Inhibition of Tumor Growth and Metastasis by an Immunoneutralizing Monoclonal Antibody to Human Vascular Endothelial Growth Factor/Vascular Permeability Factor

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ABSTRACT

We elucidated the relationship between vascular endothelial growth factor/vascular permeability factor (VEGF/VPF), which is a potent angiogenic factor, and the growth of primary and metastatic tumors using an immunoneutralizing monoclonal antibody against human VEGF/VPF. The monoclonal antibody, MV303, suppressed the growth of human umbilical vein endothelial cells (HUVEC) induced by VEGF/VPF, or VEGF/VPF, but did not inhibit its growth induced by basic fibroblast growth factor. MV303 inhibited the binding of 125I-VEGF/VPF, or VEGF/VPF, to HUVEC. We examined the effects of MV303 on tumor angiogenesis using a membrane chamber packed with the human fibrosarcoma cell line HT-1080 and implanted s.c. into BALB/c mice. The neovascularization induced by HT-1080 was inhibited by the i.v. injection of MV303 at a dose of 100 μg/mouse. Furthermore, the growth of solid tumors of s.c. implanted HT-1080 in BALB/c nude mice was almost completely inhibited by the i.v. and s.c. administration of MV303 ten times from day 1 at a dose of 100 μg/mouse (T/C values of tumor volume at day 18 were 0.20 and 0.18, respectively). Tumor growth was suppressed when MV303 was administered, even from eight days after tumor inoculation. MV303 suppressed the increase in lung weight caused by experimental metastasis with i.v. inoculation of cultured HT-1080 cells to BALB/c mice. The life spans of the mice treated with MV303 were significantly prolonged. These results indicated that VEGF/VPF played an important role in both primary and metastatic tumor growth as a tumor angiogenesis factor. MV303, an immunoneutralizing monoclonal antibody against VEGF/VPF, potently inhibited both primary and metastatic tumor growth with no marked side effects.

INTRODUCTION

Neovascularization is essential for the growth of solid tumors (1, 2). Tumor cells are thought to secrete angiogenesis factor(s) to induce neovascularization around tumors (3–5). Several angiogenesis factors such as acidic and basic fibroblast growth factor (6–8), transforming growth factor-α and -β (9, 10), angiogenin (11), angiotropin (12), tumor necrosis factor-α (13, 14), and platelet-derived endothelial cell growth factor (15, 16) have been identified.

VEGF (17–20), also known as VPF (21–24) or vasculotropin (25, 26), is also an angiogenesis factor. It specifically promotes the growth of endothelial cells and induces vascular leakage. A CDNA analysis of human VEGF/VPF shows that it has a signal peptide and is similar in structure to platelet-derived growth factor (18, 22). VEGF/VPF stimulates the growth of endothelial cells by binding to cell surface receptors (27). Two receptor-type tyrosine kinases, Flt-1 (28) and KDR (29), have been identified as high affinity receptors for VEGF/VPF in human endothelial cells (30, 31). VEGF/VPF is secreted into the culture medium by several tumor cell lines (32–35), and we (36) and Yeo et al. (37) found VEGF/VPF in the sera and pleural or peritoneal fluids of cancer patients. Tumor cells that acquired the ability to secrete high levels of VEGF/VPF by gene transformation or mutation can be grown more rapidly than their parental cells in nude mice (32, 35). The inhibition of VEGF/VPF activity by an immunoneutralizing antibody suppresses tumor growth (35, 38). Neovascularization is also necessary for the growth of metastatic tumors (39). Thus, it is important to understand whether or not inhibiting the activity of VEGF/VPF could suppress tumor growth at metastatic regions.

Here we described the generation and characterization of an immunoneutralizing monoclonal antibody, MV303, against human VEGF/VPF and its potent suppression of not only primary but also metastatic tumor growth.

MATERIALS AND METHODS

Materials. Recombinant human VEGF/VPF was purified from the culture medium of transformed yeast cells (40). An insect-cell derived recombinant human VEGF/VPF was purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant human bFGF was purchased from Oncogene Science, Inc. (Uniondale, NY).

Preparation of Monoclonal Antibody against VEGF/VPF and Mouse Immunoglobulin. Mouse melanoma cells, Sp2/0-Ag14, were fused with spleen cells from BALB/c mice that were immunized by GST-VEGF/VPF (41). BALB/c nude mice were inoculated i.p. with hybridomas, and monoclonal antibodies were purified from ascites fluids using a protein-G Sepharose column (Pharmacia, Uppsala, Sweden). Mouse immunoglobulin (IgG) was also prepared from ascites fluids from mouse plasmacytoma, MPC-31C-transplanted nude mice using a protein-G Sepharose column.

