at 37°C with a secondary antibody of 1,000-fold-diluted peroxidase-conjugated affinity purified goat anti-mouse IgG Fc fragment antibody (Jackson Immunoresearch Laboratories (JIL), PA, U.S.A.). Diaminobenzidine tetrahydrochloride (DAB) was used as a substrate and the cells were counterstained with hematoxylin. The immunocytochemical double staining with ED1 and an antibody to α -smooth muscle actin (SMA; DAKO, Tokyo) was also performed, because MT-8 and MT-9 cells showed myofibroblastic immunophenotypes [25]. After incubation with ED1 and visualization with DAB as described above, the sections were again reacted for SMA in the same manner.

ED1-, ED2- and SMA-positive cells were counted at a magnification of $\times 400$ in 200 cells of five different cultures and expressed as a percentage.

In vivo observations: One $\times 10^6$ cells of MT-8 or MT-9 were inoculated subcutaneously into 10 male F344/DuCrj rats (103–128 g, 6-week-old; Charles River Japan). Five male BALB/c nude mice (21–29 g, 7-week-old; Laboratory Animal Research Station, Yamanashi) received subcutaneous inoculations of MT-8 or MT-9 cells.

At 4 and 6 weeks after inoculation, five rats each were euthanatized after collecting blood samples under ether anesthesia. Nude mice with tumors were euthanatized 5 weeks after inoculation. Removed tumors were weighed, and fixed in 10% neutral buffered formalin and in B5 fixative only for ED2 staining [28]. They were embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE). The indirect immunoperoxidase technique was applied to deparaffinized and 0.1% trypsin-treated sections for ED1 and ED2 in the same manner as described above. For detecting nuclear-incorporation of bromodeoxyuridine (BrdU; 100 mg/kg body weight, intraperitoneal injection one hr prior to autopsy) in ED1-positive cells, the double immunohistochemistry was carried out [28]; after incubation with ED1 and visualization with DAB to yield a brown reaction product, the sections were treated with 4 N HCl at 37°C for 20 min; subsequently, they were reacted with anti-BrdU monoclonal antibody diluted 1:100 at 4°C for 14 hr and then with the secondary antibody. BrdU incorporated into nuclei was visualized black with nickel-modified DAB (Cosmobio, Tokyo).

To evaluate proliferative activity, BrdU-incorporated cells or cells with mitotic figures were counted in 1,000 cells reacting to ED1. Transplantable histiocytic sarcoma (HS) established from a spontaneous HS in an F344 rat by the present authors (in preparation) was also stained by the double immunohistochemistry for ED1 and BrdU to compare proliferative activities between MFH and HS cells. Rodent HS is a distinct tumor entity consisting of a uniform population of histiocytic cells [8].

Frozen sections from tumors produced in nude mice were fixed in cold acetone and reacted with mouse macrophage-specific monoclonal antibody (1:50) (Mac1; Sera-Lab, Tokyo) by the indirect immunoperoxidase methods using

peroxidase-conjugated goat anti-rat IgG (1:20) (JIL) as a secondary antibody.

Frozen sections from rat and mouse tumors were stained for lysosomal enzymes such as acid phosphatase (ACP; Gomori's method, pH 5.0) and non-specific esterase (NSE; the alpha-naphthyl acetate method, pH 7.0) by the methods described [24, 27].

Quantitation of rat MCP-1 by sandwich enzyme-linked immunosorbent assay (ELISA): This assay is a modification of the human MCP-1 ELISA; the detailed method has been described [23]. Samples examined were culture supernatants of MT-8 and MT-9 cells; at confluency, cells were washed twice with phosphate-buffered saline (PBS) and serum-free medium was added to the cultures. After 24-hr incubation, the supernatants were collected. Serum-free medium in the absence of cells was used as controls. Sera, which were collected at 6 postinoculation weeks from rats bearing MT-8 and MT-9 tumors or from age-matched control rats, were also measured for MCP-1.

Kinetics of ED-1-positive macrophages in rats with MT-8 and MT-9 tumors: Blood samples were collected from the abdominal aorta of rats with MT-8 and MT-9 tumors and age-matched control rats as described above. Differential leukocyte counts were calculated by observing 200 white blood cells (WBC) on May-Grünwald Giemsa-stained smears. The liver and spleen from these rats were fixed in 10% neutral buffered formalin, and their sections were stained with ED1 by the indirect immunoperoxidase method. The appearance of ED1-positive cells was assessed semiquantitatively using a scale of $\pm$ to 4+ (see footnotes to Table 1) [17, 23, 26].

