FULL PAPER *Pathology*

Expressions of Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor in Tumors Induced by Two Different Cloned Cell Lines Established from Transplantable Rat Malignant Fibrous Histiocytoma

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ABSTRACT. In order to establish base-line data on angiogenic factors in development of mesenchymal tumors, expressions of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in implanted MT-8 and MT-9 tumors, both derived from a transplantable malignant fibrous histiocytoma (MFH) in the F344 rat, were investigated by immunohistochemistry and Western blotting method. MT-8 and MT-9 tumors were developed in syngeneic rats by implant of a tumor tissue fragment. MT-8 tumors were examined on post-implantation (PI) days 3, 6, 9 and 17, and MT-9 tumors were on PI days 9, 14, 17 and 23. The growth of MT-8 tumors was faster than that of MT-9 tumors. Histologically, MT-8 tumors were features of undifferentiated sarcomas, whereas MT-9 tumors exhibited a typical storiform growth pattern of MFH. Immunohistochemically, all cells constituting MT-8 and MT-9 tumors reacted with antibodies to VEGF and bFGF, indicating production of these factors by mesenchymal neoplastic cells. However, there were no marked differences in these immunoreactions between tumors examined. Thus, the bands obtained in the Western blotting methods were densitometrically scanned. The expression levels of VEGF and bFGF gradually increased PI day 3 to 9 in MT-8 tumors and PI day 9 to 17 in MT-9 tumors. On last examination day, the levels of bFGF in both tumors and of VEGF in MT-9 tumors decreased, but the VEGF expression level in MT-8 tumors was still increased. These findings indicated that VEGF and bFGF may contribute cooperatively to angiogenesis in an early growth of mesenchymal tumor development.

KEY WORDS: angiogenesis, bFGF, F344 rat, MFH, VEGF.

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Angiogenesis or neovascularization, which is formed from preexisting blood vessels, has been considered to be intensely related to proliferation, invasion, and metastasis of tumors [7, 9]. Angiogenesis is induced by a variety of growth factors produced by neoplastic cells, macrophages infiltrated in tumor tissues or newly-formed endothelial cells themselves [8, 29]. More than 20 angiogenic factors have been reported during the last decade [3]. Particularly, vascular endothelial growth factor (VEGF) [2, 6, 24] and basic fibroblast growth factor (bFGF) [26] have been demonstrated to play a central role in angiogenesis in tumor tissues. The VEGF is a homodimeric 15-42 kDa heparin-binding glycoprotein with potent angiogenic, endothelial cell-specific mitogenic and vascular permeability-enhancing activities [19, 22]. Basic FGF is a 16.5–24.2 kDa cationic polypeptide consisting of 154 aminoacids with mitogenic activity for vascular endothelial cells [22]. Since angiogenesis plays an important role in growth, metastasis and invasion of neoplasms, chemicals capable of inhibiting angiogenesis have been developed as an anti-tumor drug in order to cure patients with advanced tumors [7, 9, 10]. Some of them can prohibit production of angiogenic factors in tumor tissues, resulting in degradation of tumor growth [10]; for example, TNP-470, synthetic analogue of fumagillin [14], has been shown to inhibit production of VEGF in human epithelial tumors [27].

To our knowledge, there has been no substantial information on relationship between production of VEGF or bFGF and growth of sarcomas in experimental rats. In order to establish base-line data, thus, the present study investigated the productions of VEGF and bFGF in rat malignant fibrous histiocytoma (MFH). MFH is the most common soft-tissue sarcoma in human adults [5]. MFH cell lines (MT-8 and MT-9) used in the present study were established from a spontaneous MFH in an aged F344 rat [38]. Tumors induced in syngeneic rats by inoculation of MT-8 or MT-9 cells show histology different to each other; MT-8 tumors are of undifferentiated sarcomas, whereas MT-9 cells develop tumors showing a storiform growth pattern typical for human MFH [38]. The two cell lines have been used as experimental models for studying on the histogenesis of MFH [35, 37, 38].

In the present study, productions of VEGF and bFGF in MT-8 and MT-9 tumors were investigated by two different methods: the immunohistochemistry and Western blotting method.

MATERIALS AND METHODS

Tumor implantation: The derivation and characteristics of MT-8 and MT-9 cells have been described [35, 37, 38]. Both the cell lines have been cultured in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/m*l*) and penicillin (100 U/m*l*). In the present study, first, MT-8 or MT-9 tumors were induced by a subcutaneous inoculation of 1×10^6 of MT-8 or MT-9 cells, respectively, into a syngeneic rat. The developed tumors (1–2 cm in diameter) were used for the following implantation.