Cell Lines. Human umbilical vein endothelial cells (HUVEC) were purchased from Kurabo (Osaka, Japan) and maintained in MCDB131 medium containing 2% FBS, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 10 μg/ml endothelial cell growth supplement, and 10 μg/ml heparin. The human fibrosarcoma cell line, HT-1080, was purchased from the American Type Culture Collection and maintained in DMEM supplemented with 10% FBS. Cells were cultured at 37°C in 5% CO2 under a humidified atmosphere.

Cell Growth Assay. HUVEC growth assay was as described (35) with some modifications. HUVEC were seeded on collagen-coated, 96-well culture plates at a density of 2 × 104 cells/well in 100 μl of medium containing 10 ng/ml VEGF/VPF, or VEGF/VPF, or bFGF with various concentrations of MV303 for 4 days. Thereafter, 10 μl of [methyl-3H]Thymidine (1.85 MBq/ml, 925 GBq/mmol; Amer sham) were added to each well and incubated for 24 h. Cells were collected on glass fiber filters using a PHD-cell harvester (Cambridge Technology, Inc., Watertown, MA). The radioactivity level was counted in a liquid scintillation counter.

HT-1080 cells were seeded on 96-well culture plates at a density of 2 × 104 cells/well in 100 μl of medium with various concentrations of MV303 for 2 days. [methyl-3H]Thymidine uptake was performed in the same manner as described above.

Binding of 125I-labeled VEGF/VPF, to HUVEC. VEGF/VPF was radiiodinated using chloramine-T (42), and 125I-labeled VEGF/VPF (about 106 cpm/ng) was purified on a PD-10 column (Pharmacia). HUVEC were cultured to subconfluence on collagen-coated, 24-well culture plates. About 6 × 106 cpm of 125I-labeled VEGF/VPF were incubated with several doses of MV303 at room temperature for 3 h, then with HUVEC at 37°C for 3 h.
After unbound radioligand was removed, the cells were solubilized in 1% SDS. The amount of 125I-labeled VEGF/VPF121 bound to HUVEC was measured using a gamma counter (COBRA II; Packard, Meriden, CT).

**Angiogenesis by Tumor Cells in Vivo.** in vivo angiogenesis was assayed by the dorsal air-sac method (43). Briefly, 1 x 10^7 cultured HT-1080 cells were suspended in PBS and packed into round-shaped cellulose ester membrane chambers with a diameter of 14 mm (pore size, 0.45 μm; Millipore, Bedford, MA) and implanted into a dorsal air sac of male BALB/c mice (SLC, Hamamatsu, Japan; day 0). MV303 (100 μg) was i.v. administered from days 1 to 3. The mice were sacrificed on day 4, and the formation of new blood vessels in s.c. regions was examined under a dissecting microscope.

**Tumor Growth in Vivo.** The HT-1080 tumor was maintained in male BALB/c nude mice (Nihon Clea Co., Tokyo, Japan). A solid tumor, measuring 2 x 2 x 2 mm, was dissected and transplanted s.c. into the abdominal region of BALB/c nude mice. MV303 (100 μg) was administered ten times on the indicated days. The control mice were administered saline or 100 μg of mouse immunoglobulin. The tumor was measured in two dimensions, and the volume was calculated using the formula, width^2 x length/2.

**Experimental Metastasis.** For the experimental metastasis model, cultured HT-1080 cells were harvested by incubation with PBS containing 0.5 mM EDTA. Viable cells were counted by trypan blue dye exclusion under a microscope. Fractions containing over 95% of viable cells were used in this experiment. Cells (1 x 10^6 cells/ml) were suspended in PBS, and 0.1 ml of the suspension was inoculated i.v. into the tail vein of BALB/c nude mice (day 0). MV303 (100 μg) was administered i.v. on days 1—2, 5—9, 12—16, and 19—23, and the lung was weighed on day 25. In another experiment, the number of metastatic foci in lungs was counted under a dissecting microscope on day 14.

