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Wide Spectrum of Antitumor Activity of a Neutralizing Monoclonal Antibody to Human Vascular Endothelial Growth Factor

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Vascular endothelial growth factor (VEGF) is known as an angiogenic factor for tumor angiogenesis. We developed a neutralizing anti-VEGF monoclonal antibody (MAb), MV833, and examined its antitumor activity against 27 human tumor cell lines transplanted in nude mice. All the tumor cell lines used in this study secreted various amounts of VEGF into culture medium *in vitro*. However, the growth of the cell lines, including three which expressed VEGF receptor, was not affected by exogenously added MV833 *in vitro*. All tumor cell lines including colon, lung, breast, pancreas, and melanoma, grew subcutaneously in nude mice. The growth of HeLa/v5, which had been transformed by human $VEGF_{121}$ gene and secreted large amounts of VEGF, was significantly faster than that of the control vector transformant. Although the amounts of VEGF secreted from two HeLa transformants differed greatly, MV833 completely inhibited the growth of both tumors. Moreover, the growth of the other 25 human tumor cell lines transplanted into nude mice was also strongly suppressed by MV833. Neither the amount of VEGF secreted from each tumor cell line *in vitro* nor the expression of VEGF receptor correlated with the antitumor activity of MV833. MV833, administered when tumor volumes reached 400 mm^3, completely inhibited the growth of some tumor lines. The results show VEGF to be a critical angiogenic factor for many tumors. VEGF-neutralizing antibody could be applicable as an antitumor agent for a wide range of tumors.

Key words: VEGF — Monoclonal antibody — Antitumor activity — Nude mice — Xenograft

In solid tumors, neovascularization is a prerequisite for growth.[1, 2] Tumor cells are thought to secrete angiogenic factor(s) that induce neovascularization around the tumors.[3–5] Vascular endothelial growth factor (VEGF) is a secretable angiogenic factor also known as vascular permeability factor (VPF) or vasculotropin.[6–8] It specifically promotes the growth of endothelial cells and induces vascular leakage. VEGF protein or its mRNA has been detected in specimens from many cancer patients.[9–17] VEGF has also been detected in the conditioned medium of many tumor cell lines from different organs,[18] in sera,[19, 20] and in pleural and peritoneal fluid of cancer patients.[21] These findings suggest that VEGF is secreted from a variety of solid tumors *in vivo* and acts as a tumor angiogenic factor (TAF). We have previously established two neutralizing anti-VEGF monoclonal antibodies (MAbs), MV101 and MV303. These MAbs inhibited the growth of human solid tumor xenografts in nude mice.[22, 23] We therefore tried to develop another neutralizing anti-VEGF MAb with more potent antitumor activity than MV101 or MV303. We obtained more than 140 clones of hybridomas, producing anti-VEGF MAbs, from mice immunized with recombinant human $VEGF_{121}$. Among them, 26 clones showed neutralizing activity and MV833 possessed the most potent antitumor activity *in vivo*.[24] Here we describe the antitumor activity spectrum of MV833 against 27 human tumors transplanted in nude mice and show that anti-VEGF antibody has the potential to inhibit the growth of many kinds of solid tumors.

MATERIALS AND METHODS

Preparation of an anti-human VEGF MAb, MV833 BALB/c mice (8-week-old, female) were immunized with recombinant human $VEGF_{121}$.[25] Spleen cells were fused with mouse myeloma Sp2/O-Ag14 and hybridomas were cloned by limiting dilution.[24] Hybridoma cells were inoculated i.p. in BALB/c nude mice, and MV833 was purified from ascites fluid using a protein-A column (MAPS-II; Bio-Rad, Cambridge, CA).

Cell lines A375, G361, WM-115 (melanoma), LS174T, LS180, HCT116, HT-29, SW480, LoVo (colon carcinoma), PC-3 (prostate carcinoma), PLC/PRF/5 (hapatoma), MIA PaCa-2 (pancreatic carcinoma), A-673 (rhabdomyosarcoma), HT-1080 (fibrosarcoma), and A-431 (epidermoid carcinoma) were supplied by American Type Culture Collection through Dainippon Pharmaceutical Co. (Osaka). Two lung carcinoma cell lines, A549 and PC-14 were purchased from RIKEN Cell Bank (Tsukuba). Two pancreas carcinoma cell lines, BXPC-3 and ASPC-1, were kindly donated by Dr. Toi (Tokyo Metropolitan Komagome Hospital, Tokyo). Four ovarian carcinoma cell