Animals used in the implantation study were sixteen F344/DuCrj male rats that were obtained from Charles River Japan Inc. (Hino, Japan) at the age of 6 weeks. The rats were kept individually in clear polycarbonate cages in an air-conditioned room at a room temperature of 23 ± 3°C and a relative humidity of 50 ± 20% and with a 12-hr light dark cycle (08:00 to 20:00). Tap water and food for rats (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) were available *ad libitum*.

After one week-acclimatization period, the animals were divided into two groups (MT-8 or MT-9 implant) with eight rats. A tissue fragment (2 mm in diameter) of MT-8 or MT-9 tumor was transplanted subcutaneously in the interscapular region of rats through a trochar of 2 mm in diameter under light anesthesia with ether. After the implantation, in order to obtain samples of the same size between MT-8 and MT-9 tumors, the major (a) and minor (b) axes of a developing tumor were measured in mm with a caliper, and the tumor volume was estimated by the formula $a \times b^2/2$ [34]. Out of eight rats in MT-8 implant group, two rats were sacrificed each on post-implantation (PI) days 3, 7, 9 and 17. Rats in MT-9 implant group were sacrificed, two each, on PI days 9, 14, 17 and 23. Tumors on each PI day were weighed, and minced into some pieces for detection of angiogenic factors by the immunohistochemistry and Western blotting method.

Immunohistochemical Analysis: A portion of excised tumors was fixed with 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 μm in thickness. The sections were stained with hematoxylin-eosin (HE), and immunostained with an antibody against VEGF or bFGF. The immunostaining was performed with the LSAB kit (Dako Corp., Tokyo, Japan) [33]. The primary antibodies used were rabbit polyclonal anti-rabbit VEGF antibody (× 400; Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) and mouse monoclonal anti-bovine bFGF antibody (× 400; Upstate Biotech, Lake Placid, NY, U.S.A.). After treatment for 30 min with 3% methyl alcohol added hydrogen peroxide to quench endogeneous peroxidase, the sections were incubated with microwave at 800 W for 4 min × 4 in 0.01 M phosphate-buffered saline (PBS) (pH7.2). Following treatment for 5 min with a protein blocking agent (Dako Corp., Tokyo, Japan), the sections were reacted for 48 hr at 4°C with a primary antibody to VEGF or bFGF. Incubation with biotinylated goat anti-mouse or rabbit IgG for 10 min followed. Final incubation was carried out for 10 min with labeled streptavidin, and positive reactions were visualized with 3,3'-diaminobenzidine (DAB) for 10 min. Sections were lightly counterstained with hematoxylin. Negative controls were reacted with PBS and mouse or rabbit nonimmune IgG instead of primary antibodies.

VEGF and bFGF Determination by the Western Blotting Method: Tissue samples for the Western blotting method were weighed, diced into very small pieces using a clean razor blade, and then dissolved in an ice cold Lysis buffer (10 mM Tris-Cl (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 0.2% Triton-X-100, 100 μg/m*l* PMSF, 1 μg/m*l* aprotinin). Thereafter, the tissue was disrupted and homogenized with a homogenizer, maintaining the temperature at 4°C throughout all procedures. The tubes were allowed to stand on ice for about 30 min and then, centrifuged at 15,000 rpm for 20 min at 4°C. Removed supernatants were microfuged again in order to pellet out any insoluble material/debris. The clear supernatant fluid obtained was the total tissue lysate. The total protein was determined by Bradford assay using bovine serum albumin supplied with the kit (Amersham International, Buckinghamshire, UK) as a standard [1]. Briefly, a set of standards is created from a stock of protein whose concentration is known. The Bradford values obtained for the standard are then used to construct a standard curve to which the unknown values obtained can be compared to determine their concentration.