**RESULTS**

**Effect of MV303 on the Growth of HUVEC and HT-1080 Cells in Vitro.** We immunized BALB/c mice with GST-VEGF/VPF121, and obtained 28 hybridomas producing the monoclonal antibodies against VEGF/VPF121. MV303 was an immunoneutralizing monoclonal antibody against VEGF/VPF121, and it belonged to the IgG2a subclass. Fig. 1a shows the immunoneutralizing activity of MV303 on the growth of HUVEC induced by VEGF/VPF or bFGF. MV303 (1 μg/ml) completely inhibited the growth of HUVEC, which was stimulated by 10 ng/ml of VEGF/VPF121 or VEGF/VPF165. In contrast, MV303 did not affect the growth of HUVEC induced by 10 ng/ml of bFGF. These results indicated that MV303 recognized VEGF/VPF121 and VEGF/VPF165, but not bFGF, and that the immunoneutralizing activity of MV303 was specific for VEGF/VPF. MV303 did not exhibit any direct inhibitory activity, even at 100 μg/ml, on the growth of cultured human fibrosarcoma HT-1080 cells in vitro (Fig. 1b).

**Effect of MV303 on the Binding of 125I-labeled VEGF/VPF121 to HUVEC.** We investigated the mechanism of the neutralizing activity of MV303 by means of a radio receptor assay using 125I-labeled VEGF/VPF121 and HUVEC. MV303 inhibited the binding of 125I-VEGF/VPF121 to HUVEC in a dose-dependent manner (Fig. 2). Thus, the mechanism of inhibition by MV303 on mitogenic activity of VEGF/VPF to HUVEC was considered to be that MV303 bound VEGF/VPF and inhibited the binding of VEGF/VPF to its receptors.

**Inhibition of Tumor-Induced Neovascularization by MV303.** To visualize the neovascularization induced by tumors, we selected HT-1080 human fibrosarcoma cells because they produce high levels of VEGF/VPF (35). Four days after implanting of the tumor cells, which were packed into membrane chambers, neovascularization from surrounding blood vessels was evident in the region touched by the chamber containing HT-1080 cells (Fig. 3, left). Three i.v. administrations of 100 μg of MV303 (Fig. 3, middle) suppressed the neovascularization induced by HT-1080 cells to the level of the control (no tumor cells; Fig. 3, right). These results indicated that MV303 inhibited neovascularization induced by tumor cells in vivo by suppressing VEGF activity.

**Antitumor Activity of MV303 against HT-1080.** Fig. 4 shows the antitumor activity of MV303. The growth of HT-1080 that was transplanted s.c. into the abdominal region of BALB/c nude mice were significantly suppressed by 10 i.p. administrations of MV303 (Fig. 4a; P < 0.01, from days 11 to 20). But the administration of the same dose of mouse immunoglobulin (MOPC-31C) had no effect. On day 20, the T/C value of tumor volume in mice administered MV303 or mouse immunoglobulin against saline control was 0.26 or 0.88, respectively. This finding shows that the antitumor activity of MV303 was not dependent on the nonspecific action of the administration of mouse immunoglobulin. Thus, in the following experiments, we administered only saline as a control to examine the activity of MV303. Fig. 4b also shows the antitumor activity of MV303 by other administration routes. The administration of MV303 both s.c. and i.v. more potently suppressed the growth of HT-1080. On day 18, the T/C value...
ANITITUMOR ACTIVITY OF ANTI-VEGF MoAb

MV303-treated mice had significantly prolonged life span. The weights of the mice treated with MV303 were similar to those of the control mice at day 14 (Table 1), and they appeared as healthy as the control mice during the experiment, suggesting that no severe side effects were caused by MV303.

DISCUSSION

We raised monoclonal antibodies against human VEGF/VPF,121 by immunizing mice with a GST-VEGF/VPF,121 conjugate protein produced in Escherichia coli (41). Among them, MV303 had high neutralizing activity against VEGF/VPF. The antibody blocked the growth of HUVEC induced by VEGF/VPF,121 or VEGF/VPF,165 at doses over 0.2 µg/ml (Fig. 1). However, it did not affect the growth of HUVEC induced by bFGF, suggesting that its neutralizing activity was specific for VEGF/VPF. The four known isoforms of VEGF/VPF consist of 121, 165, 189, and 206 amino acids (44, 45). The former two are secreted as soluble proteins, and the latter two are tightly bound to the extracellular matrix (45). Since we used the shortest isoforms as the antigen, the monoclonal antibodies should have reacted with all types of isoforms of VEGF/VPF. Indeed, MV303 reacted with not only VEGF/VPF,121 but also VEGF/VPF,165 (Fig. 1). The shortest isoforms possessed the full activities of angiogenesis and vascular permeability (40).

MV303 inhibited the binding of 125I-labeled VEGF/VPF,121 to HUVEC (Fig. 2). Two types of receptors for VEGF/VPF have been identified as Flt-1 and KDR (30, 31), and they have different signal transduction properties in HUVEC (46). MV303 is thought to block the binding of VEGF/VPF to both types of receptors by forming an immunocomplex with them, although this should be further clarified.