Colony formation of rat bone marrow (BM) cells in semisolid agar: Sera used were collected from rats with MT-8 and MT-9 tumors at 6 postinoculation weeks or age-matched control rats. The serum samples were sterilized by filtration (0.45 μ m). BM cells were obtained from the femur of four 15-week-old normal male F344 rats, and processed through a nylon mesh funnel to make a single cell suspension as described [1]. The colony formation assay in semisolid agar was performed by the methods described [1, 19]; briefly, suspensions of 10^3 BM cells in the growth medium containing 0.3% Special Agar Noble (Difco Lab., MI, U.S.A.) and 10% serum samples were poured into the agar medium which had been hardened by adding 0.6% agar into the growth medium in 35 $\times$ 10 mm tissue culture dishes. Thereafter, the agar cultures were incubated for 15 days at 37°C, and colonies containing more than 50 cells were counted under the inverted microscope. After observation, agar cultures were placed on glass slides, dried, and stained with ED1 and NSE as described above.

RESULTS AND DISCUSSION

Heterogeneous immunophenotypes of rat MFH cloned cells: It has been reported that cultured cells from human MFH showed functional and morphologic features of histiocytes [11, 12, 19]. These human cell lines and

Fig. 1. MT-9 cells. a) Alpha-smooth muscle actin (SMA)-positive cells showing filamentous reactions (arrowheads) differ from cells reacting to rat macrophage-specific antibody (ED1) (large arrow) with granular reactions. Smaller arrows point to cells that are negative for SMA and ED1. Double immunocytochemistry, $\times 550$. b) Two cells reacting to rat histiocyte-specific antibody (ED2). $\times 550$.

experimentally-induced rat MFH cells [15] failed to react to antibodies directed against macrophages/histiocytes. In the present study, however, cells reacting to ED1 (Fig. 1a) and ED2 (Fig. 1b) were observed in both MT-8 and MT-9 cells; in different cultures, the positive rates in MT-8 cells ranged from 2.5% to 12.5% with ED1 and from 16.0% to 26.0% with ED2; those in MT-9 cells from 6.0% to 40.0% with ED1 and from 19.5% to 27.0% with ED2. These findings suggest that MT-8 and MT-9 cells might be related to the rat monocyte/macrophage lineage. In the authors' previous study, some MT-8 and MT-9 cells were immunoreactive for SMA [25]. The SMA-positive cells were interpreted as myofibroblastic cells that have been considered to be phenotypically-modulated fibroblasts [18]. Takeya *et al.* [21] recently reported that both human MFH and fibrosarcoma cell lines reacted with anti-myeloid antibodies such as CD13 and CD68 (KP-1), indicating immunophenotypic similarities between MFH cells and fibroblasts. In the present examinations, SMA-positive cells showing filamentous reactions were seen in 9.0% to 31.0% of MT-8 cells and in 15.5% to 25.0% of MT-9 cells. Interestingly, the immunocytochemical double staining revealed that SMA-positive cells differed from cells reacting to ED1 (Fig. 1a); no cells double positive for ED1 and SMA were found in both cell lines. By chromosomal analysis of human MFH, histiocytic and fibroblastic cells have been shown to be separately originated from a single clone [7]. It is conceivable that under cultural conditions MT-8 and MT-9 cells could express either myofibroblastic or histiocytic antigenic markers. This may also imply that MFH cells have a pluripotential differentiation [4, 6, 9, 22].

Heterogeneous expressions of ED1 and ED2 antigens in rat MFH cloned cells: Subcutaneous tumors induced in syngeneic rats or nude mice by MT-8 and MT-9 cells were examined. In syngeneic rats, MT-8 cells produced undifferentiated sarcomas differing from MFH, whereas heterotransplantation of MT-8 produced tumors showing a mixed histology of undifferentiated sarcoma and storiform

type MFH (Fig. 2a). The storiform growth pattern was a constant histology of tumors induced by homo- and heterotransplantations of MT-9 (Fig. 2b). In homotransplantation of MT-9, ED1-, ACP- and NSE-positive histiocytic cells were frequently observed in the tumors (Fig. 2b), whereas the positive cells were barely seen in MT-8-induced undifferentiated sarcomas. To see whether ED1-positive cells in MT-9 tumors have proliferative capacity, the immunohistochemical double staining for BrdU and ED1 was performed in comparison with HS. The percentage of BrdU-incorporated cells in ED1-positive cells was 1.5% in MT-9 tumors (Fig. 3a) and 2.8% in HS (Fig. 3b). On the other hand, 2.7% and 4.0% of ED1-positive cells were detected with mitotic figures in MT-9 tumors (Fig. 2b) and HS, respectively. A possible proliferation of ED1-positive cells was indicated in MT-9 tumors, and that was similar generally to HS consisting solely of neoplastic histiocytes. The histiocytic cells in MT-9 tumors might be derived mostly from cells reacting to ED1 *in vitro*.