All the samples with equal amounts of protein (10 μg/m*l*) were mixed in sodium dodecyl sulfate (SDS) buffer [50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% sodium dodecyl sulfate (electrophoresis grade), 0.1% bromophenol blue, 10 % glycerol] in the ratio of 1:1, and boiled for 3 min to denature the protein. Human recombinant antigens of VEGF (21 kDa; Santa Cruz Biotech, Santa Cruz, CA, U.S.A.) and bFGF (17.5 kDa; Upstate Biotech, Lake Placid, NY, U.S.A.) were used as the positive control antigen. Each antigen (1 *n*g) was loaded on a lane. Then, the samples were loaded on each lane and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 4–20% acrylamide gradient gel) with a Tris/glycine running buffer [25 mM Tris, 250 mM glycine (electrophoresis grade) (pH 8.3), 0.1 % SDS]. For the Western blotting, the separated proteins were transferred to a polyvinylidine difluoride (PVDF) membrane (0.2 μm) by using wet electrotransfer (Bio-Rad transfer unit, CA, U.S.A.) for 1 hr at 100 V. The Western blots were carried out as described by Towbin *et al.* [31], with slight modifications. After the transfer, the membranes were blocked with 10% of a commercially available blocker, Block Ace (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) in PBS at room temperature for 1 hr, then incubated at 4°C overnight with primary antibodies (1 μg/m*l*) for VEGF or bFGF in PBS. The blots were then washed with Tris-PBS (5 min × 3, 1 min × 2), and incubated with a secondary antibody (1:2,000) at room temperature for 2 hr. The secondary antibodies used were a horseradish peroxidase (HRP) conjugated anti-mouse IgG for anti-bFGF antibody and a HRP conjugated anti-rabbit IgG for anti-VEGF antibody. The blots were washed thoroughly with Tris-PBS and the signal-detected bands were then visualized with Western blotting analysis system (ECL™; Amersham International). The blots were densitometrically scanned using an imaging densitometer (Model GS-700, BIO-RAD, CA, U.S.A.) and each optical density was measured by the Power Macintosh and PC image analysis software (Model GS-700, BIO-RAD). The measurements were carried out 2 times, and the optical density was shown as a percent of total volumes obtained from all the samples.

RESULTS

Tumor growth: As shown in Fig. 1 (a. c), the growth of MT-8 tumors was faster than that of MT-9 tumors; on PI day 17, the weight of MT-8 tumors was about three times greater than that of MT-9 tumors (Figs. 1b, 1d). MT-8 tumors showed

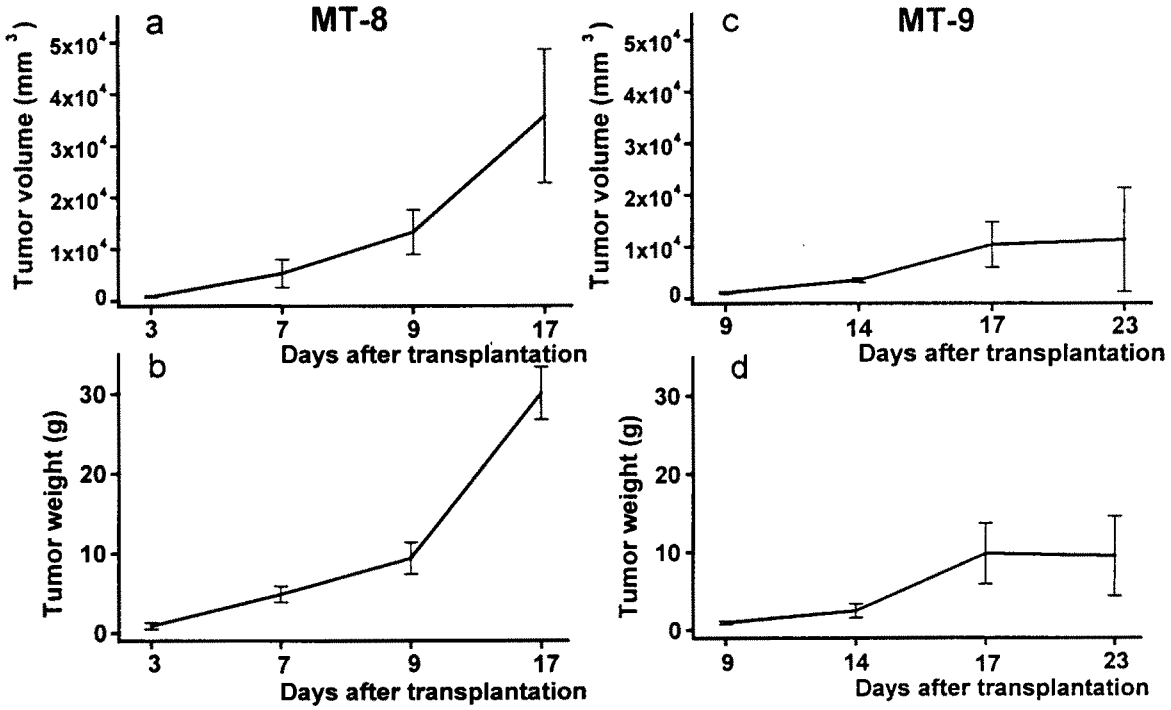


Fig. 1. Growth curves in volume and weight of MT-8 (a, b) and MT-9 (c, d) tumors, derived from a transplantable rat malignant fibrous histiocytoma (MFH). Bars represent the mean ± SD.

quick growth from PI day 9 to PI day 17, whereas MT-9 tumors grew slowly until PI day 23 (Fig. 1).