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lines, SKOV-3, NOS2, NOS4 and HRA, were a gift from Dr. Okamoto (Nagoya University of Medicine, Nagoya). MX-1 (breast carcinoma) and LX-1 (lung carcinoma) were obtained from Dr. Tashiro (Cancer Chemotherapy Center, Foundation for Cancer Research, Tokyo). HeLa/v5 and HeLa/c were transformed with a human $VEGF_{121}$ gene-bearing or control vector, respectively.[18)]

Cell growth *in vitro* Cells ($1-4\times10^4$) were seeded on 24-well culture plates in 400 μl of Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10 % FBS (fetal bovine serum) with or without 100 μg/ml of MV833 at 37°C under a humidified atmosphere containing 5% CO_2. After 4 days of culture, the cells were harvested after trypsinization and viable cells were counted under a microscope as trypan blue dye-excluding cells.

Radio receptor assay Cells (5×10^4) were seeded on 96-well culture plates, cultured for 24 h, washed with the medium and incubated with 5×10^4 cpm of ^{125}I-labeled $VEGF_{165}$ (66 TBq/mmol; Amersham, Buckinghamshire, UK) at 37°C for 3 h with or without a 100-fold amount of unlabeled $VEGF_{165}$ (R&D Systems Inc., Minneapolis, MN). Unbound radioligand was removed, and the cells were solubilized in 1% SDS (sodium dodecyl sulfate). The amount of ^{125}I-labeled $VEGF_{165}$ specifically bound to tumor cells was measured using a γ counter (COBRA II; Packard, Meridian, CT).

Detection of VEGF in conditioned media of cultured human tumor cells For preparation of the conditioned media, cells (7.5×10^4) were cultured in 24-well culture plates in 500 μl of D-MEM for 48 h. Amounts of VEGF in the conditioned media were determined by sandwich-type ELISA as described.[19)]

Angiogenesis induced by tumor cells *in vivo* *In vivo* angiogenesis was assayed by the dorsal air-sac method.[22)] Briefly, 1×10^7 cultured HT-1080 cells were packed into membrane chambers and implanted into a dorsal air sac of BALB/c mice (day 0). MV833 (100 μg) was administered i.v. from days 1 to 3. The mice were killed on day 4, and

Table I. Characteristics of Tumor Cell Lines Used

Cell line	Type	VEGF production[a)]	VEGF receptor[b)]
A375	Melanoma	+	+
G361	Melanoma	+	−
WM-115	Melanoma	++	+
MX-1	Breast carcinoma	+	−
PC-14	Lung carcinoma	+++	−
A549	Lung carcinoma	++	−
LX-1	Lung carcinoma	+	−
LS174T	Colon carcinoma	+++	−
LS180	Colon carcinoma	+++	−
HCT116	Colon carcinoma	++	−
HT-29	Colon carcinoma	+	−
SW480	Colon carcinoma	+	−
LoVo	Colon carcinoma	++	−
PLC/PRF/5	Hepatoma	++	−
MIA PaCa-2	Pancreas carcinoma	+	−
BXPC-3	Pancreas carcinoma	++++	−
ASPC-1	Pancreas carcinoma	+	−
PC-3	Prostate carcinoma	+	+
SKOV-3	Ovarian carcinoma	++	−
NOS2	Ovarian carcinoma	+	−
NOS4	Ovarian carcinoma	++	−
HRA	Ovarian carcinoma	+	−
A-673	Rhabdomyosarcoma	+++	−
HT-1080	Fibrosarcoma	++++	−
A-431	Epidermoid carcinoma	+++	−
HeLa/v5	Cervix carcinoma	++++	−
HeLa/c	Cervix carcinoma	+	−

a) VEGF production was scored based on the amount of VEGF in the conditioned medium by sandwich ELISA system. +, 0.05−0.5; ++, 0.5−1.0; +++, 1.0−1.5; ++++, >1.5 ng/ml.
b) VEGF receptor was analyzed by assay of specific binding of ^{125}I-labeled $VEGF_{165}$ to tumor cells.

the formation of new blood vessels in s.c. regions was examined under a dissecting microscope.