We have demonstrated previously that a s.c. injection of a polyclonal antibody against human VEGF/VPF,121 blocked the growth of HeLa/v5-transfected cells with human VEGF/VPF,121 cDNA, which secreted high amounts of VEGF/VPF,121, as well as the growth of human hepatoma PLC/PRF/5 cells in nude mice (35). Kim et al. (38) have reported that i.p. administration of an immunoneutralizing monoclonal antibody for human VEGF/VPF,165 partially inhibits the growth of tumors in nude mice, and they discussed the presence of additional angiogenic factors such as bFGF or acidic fibroblast growth factor. In this study, we demonstrated that not only i.p. but also s.c. and i.v. injections of MV303 suppressed the growth of solid tumors implanted s.c. into nude mice (Fig. 4). We also assessed the effect of MV303 on tumor metastasis in an experimental system in which...
neovascularization at the surrounding regions and/or inside solid tumors. Stimulation of host immune systems such as antibody-dependent, cell-mediated cytotoxicity or complement-dependent cytotoxicity might contribute to the decrease in the number of metastatic foci (Table 1).

bFGF is also a tumor angiogenic factor (8, 47). Immunoneutralizing antibodies against bFGF suppressed tumor growth (47, 48). On the contrary, other groups reported that immunoneutralizing antibodies against bFGF had little or no effect on tumor growth (49—51). This discrepancy might be due to the tumor cells they used. Unlike bFGF, VEGF/VPF has a signal peptide, which gives it the ability to act as a paracrine mediator. Indeed, all of the cultured tumor cell lines we examined secreted VEGF/VPF into the medium (35). The immunoneutralizing monoclonal antibody, MV303, almost completely blocked the growth of solid tumors after ten i.v. and s.c. administrations of 100 μg/mouse and significantly prolonged their life span (Figs. 4b and 5). These finding indicated that VEGF/VPF is the most important mediator of tumor angiogenesis.

We showed that the inhibiting activity of VEGF/VPF suppresses the growth of primary and metastatic solid tumors, indicating that VEGF/VPF can be a target of antitumor agents. Contrary to cytotoxic

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of foci at 14 days</th>
<th>Lung weight at 25 days</th>
<th>Body weight at 25 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>152.6 ± 60.0f</td>
<td>695.7 ± 309.1</td>
<td>25.18 ± 1.30</td>
</tr>
<tr>
<td>MV303</td>
<td>98.9 ± 33.5f</td>
<td>258.4 ± 67.1f</td>
<td>25.17 ± 1.36f</td>
</tr>
</tbody>
</table>

- Metastatic foci were counted on day 14; n = 8.
- Lung weights were measured on day 25; n = 7.
- The mice were weighed on day 14; n = 10.
- Mean ± SD.
- P < 0.05 versus saline control, calculated by the t test.
- P < 0.01 versus saline control, calculated by the t test.
- Not significant versus saline control.

Table 1: Effects of MV303 on the experimental metastasis model using HT-1080 cells

Fig. 4. Inhibition of tumor growth by MV303. HT-1080 tumor was inoculated into BALB/c nude mice on day 0. a. MV303 or mouse immunoglobulin (MOPC-31C) (100 μg) was administered i.p. on days 1—4, 7—11, and 14. b. MV303 (100 μg) was administered s.c. or i.v. on days 1—4, 8—11, 14, and 15; in another experiment, s.c. administration was on days 8—11, 14—18, and 21. The control group was administered with saline on days 1—4, 8—11, 14, and 15. Mean values are averages of five tumors; bars, SD. Arrows, times of injection.

Fig. 5. Prolonged survival time of HT-1080 tumor-bearing mice induced by MV303. Cultured HT-1080 cells were inoculated i.v. into BALB/c nude mice on day 0. Saline or 100 μg MV303 was administered i.v. on days 1—2, 5—9, 12—16, and 19—23. Each group comprised 10 mice. Statistical significance: P < 0.001 versus saline control, calculated using the Cox-Mantel test. Arrows, times of injection.

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antitumor drugs, MV303 did not exert appreciable side effects during the experiments, and it was effective not only when it was administered from day 1 but also when treatment was initiated 8 days after tumor inoculation, when solid tumors started to grow (Fig. 4B). These are good advantages for VEGF/VPF inhibitors when compared to cytotoxic antitumor drugs. We are currently studying the optimal time schedule and treatment doses of MV303.

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