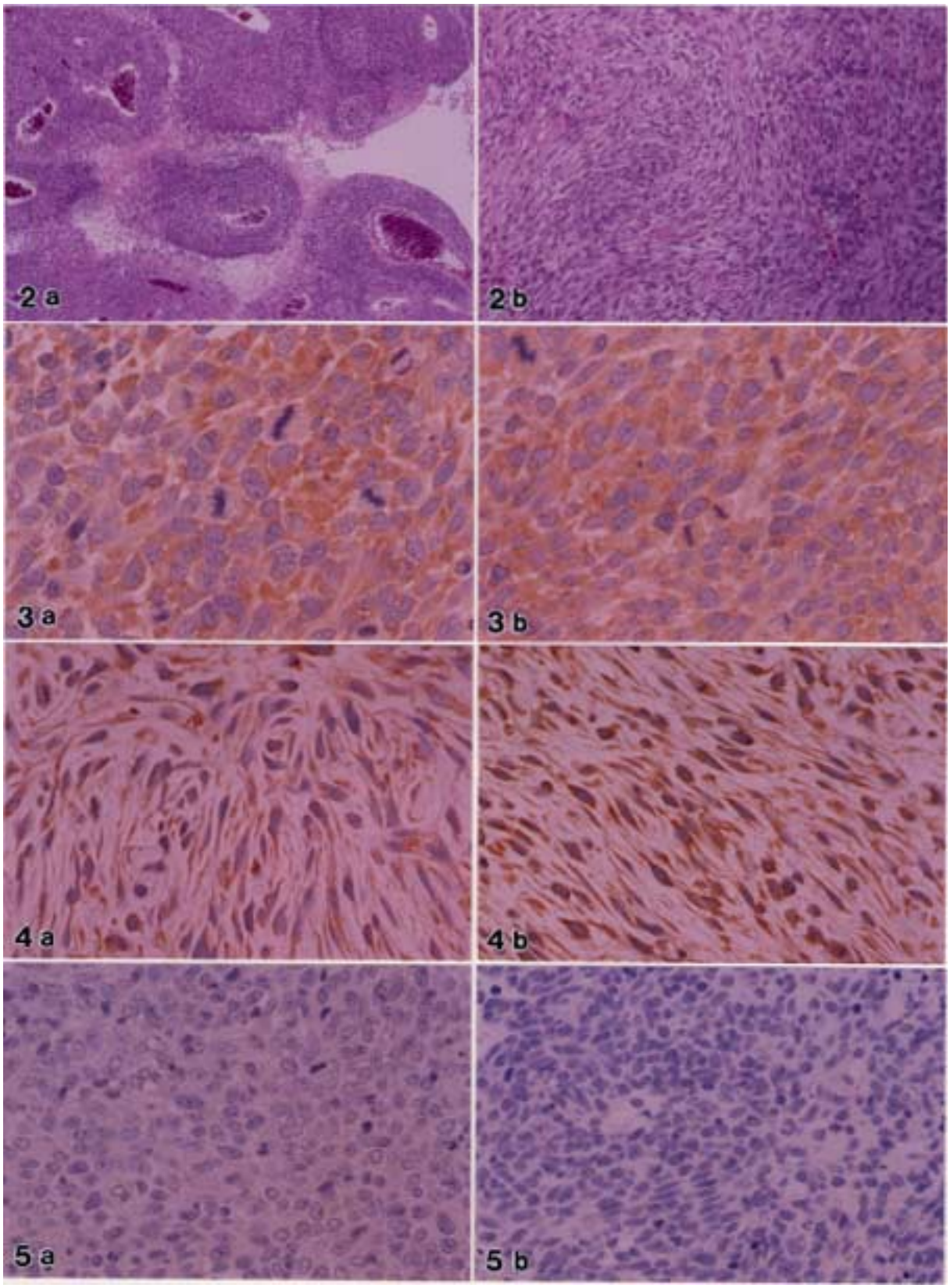
Light microscopic findings: Histologically, MT-8 tumors exhibited uniformly features of undifferentiated sarcoma consisting of round to fusiform cells with scanty cytoplasm and hyperchromatic nuclei; these cells were arranged mainly in organoid structures (Fig. 2a). In contrast, MT-9 tumors showed a storiform growth pattern consisting of histiocytic cells and elongated fibroblastic cells as well as a moderate amount of collagen fibers (Fig. 2b).

Immunohistochemically, MT-8 tumor-constituting cells reacted moderately to strongly for VEGF (Fig. 3a) and bFGF (Fig. 3b). There were no marked differences in the reactivities between VEGF and bFGF immunostainings, or among tumors examined on each PI day. In MT-9 tumors, all cells were positive for VEGF (Fig. 4a) and bFGF (Fig. 4b) like MT-8 tumors; no differences in the immunostainings were seen between VEGF and bFGF or among tumors examined. In negative controls incubated with normal mouse IgG or normal rabbit IgG instead of primary antibodies, no positive reactions developed (Figs. 5a, 5b).