Tumor growth *in vivo* All the human tumors were maintained by serial transplantations in the s.c. region of BALB/c nude mice (Charles River Japan Inc., Kanagawa) housed in specific pathogen-free conditions. Tumors were taken from mice, cut into 2×2×2-mm pieces, and transplanted s.c. into the abdominal region of native nude mice using a trocar. MV833 was administered i.v. through a lateral tail vein every 4 days until the experiments were terminated. The tumor was measured in two dimensions, and volume was calculated using the formula volume= width^2×length/2. The control mice were administered the same volume of PBS (phosphate-buffered saline) (0.2 ml/injection).

RESULTS

Secreted VEGF levels and VEGF receptor expression *in vitro* Table I shows the characteristics of all the human tumor cell lines used in this study. VEGF was detected in the conditioned media from all the human tumor cell lines, though the amounts differed; PC-14, LS180, LS174T, BXPC-3, A-673, HT-1080 and HeLa/v5 secreted large amounts of VEGF into the culture medium while A375, G361, SW480, ASPC-1, NOS2, HRA, LX-1, MX-1, PC3, HT-29 and HeLa/c secreted low levels of VEGF. No relationship between the level of VEGF secreted and the origin of the tumors was observed. ^{125}I-labeled VEGF bound to A375, WM-115 and PC3, indicating expression of VEGF receptors on their cell surface.

Effects of MV833 on the growth of human tumor cells *in vitro* MV833 (100 μg/ml) did not exhibit any direct inhibitory activity on the *in vitro* growth of cultured human tumor cells, including A375, WM-115 and PC3, that express the VEGF receptor (Table II).

Inhibition of tumor-induced neovascularization by MV833 Tumor induced neovascularization was examined by implanting HT-1080 cells, packed into membrane chambers, in mice. Neovascularization was evident in the region adjacent to the chamber containing HT-1080 cells after 4 days (Fig. 1). Three i.v. administrations of MV833 (100 μg) suppressed the neovascularization induced by HT-1080 cells to the level of the control (no tumor cells). These results indicated that MV833 neutralized the activity of VEGF secreted from tumor cells and inhibited tumor-induced neovascularization *in vivo*.

The growth of human *VEGF$_{121}$*-transformed HeLa cell line and its inhibition by MV833 *in vivo* The human cervix tumor cell line, HeLa/v5, which was transformed by human *VEGF$_{121}$* gene, secreted higher levels of VEGF than HeLa/c transformed by control vector, *in vitro* (Table I). The growth of solid tumor of HeLa/v5 s.c. transplanted in nude mice was significantly faster than that of HeLa/c (Fig. 2; open symbols). From day 28 to day 70 after the transplantation, the tumor volumes of HeLa/v5 and HeLa/c increased 11.7- and 5.0-fold, respectively. The growth of both HeLa/v5 and HeLa/c tumors was almost completely suppressed by the administration of MV833 (Fig. 2; closed symbols).

Table II. Effect of MV833 on the Growth of Human Tumors *in vitro* and *in vivo*

Cell line	*In vitro* cell growth (%)	*In vivo* T/C minimum
A375	101.1	0.31
G361	104.2	0.30
WM-115	101.1	0.30
MX-1	96.3	0.13
PC-14	109.4	0.33
A549	107.1	0.27
LX-1	99.1	0.07
LS174T	89.5	0.09
LS180	111.2	0.10
HCT116	106.3	0.17
HT-29	116.6	0.39
SW480	106.4	0.10
LoVo	97.5	0.16
PLC/PRF/5	100.0	0.30
MIA PaCa-2	102.4	0.17
BXPC-3	102.2	0.20
ASPC-1	98.7	0.38
PC-3	102.0	0.18
SKOV-3	93.6	0.18
NOS2	102.3	0.20
NOS4	119.4	0.20
HRA	106.0	0.33
A-673	96.9	0.04
HT-1080	97.6	0.22
A-431	93.0	0.30
HeLa/v5	90.5	0.09
HeLa/c	92.5	0.18

Antitumor activity of MV833 against various kinds of human tumors s.c. transplanted in nude mice The solid tumors of 25 native human tumor cell lines grew s.c. in nude mice. Repeated i.v. administration of 100 μg of MV833 (every 4 days) strongly suppressed the growth of all human tumors. The antitumor activity of MV833 is summarized in Table II in terms of minimum T/C (T/C_{min}) (T, treatment with MV833; C, control) values. All T/C_{min} values were below 0.4. MV833 showed antitumor activities against MX-1, LX-1, LS174T, LS180, SW480 and A-673, suppressing their growth almost completely (T/C_{min} values below 0.15). Typical examples for six colon tumors are shown in Fig. 3. The growth of five of these colon tumors was markedly suppressed by MV833. The origin of the tumors, level of VEGF production, and