In contrast, no ED2-positive cells were detectable within tumors of homotransplants of MT-8 and MT-9 cells, although the positive cells were frequently seen in surrounding tissues of the tumors (Fig. 2c). The ED2-positive cells have been regarded as non-neoplastic, reactive tissue macrophages [23]. Furthermore, despite positive reactions to ED1 and ED2 *in vitro*, no cells reacting to these antibodies were found in tumors produced in nude mice by MT-8 and MT-9 cells. These findings indicate that immunoreactivities of MT-8 and MT-9 cells to ED1 and ED2 were inconsistent between *in vitro* and *in vivo* or between homo- and hetero-transplants. ED2 represents a differentiation antigen of the rat monocyte/macrophage lineage [2], and even in HS cells, reactivity to ED1 varied from negative to strong positive (Fig. 3b). These observations suggest that the immunophenotypes of MT-8 and MT-9 cells might be easily altered by cultural conditions and microenvironmental conditions *in vivo*. Phenotypic modulation of a cell line derived from human MFH has also

Fig. 2. a) Tumor induced in a nude mouse by MT-8 cells consists of mixture of undifferentiated sarcoma areas (U) and a storiform growth pattern (S). HE, $\times 70$. b, c) Tumors induced in a syngeneic rat by MT-9 cells; b) Many ED1-positive cells arranged in a storiform pattern and ED1-positive cells showing mitotic figures (metaphase, arrows) are seen. $\times 280$; c) ED2-positive cells are seen outside the tumor tissue (T). $\times 280$.

Fig. 3. Double immunohistochemistry for bromodeoxyuridine (BrdU) and ED1 in MT-9 homotransplanted tumor (a) and histiocytic sarcoma (HS) (b); double positive cells (large arrows) for BrdU and ED1 are seen; only BrdU-positive cells (small arrowheads) or cells reacting to ED1 (small arrows) are present; there are also cells negative to ED1 (large arrowheads), indicating heterogeneous reactions of HS cells to ED1. $\times 350$. c) MT-9 heterotransplanted tumor. Cells positive to mouse-macrophage-specific antibody (Mac1) are seen (arrows). $\times 350$.

been reported [14]; the causes of such alterations have been sought in different microenvironmental conditions induced by cell-to-cell and cell-to-matrix interactions [13, 14]; these factors remain to be investigated.

Intratumoral macrophage infiltration, and possible

productions of MCP-1 and other bioactive molecules by rat MFH cloned cells: It has been demonstrated in studies using xenotransplants of experimentally-induced rat MFH and human MFH that histiocytic cells in MFH were non-neoplastic, but rather infiltrating macrophages attracted by

tumor-derived MCP-1 [15, 21]. In heterotransplantations of MT-8 and MT-9, ACP- and NSE-positive histiocytic cells were present mostly in the storiform growth areas of the tumors, and these histiocytic cells reacted only with Mac1 (Fig. 3c), indicating infiltration of macrophages of host origin into tumor tissues.

The MCP-1 level was 1.7 ± 0.3 ng/ml in MT-9 culture supernatants and none were detected in MT-8 culture supernatants and control media. By using the same ELISA, Yamashiro *et al.* [23] have shown high levels of MCP-1 in culture supernatants of rat glioma (9L) (154.2 ± 9.2 ng/ml) and mammary carcinoma (Ad-2) (101.2 ± 4.8 ng/ml) cell lines and low level (0.84 ± 0.07 ng/ml) in MT-P cells (the parent line of MT-8 and MT-9 [26, 27]). Reflecting these results, many blood monocyte-derived macrophages infiltrated into 9L- and Ad-2-transplanted tumors, but few macrophages were seen in MT-P-induced tumors [23]. As compared with 9L and Ad-2 [23], a quantity of MCP-1 produced by cultured MT-8 and MT-9 cells seemed not sufficient to attract macrophages to tumor tissues. On the contrary, serum levels of MCP-1 in rats with MT-8 and MT-9 tumors at 6 postinoculation weeks were 15.9 ± 1.0 and 51.8 ± 1.2 ng/ml, respectively, being 4.8- and 15.2-fold greater than that in control rats (3.4 ± 0.2 ng/ml). Although the source of MCP-1 production remains to be determined, high levels of MCP-1 were demonstrated in sera of rats with MT-8 and MT-9 tumors.