Western blotting: As shown in Fig. 6, in MT-8 and MT-9 tumors, bands for VEGF and bFGF appeared at 21 kDa and 17.5 kDa positions, respectively; these positions were corresponding to those reported previously [16, 20] and positive controls of commercially available antigens for VEGF (Fig. 6a, line 1) and bFGF (Fig. 6b, line 1). The expressed bands were different in the density between examination points. Thus, the densitometry was measured, and the data obtained are shown in Fig. 7. In MT-8 tumors, expression level of VEGF increased with tumor growth until PI day 17, whereas that of bFGF increased until PI day 9 but decreased on PI day 17. In MT-9 tumors, the expressions for VEGF and bFGF increased with tumor growth until PI day 17, but on PI day 23, those decreased.

DISCUSSION

A variety of angiogenic factors have been reported, and it has been suggested that the expression levels of these factors are variable among tumor types or depending on tumor growth [19]. VEGF and bFGF both are heparin-binding factors with potent angiogenic properties [4]. Recently, it has been reported that VEGF may be an important factor responsible for the induction of angiogenesis in malignant epithelial tumors such as stomach and colorectal cancers [18, 30]. Furthermore, it was reported in human lung cancers and brain astrocytomas that VEGF and bFGF may act cooperatively in neovascularization in these tumor tissues [17, 25]. In the present study, immunohistochemically, although infiltrating macrophages might be involved [36], all cells constituting MT-8 and MT-9 tumors reacted to VEGF and bFGF, indicating that neoplastic mesenchymal cells can produce these angiogenic factors in developing tumors. However, there were no marked differences in immunoreactivities for VEGF or bFGF between tumors examined. Therefore, we tried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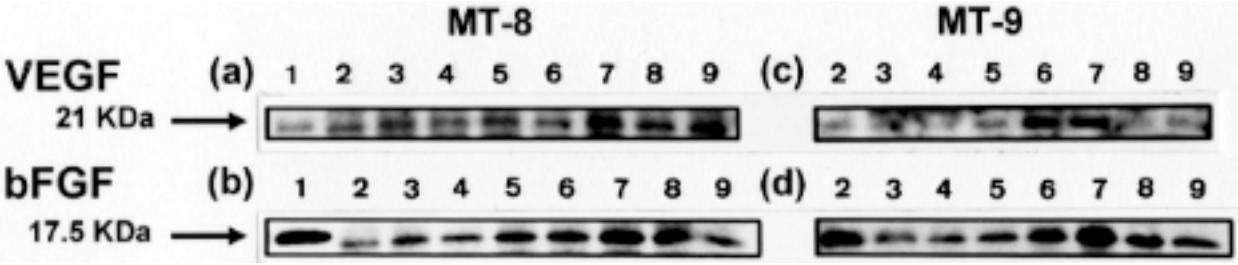

Fig. 6. Western blotting analysis of VEGF and b FGF. The cell lysates of both MT-8 (a, b) and MT-9 (c, d) were run on SDS-PAGE (15/25%) and transferred to two sets of membranes. One set (a, c) was probed with an anti-VEGF polyclonal antibody and second set was probed with an anti-basic FGF monoclonal antibody (b, d) using an ECL Western blotting kit (Amersham International). Line 1 shows the band of a commercially available antigen for VEGF or bFGF as positive control. Other lines show the bands of 3 (lines 2 and 3), 7 (lines 4 and 5), 9 (lines 6 and 7), 17 (lines 8 and 9) days after transplantation of MT-8 (a, b), and 9 (lines 2 and 3), 14 (lines 4 and 5), 17 (lines 6 and 7) and 23 (lines 8 and 9) days after transplantation of MT-9 (c, d). The 17.5 (b FGF) and 21 kDa (VEGF) bands are shown by arrows.