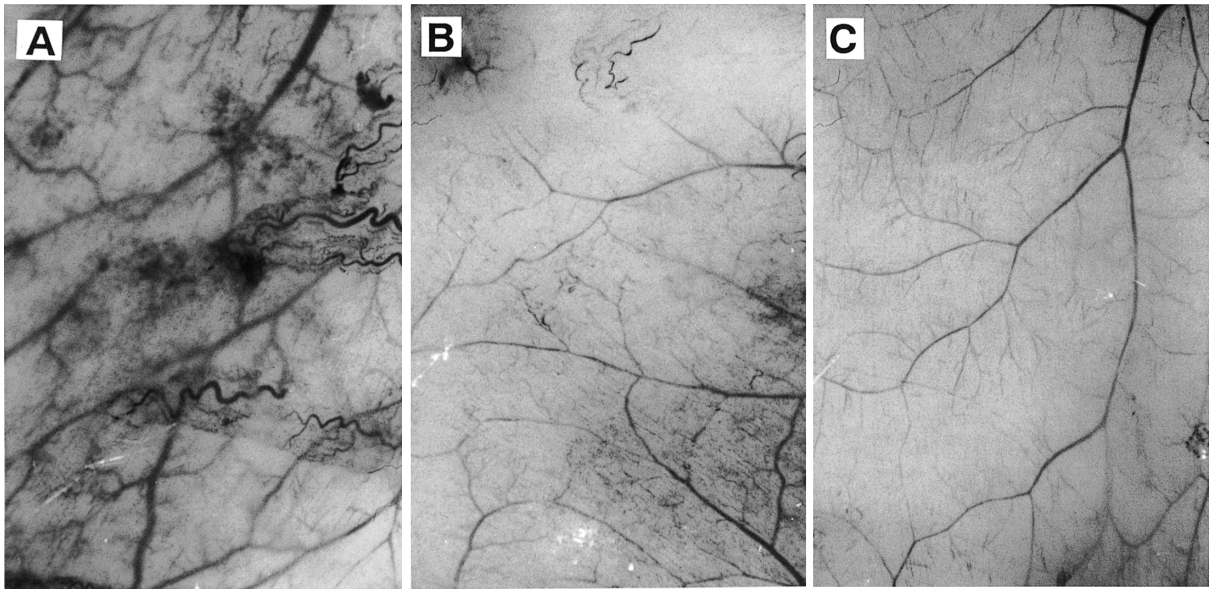

Fig. 1. Inhibition of tumor-induced angiogenesis by MV833. Chambers packed with HT-1080 cells were implanted s.c. into the abdominal region of BALB/c mice on day 0. PBS (A) or 100 μg of MV833 (B) was i.v. administered from days 1 to 3. The control chamber (C) contained PBS instead of HT-1080 cells. Mice were killed, and the s.c. region was photographed on day 4.

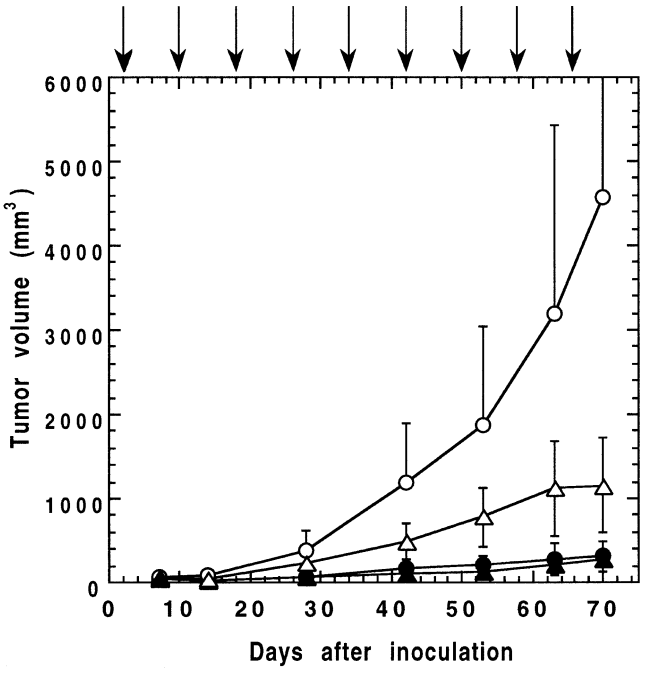

Fig. 2. The tumor growth of two HeLa cell lines and its inhibition by MV833 *in vivo*. HeLa/v5 (●, ○) and HeLa/c (▲, △) tumors were cut into 2×2×2-mm pieces, and transplanted into nude mice. MV833 (100 μg; ●, ▲) or PBS (○, △) was administered i.v. every 4 days. The volumes of tumors were monitored by caliper measurement. Arrows, injections.

