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

Makoto Asano, Ayako Yukita and Hideo Suzuki

Bioscience Research Department, Tsukuba Research Laboratory, Toagosei Co., Ltd., 2 Ohkubo, Tsukuba, Ibaraki 300-2611

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 \textit{in vitro}. However, the growth of the cell lines, including three which expressed VEGF receptor, was not affected by exogenously added MV833 \textit{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 \textit{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 \textit{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. 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 vasulotropin.$^{6-8}$ 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 \textit{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 \textit{VEGF}_{121}. Among them, 26 clones showed neutralizing activity and MV833 possessed the most potent antitumor activity \textit{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 \textit{VEGF}_{121}$^{25}$ Spleen cells were fused with mouse myeloma Sp2/O-Ag14 and hybridomas were cloned by limiting dilution.$^{20}$ Hybridomas 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
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 VEGF121 gene-bearing or control vector, respectively.18)

**Cell growth in vitro** Cells (1–4×10^4) 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% CO2. 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 125I-labeled VEGF165 (66 TBq/mmol; Amersham, Buckinghamshire, UK) at 37°C for 3 h with or without a 100-fold amount of unlabeled VEGF165 (R&D Systems Inc., Minneapolis, MN). Unbound radioligand was removed, and the cells were solubilized in 1% SDS (sodium dodecyl sulfate). The amount of 125I-labeled VEGF165 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

| Cell line   | Type               | VEGF production | VEGF receptor |
|-------------|--------------------|-----------------|---------------|
| A375        | Melanoma           | +               | +             |
| G361        | Melanoma           | +               | –             |
| WM-115      | Melanoma           | ++              | –             |
| PC-14       | Lung carcinoma     | +               | –             |
| MX-1        | Breast 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   | +               | –             |

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**Table I. Characteristics of Tumor Cell Lines Used**

*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 125I-labeled VEGF165 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×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. 125I-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).

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 shown significantly faster than that of 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                |
| MiaPaCa-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                |
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 $2\times2\times2$-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.
neovascularization (Fig. 1). HeLa/v5, which was transfected with human VEGF 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.

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

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.
express VEGF receptor, though their growth was not affected by VEGF in vitro.\textsuperscript{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\textsubscript{165}-specific receptor known as neuropilin-1.\textsuperscript{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.\textsuperscript{26, 33, 34} Millauer et al. showed the inhibitory activity of a dominant-negative Flk-1 mutant.\textsuperscript{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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