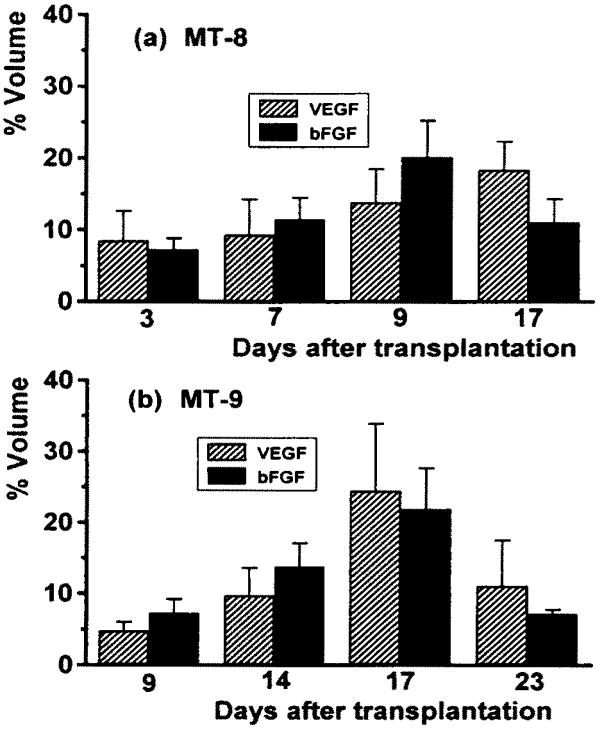

Fig. 7. Densitometric analysis for VEGF and bFGF. Volumes of each samples were evaluated by densitometric analysis of the doublets. Results are expressed as a percent (mean ± SD) of total volumes obtained from samples.

Western blotting method for quantitative information. The analyses revealed differences in expression levels for VEGF and bFGF depending on tumor growth. Both analyses (the immunohistochemistry and the Western blotting method) should be needed to evaluate the exact productions for such angiogenic factors in tumor tissues.

During the observation period, the expression levels for VEGF and bFGF gradually increased until PI day 9 in MT-8 tumors and until PI day 17 in MT-9 tumors (Fig. 7). Goto *et al.* [11] and Pepper *et al.* [21] reported that there is a potent synergism between VEGF and bFGF in the induction of angiogenesis *in vitro*. These findings indicate that VEGF and bFGF might contribute cooperatively to angiogenesis in an early growth of mesenchymal tumors. There appear to be some differences in biological nature between VEGF and bFGF. Trophoblastic cells play an important role in extensive angiogenesis occurring in very early gestation periods, mainly by releasing bFGF but not VEGF [13]. It is also known that bFGF can activate DNA synthesis for mesodermal cells such as fibroblasts and smooth muscles, resulting in active proliferation of tumors derived from these cells [22]. The bFGF expressions in MT-8 and MT-9 tumors increased until PI days 9 and 17, respectively, but thereafter, the expressions decreased on PI days 17 and 23, respectively (Fig. 1). This may mean the presence of saturation level of bFGF in tumor development. Since bFGF has greater mitogenic activity for vascular endothelial cells [13, 22], angiogenesis in an early growth of MFH might be caused mainly by bFGF. Indeed, the sprouting angiogenesis in both MT-8 and MT-9 tumors was demonstrated in the early growth by scanning electron microscopical observations using corrosion casts [32]. The sprout-

Fig. 2. Histology of MT-8 and MT-9 tumors. MT-8 tumor shows organoid structures which consist of round to fusiform cells (a). MT-9 tumor consists of oval to spindle cells arranged in a storiform growth pattern (b). H & E. a, × 33; b, × 83.

Fig. 3. Immunohistochemical staining for VEGF (a) and bFGF (b) in MT-8 tumor on day 17 after transplantation. Positive reactions are seen in cytoplasm. Counterstained with hematoxylin. × 330.

Fig. 4. Immunohistochemical staining for VEGF (a) and bFGF (b) in MT-9 tumor on day 23 after transplantation. Positive reactions are seen in cytoplasm. Counterstained with hematoxylin. × 330.

Fig. 5. No positive reaction is noted when species-specific IgG (control) was substituted for the primary antibody in MT-8 tumors. a, normal rabbit IgG (negative control in VEGF immunostaining); b, normal mouse IgG (negative control in bFGF immunostaining). Counterstained with hematoxylin. × 330.

ing angiogenesis is indicative of active proliferation of vascular endothelial cells [12, 15, 28]. Moreover, it was reported that bFGF is implicated in the early phase of tumorigenesis and vascularity of N-nitrosoethylurea-induced brain tumors in rats [23].

In spite of decreased expression in bFGF on PI day 17 in MT-8 tumors, VEGF expression level was still increased; in MT-9 tumors, the expression level of VEGF was decreased on PI day 23 (Fig. 7). There was a difference in VEGF expression on the last examination point between MT- 8 (PI day 17) and MT-9 (PI day 23) tumors. As shown in Fig. 1, MT-8 tumors rapidly grew from PI day 9 to PI day 17, whereas MT-9 tumors still showed slow growth on PI day 23. The rapid growth of MT-8 tumors might have been caused partly by high production of VEGF, because VEGF has mitogenic capacity for endothelial cells as well as vascular permeability-enhancing activities resulting in intercellular exudation [19]. Indeed, MT-8 tumors were more edematous, and had often necrotic areas in the tumor tissues; such findings were rarely seen in MT-9 tumors examined [38], and MT-9 tumors were firm on gross findings. Cell characteristics are different between MT-8 and MT-9 cells, although both cell lines were induced from a rat MFH [38]. It has been considered that MT-8 cells are at more primitive stage in mesenchymal differentiation than MT-9 cells [35]. Indeed, MT-8 and MT-9 tumors were different histologically from each other [35, 37, 38]. It is interesting to pursue the relationship between VEGF expression and differentiation stages of mesenchymal cells or mesenchymal tumors showing different histology.