expression of VEGF receptors were not correlated with the strength of the antitumor activity of MV833.

In some tumor lines, treatment with MV833 was delayed until the tumor volume had reached 400–500 mm^3; almost complete suppression was observed after the treatment (Fig. 4).

DISCUSSION

Neovascularization is necessary for the growth of solid tumors. Solid tumors are thought to secrete TAF, thereby inducing new vessel formation for explosive growth. It is becoming clear that VEGF plays the most important role in tumor angiogenesis among several angiogenic factors so far characterized.

We previously obtained two VEGF-neutralizing monoclonal antibodies, MV101 and MV303, by immunization of mice with human $VEGF_{121}$ and established their potential as therapeutic agents for solid tumors.[22, 23] Next, we wished to establish another neutralizing antibody which exhibited higher activity than MV101 and MV303, and we selected MV833 from over 140 clones of hybridomas producing anti-VEGF MAb.[24] In this paper, we deal with the role of VEGF in tumor growth *in vivo* and the anti-angiogenic and antitumor activity of MV833 against various kinds of tumors implanted in nude mice.

I.v. administrations of MV833 to HT-1080 tumor cell-implanted nude mice clearly inhibited tumor cell-induced

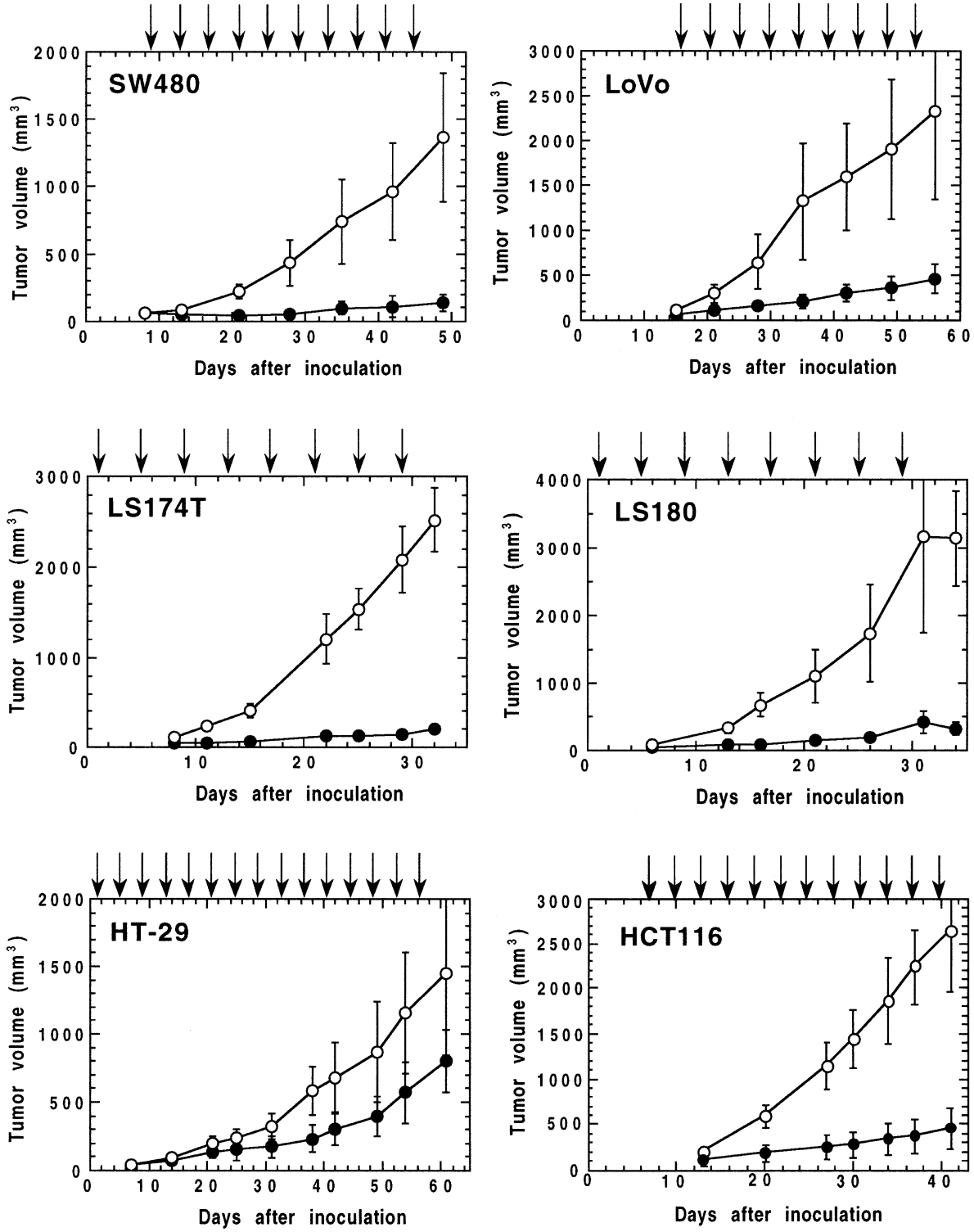

Fig. 3. Inhibitory effect of MV833 on the growth of human colon tumors *in vivo*. Human colon tumors maintained s.c. in nude mice were cut into 2×2×2-mm pieces, and transplanted into native nude mice. MV833 (100 μg; ●) or PBS (○) was administered i.v. every 4 days until the experiments were terminated. The volumes of tumors were monitored by caliper measurement. Arrows, injections.