In addition to MCP-1, production of leukocyte stimulating factors by the tumor has recently been reported in human MFH [21]. These factors have been proposed to play an additional role to MCP-1 on monocyte/macrophage influx into tumor tissues. Thus, the kinetics of ED1-positive macrophages was investigated in rats with the tumors. As shown in Table 1, at 6 postinoculation weeks, WBC number and differential count of monocytes were significantly higher

in rats with MT-8 and MT-9 tumors than in control rats. Many ED1-positive macrophages appeared in the liver (Figs. 4a, 4b) and spleen (Figs. 4c, 4d) of rats with MT-8 and MT-9 tumors (Table 1), although some of the positive cells might be tissue macrophages (Kupffer cells). These findings indicate that there was the notably increased number of circulating monocytes, resulting in the abnormal increment of macrophages in rats bearing MT-8 and MT-9 tumors. Therefore, the colony formation assay using rat BM cells was performed to confirm the presence of factors stimulating macrophage proliferation. The numbers of colonies in semisolid agar 7, 10 and 15 days after inoculation of rat BM cells were significantly increased by adding sera from rats with MT-8 and MT-9 tumors than by adding normal rat sera, although there was no significant difference between MT-8- and MT-9-tumor-bearing rats (Table 2). More than half of colony-forming cells reacted to NSE and ED1, indicating that proliferating cells were of the monocyte/macrophage lineage. MT-8 and MT-9 tumors might have produced bioactive molecules such as colony stimulating factors. These factors together with MCP-1 could be responsible for proliferation of macrophages and their influx into tumor tissues. Particularly in MT-9 tumors, some of ED1-positive cells might be derived from blood monocytes.

In conclusion, the present study pointed out a possibility that MT-8 and MT-9 tumors may contain non-neoplastic, host-derived infiltrating macrophages induced by MCP-1 and factors stimulating proliferation of monocytes/macrophages, besides that neoplastic cells derived from rat MFH could heterogeneously express ED1 and ED2 antigens. It appears that there are heterogeneities in the origin and immunophenotypes of "histiocytic" cells in MFH [13]. Such heterogeneous properties of MFH-constituting cells might contribute to endless, unstable histology of MFH.

Table 1. The kinetics of monocytes/macrophages in rats bearing MT-8 and MT-9 tumors and control rats

W ^{a)}	Cell	N ^{b)}	BW ^{c)}	Tumor weight (g)	WBC ^{d)} (10 ² /mm ³)	MC(%) ^{e)}	ED1-positive cells ^{f)}	
							Liver	Spleen
4	C ^{g)}	5	249 $\pm$ 10 ^{h)}	—	79 $\pm$ 9	1.4 $\pm$ 1.0	$\pm$	1+ – 2+
	MT-8	5	257 $\pm$ 11	25.3 $\pm$ 6.2	88 $\pm$ 20	4.6 $\pm$ 3.4	$\pm$	1+ – 2+
	MT-9	5	263 $\pm$ 6*	26.0 $\pm$ 2.8	86 $\pm$ 11	1.1 $\pm$ 0.7	$\pm$ – 1+	2+ – 3+
6	C	5	275 $\pm$ 16	—	74 $\pm$ 11	1.7 $\pm$ 0.6	$\pm$	1+ – 2+
	MT-8	5	282 $\pm$ 14	62.7 $\pm$ 19.4	109 $\pm$ 27*	5.6 $\pm$ 2.1*	1+ – 2+	2+ – 3+
	MT-9	5	330 $\pm$ 15***	81.9 $\pm$ 14.5	246 $\pm$ 40***	9.1 $\pm$ 5.6*	2+ – 3+	3+ – 4+

a) Weeks after subcutaneous inoculation of MT-8 or MT-9 cells.

b) Number of rats examined.

c) Body weight at autopsy.

d) White blood cell count.

e) Differential count of monocytes in blood smear.

f) Rat macrophage-specific antibody (ED1)-positive cells; $\pm$, a few cells; 1+, small number of cells in occasional areas; 2+, small number of cells in scattered areas; 3+, many cells in scattered areas; 4+, many cells throughout tissue.

g) Age-matched control rats.

h) Figures represent mean $\pm$ S.D.

Significantly different from controls by Student's *t* test; * <0.05, *** <0.001.

Fig. 4. Many ED1-positive macrophages are seen in the liver (3+) (a) and spleen (3+) (c) of a rat with MT-9 tumor, whereas the positive cells are rarely seen in the liver (±) (b) and spleen (1+) (d) of a control rat. Arrowheads indicate some ED1-positive cells. a and b, $\times 350$; c and d, $\times 60$.

Table 2. Colony formation assay in semisolid agar^{a)}

Serum ^{b)}	Days after inoculation of rat bone marrow cells		
	7	10	15
Control	2.8 $\pm$ 0.8 ^{c)}	6.2 $\pm$ 0.8	8.0 $\pm$ 1.4
MT-8	6.4 $\pm$ 2.3*	12.2 $\pm$ 1.6**	14.2 $\pm$ 4.9*
MT-9	8.6 $\pm$ 3.0*	12.6 $\pm$ 3.2**	18.4 $\pm$ 5.7*

a) Methods are described in Text.

b) Ten percent serum from rats with MT-8 and MT-9 tumors or from age-matched control rats was added to semisolid agar.

c) Mean $\pm$ S.D. of colonies counted in five different cultures. Significantly different from controls by Student's *t* test;

* <0.05 , ** <0.01 .

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