In conclusion, the present study demonstrated using transplantable MT-8 and MT-9 tumors derived from a rat MFH that the expressions of VEGF and bFGF increased in an early growth of these tumors after implantations. These factors may contribute to tumor development by promoting angiogenesis [17, 30]. The base-line data obtained in the present study may provide useful information for biological behaviors of MFH as well as for development of anti-tumor drugs with potential inhibition of neovascularization.

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REFERENCES

1. Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248–254.
2. Connolly, D.T., Heuvelman, D.M., Nelson, R., Olander, J.V., Eppley, B.L., Delfino, J.J., Siegel, N.R., Leimgruber, R.M. and Feder, J. 1989. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J. Clin. Invest*. 84: 1470–1478.
3. Dellian, M., Witwer, B.P., Salehi, H.A., Yuan, F. and Jain, R.K. 1996. Quantitation and physiological characterization of angiogenic vessels in mice. *Am. J. Pathol.* 149: 59–71.
4. Dirix, L.Y., Vermeulen, P.B., Hubens, G., Benoy, I., Martin, M., Pooter, C.D. and Van Oosterom, A.T. 1996. Serum basic fibroblast growth factor and vascular endothelial growth factor and tumour growth kinetics in advanced colorectal cancer. *Ann. Oncol.* 7: 843–848.
5. Enzinger, F.M. and Weiss, S.W. 1995. Malignant fibrohistiocytic tumors. pp. 351–380. *In:* Soft Tissue Tumors, 3rd ed. (Enzinger, F.M. and Weiss, S.W. eds.), CV Mosby, St. Louis, MO.
6. Ferrara, N. and Henzel, W.J. 1989. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 161: 851–858.
7. Folkman, J. 1971. Tumor angiogenesis: Therapeutic implications. *New Engl. J. Med.* 285: 1182–1186.
8. Folkman, J. 1992. The role of angiogenesis in tumor growth. *Cancer Biol.* 3: 65–71.
9. Folkman, J. 1994. Angiogenesis and breast cancer. *J. Clin. Oncol.* 12: 441–443.
10. Folkman, J. 1995. Clinical applications of research on angiogenesis. *New Engl. J. Med.* 333: 1757–1763.
11. Goto, F., Goto, K., Weindel, K. and Folkman, J. 1993. Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. *Lab. Invest.* 69: 508–517.
12. Grunt, T.W., Lametschwandtner, A., Karrer, K. and Staindl, O. 1986. The angioarchitecture of the Lewis lung carcinoma in laboratory mice (A light microscopic and scanning electron microscopic study). *Scanning Electron Microsc.* 2: 557–573.
13. Hamai, Y., Fujii, T., Yamashita, T., Kozuma, S., Okai, T. and Taketani, Y. 1998. Evidence for basic fibroblast growth factor as a crucial angiogenic growth factor, released from human trophoblasts during early gestation. *Placenta* 19: 149–155.
14. Ingber, D., Fujita, T., Kishimoto, S., Sudo, K., Kanamaru, T., Brem, H. and Folkman, J. 1990. Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumour growth. *Nature* (*Lond)* 348: 555–557.
15. Konerding, M.A., Miodonski, A.J. and Lametschwandtner, A. 1995. Microvascular corrosion casting in the study of tumor vascularity: A review. *Scanning Microsc.* 9: 1233–1244.
16. Landau, C., Jacobs, A.K. and Haudenschild, C.C. 1995. Intrapericardial basic fibroblast growth factor induces myocardial angiogenesis in a rabbit model of chronic ischemia. *Am. Heart J.* 129: 924–931.
17. Mattern, J., Koomägi, R. and Volm, M. 1997. Coexpression of VEGF and bFGF in human epidermoid lung carcinoma is associated with increased vessel density. *Anticancer Res.* 17: 2249–2252.
18. Nakata, S., Ito, K., Fujimori, M., Shingu, K., Kajikawa, S., Adachi, W., Matsuyama, I., Tsuchiya, S., Kuwano, M. and Amano, J. 1998. Involvement of vascular endothelial growth factor and urokinase-type plasminogen activator receptor in microvessel invasion in human colorectal cancers. *Int. J. Cancer (Pred. Oncol.)* 79: 179–186.
19. Nguyen, M. 1997. Angiogenic factors as tumor markers. *Invest. New Drugs* 15: 29–37.
20. Papavassiliou, E., Gogate, N., Proescholdt, M., Heiss, J.D., Walbridge, S., Edwards, N.A., Oldfield, E.H. and Merrill, M.J. 1997. Vascular endothelial growth factor (Vascular permeability factor) expression in injured rat brain. *J. Neurosci. Res.* 49: 451–460.
21. Pepper, M.S., Ferrara, N., Orci, L. and Montesano, R. 1992.

Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis *in vitro*. *Biochem. Biophys. Res. Commun.* 189: 824–831.

22. Pötgens, A.J.G., Westphal, H.R., de Waal, R.M.W. and Ruiter, D.J. 1995. The role of vascular permeability factor and basic fibroblast growth factor in tumor angiogenesis. *Biol. Chem. Hoppe-Seyler* 376: 57–70.
23. Segal, D.H., Germano, I.M. and Bederson, J.B. 1997. Effects of basic fibroblast growth factor on *in vivo* cerebral tumorigenesis in rats. *Neurosurgery* 40: 1027–1033.
24. Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S. and Dvorak, H.F. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219: 983–985.
25. Shim, J.W., Koh, Y.C., Ahn, H.K., Park, Y.E., Hwang, D.Y. and Chi, J.G. 1996. Expression of bFGF and VEGF in brain astrocytoma. *J. Korean Med. Sci.* 11: 149–157.
26. Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J. and Klagsbrun, M. 1984. Heparin affinity: Purification of a tumor derived capillary endothelial cell growth factor. *Science* 223: 1296–1299.
27. Shishido, T., Yasoshima, T., Denno, R., Mukaiya, M., Sato, N. and Hirata, K. 1998. Inhibition of liver metastasis of human pancreatic carcinoma by angiogenesis inhibitor TNP-470 in combination with cisplatin. *Jpn. J. Cancer Res.* 89: 963–969.
28. Skinner, S.A., Frydman, G.M. and O'brien, P.E. 1995. Microvascular structure of benign and malignant tumors of the colon in humans. *Dig. Dis. Sci.* 40: 373–384.
29. Sunderkotter, C., Steinbrink, K., Goebeler, M., Bhardwaj, R. and Sorg, C. 1994. Macrophages and angiogenesis. *J. Leukoc. Biol.* 55: 410–422.
30. Takahashi, Y., Ellis, L.M., Ohta, T. and Mai, M. 1998. Angiogenesis in poorly differentiated medullary carcinoma of the stomach. *Jpn. J. Surg.* 28: 367–372.
31. Towbin, H., Staehelin, T. and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* 76: 4350–4354.
32. Tsunenari, I., Yamate, J., Iwaki, M., Kuwamura, M., Kotani, T. and Sakuma, S. 1997. Angioarchitecture of tumors induced by two different cloned cell lines established from a transplantable rat malignant fibrous histiocytoma. *Scanning Microsc.* 11: 473–482.
33. Tsunenari, I., Yamate, J. and Sakuma, S. 1997. Poorly differentiated carcinoma of the parotid gland in a six-week-old Sprague-Dawley rat. *Toxicol. Pathol.* 25: 225–228.
34. Yamate, J., Tajima, M., Shibuya, K., Ihara, M. and Kudow, S. 1989. Morphologic characteristics of a transplantable tumor derived from a spontaneous malignant fibrous histiocytoma in the rat. *Jpn. J. Vet. Sci.* 51: 587–596.
35. Yamate, J., Tajima, M., Shibuya, K., Kuwamura, M., Kotani, T. and Sakuma, S. 1996. Phenotypic modulation in cisplatin-resistant cloned cells derived from transplantable rat malignant fibrous histiocytoma. *Pathol. Int.* 46: 557–567.
36. Yamate, J., Tajima, M., Shibuya, K., Kuwamura, M., Kotani, T., Sakuma, S. and Takeya, M. 1996. Heterogeneity in the origin and immunophenotypes of "Histiocytic" cells in transplantable rat malignant fibrous histiocytoma. *J. Vet. Med. Sci.* 58: 603–609.
37. Yamate, J., Tajima, M., Shibuya, K. and Saitoh, T. 1994. Enhancement of lipid phagocytosis by cloned cells derived from rat malignant fibrous histiocytoma under hyperlipemic conditions. *Pathol. Int.* 44: 735–744.
38. Yamate, J., Tajima, M., Togo, M., Shibuya, K., Ihara, M. and Kudow, S. 1991. Heterogeneity of cloned cell lines established from a transplantable rat malignant fibrous histiocytoma. *Jpn. J. Cancer Res.* 82: 298–307.