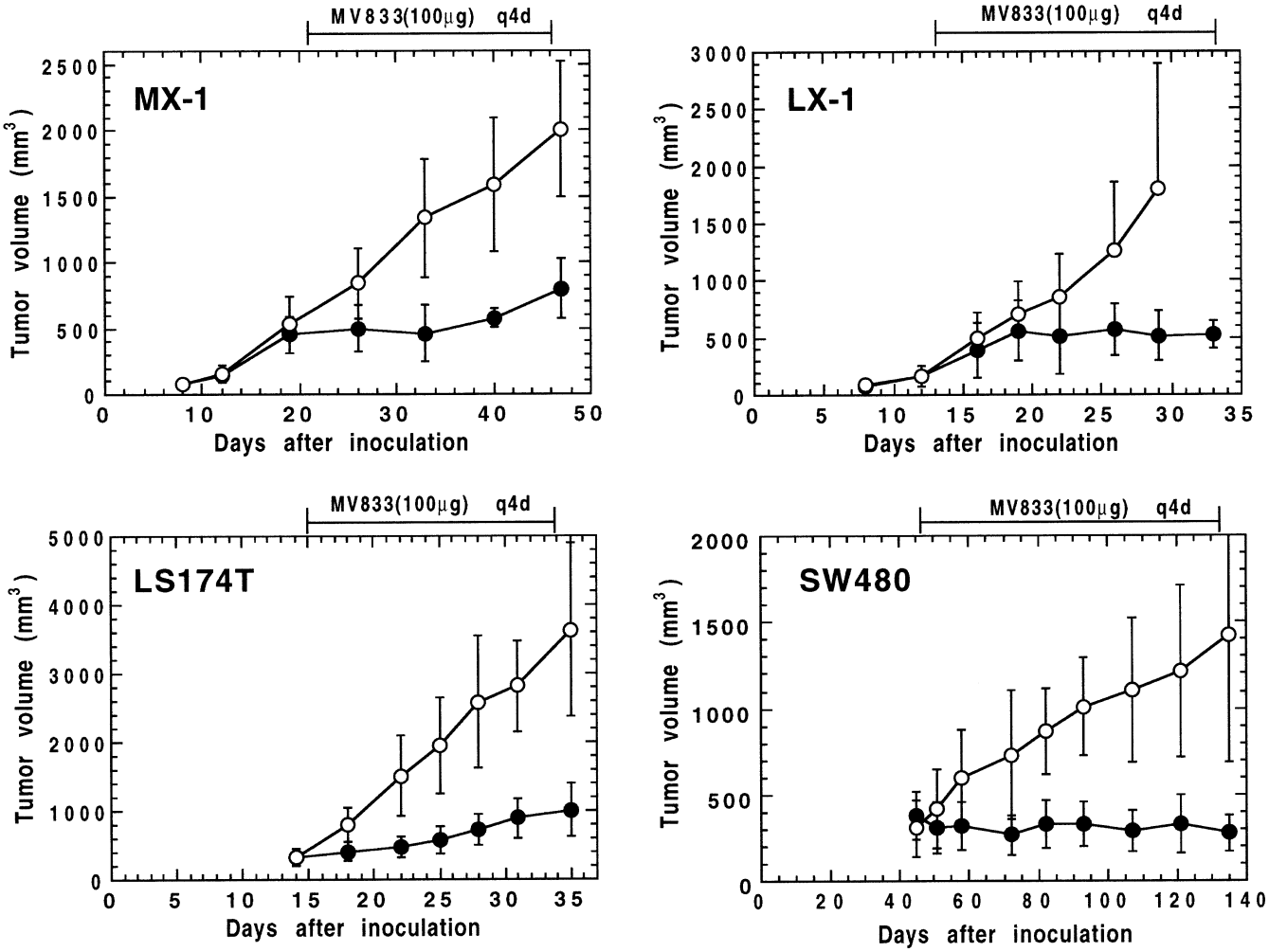

Fig. 4. Inhibition of advanced growing tumor by MV833 *in vivo*. Human tumor maintained s.c. on nude mice was cut into 2×2×2-mm pieces, and transplanted into native nude mice. MV833 (100 μg; ●) or PBS (○) was administered i.v. every 4 days from the time indicated until the experiments were terminated (above bar). The volumes of tumors were monitored by caliper measurement.

neovascularization (Fig. 1). HeLa/v5,[18] which was transfected with human $VEGF_{121}$ gene and secreted relatively high levels of VEGF, acquired the ability to grow rapidly in nude mice (Fig. 2), although its doubling time *in vitro* was not changed from that of the parental cell line, supporting an important role of VEGF in tumor growth *in vivo*. The 27 human tumor cell lines originating from various solid tumors, including colon, lung, breast, pancreas, and melanoma, secreted various levels of VEGF into the culture media, though MV833 did not inhibit their growth *in vitro* (Table I). The growth of tumors that produced high levels of VEGF (LS180, BXPC-3, A-673, and HT-1080) was relatively fast. MV833 strongly blocked the growth of all kinds of tumors, despite differences in the ability to secrete VEGF *in vitro* (Tables I and II). These results again support the idea that VEGF is the most important and generally used TAF among many angiogenic factors.

In some tumors (HT-29, ASPC-1, HRA and PC-14), the inhibition by MV833 was somewhat weak. This could be explained by limited distribution of MV833 in these solid tumors and/or the possible existence of an additional TAF in these tumors, as Kim *et al*. predicted based on experiments using an anti-VEGF MAb.[26]

Two VEGF receptors (Flt-1 and Flk-1/KDR) were expressed specifically on the surface of endothelial cells[27, 28] and only Flk-1/KDR is known as a functional transducer of VEGF's mitogenic signal in endothelial cells.[29] Some tumor cell lines (melanoma, ovarian carcinoma and megakaryoblastic leukemia) were reported to

express VEGF receptor, though their growth was not affected by VEGF *in vitro*.[17, 30, 31] We also observed the expression of VEGF receptors in A375, WM-115 and PC-3 cells (Table I). Recently, Soker *et al*. showed that PC-3 cells expressed the $VEGF_{165}$-specific receptor known as neuropilin-1.[32] The functions of VEGF receptors expressed on these tumor cells are not clear.

Kim *et al*., Warren *et al*., and Melnyk *et al*. also reported antitumor activity of VEGF-neutralizing antibodies against several human tumors.[26, 33, 34] Millauer *et al*. showed the inhibitory activity of a dominant-negative Flk-1 mutant.[35, 36]

In this study, we established the strong inhibitory activity of MV833, a neutralizing MAb to human VEGF, against various kinds of solid tumors. The inhibitory spectrum of MV833 was very wide and the degree of inhibition was very large. Moreover, strong inhibition of tumor growth was observed even after delayed administration in some tumor lines tested (Fig. 4). Unlike cytotoxic antitumor agents, MAb is expected to produce few side-effects and should not induce resistant cells, because it does not work directly on the tumor cells.

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