

The Therapeutic Efficacy of Anti – Vascular Endothelial Growth Factor Antibody, Bevacizumab, and Pemetrexed against Orthotopically Implanted Human Pleural Mesothelioma Cells in Severe Combined Immunodeficient Mice

Qi Li,^{1,3} Seiji Yano,^{1,3} Hirokazu Ogino,¹ Wei Wang,^{1,3} Hisanori Uehara,² Yasuhiko Nishioka,¹ and Saburo Sone¹

Abstract Purpose: Malignant pleural mesothelioma (MPM) is an aggressive malignancy, which has a poor prognosis with a median survival of less than 1 year. The vascular endothelial growth factor (VEGF) has been reported to be an ideal therapeutic target, and a multitargeted antifolate, pemetrexed, has been clinically used for the treatment of MPM.

Experimental Design: We examined the therapeutic efficacy of the antihuman VEGF neutralizing antibody, bevacizumab, in combination with pemetrexed against two different human MPM cells, EHMES-10 and MSTO-211H, orthotopically inoculated into severe combined immunodeficient mice.

Results: Bevacizumab inhibited a VEGF-induced proliferation of the human endothelial cells in a dose-dependent manner, but it had no effect on the proliferation of the two MPM cell lines *in vitro*. The orthotopically inoculated EHMES-10 cells (VEGF high expressing) produced thoracic tumors and a large volume of bloody pleural effusion, whereas the MSTO-211H cells (VEGF low expressing) produced thoracic tumors and a small volume of bloody effusions. Treatment with bevacizumab effectively inhibited the production of thoracic tumors and dramatically prevented the production of pleural effusion by the EHMES-10 cells but not the MSTO-211H cells. Treatment with bevacizumab reduced the number of enlarged tumor-associated vessels and proliferating tumor cells. Moreover, treatment with bevacizumab in combination with pemetrexed more effectively suppressed the formation of the pleural effusion and prolonged the survival compared with the control and monotherapy in the EHMES-10 cell – bearing severe combined immunodeficient mice.

Conclusions: These results suggest that the combined use of bevacizumab and pemetrexed may therefore be promising for controlling the progression of MPM highly expressing VEGF.

Malignant pleural mesothelioma (MPM) grows aggressively with dissemination in the thoracic cavity and frequently produces a malignant pleural effusion (1). More than 60% of patients with MPM commonly present with a pleural effusion

associated with breathlessness, often accompanied by chest-wall pain, which compromises their quality of life (2). This tumor was once considered rare, but its incidence is increasing worldwide, which can be associated with asbestos exposure and potentially with the SV40 tumor virus. MPM is refractory to conventional chemotherapy and radiotherapy, and it also has a poor prognosis (median survival time from onset is ~ 1 year). Recently, the multitargeted antifolate pemetrexed has been approved as the front-line agent in combination with cisplatin for the treatment of mesothelioma (3). Nonetheless, the overall outcome of this disease remains very poor. Therefore, novel effective therapies are necessary for improving the prognosis of this disease.

Angiogenesis is crucial for the growth and metastasis of solid tumors, to deliver oxygen and nutrients to the expanding tumor mass (4), and several reports have shown an inverse correlation between the microvessel density in tumors and the survival of the patients with various cancers, including MPM (5–7). Among the many reported angiogenic factors, the vascular endothelial growth factor (VEGF) is the most potent endothelial cell – specific mitogen associated with tumor neovascularization.

Authors' Affiliations: Departments of ¹Internal Medicine and Molecular Therapeutics; ²Molecular and Environmental Pathology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima; and ³Division of Medical Oncology, Molecular and Cellular Targeting Translational Oncology Center, Cancer Research Institute, Kanazawa University, Ishikawa, Japan Received 2/28/07; revised 6/20/07; accepted 6/29/07.

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Requests for reprints: Saburo Sone, Department of Internal Medicine and Molecular Therapeutics, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan. Phone: 81-88-633-7127; Fax: 81-88-633-2134; E-mail: sson@clin.med.tokushima-u.ac.jp.

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It also has significant effects on vascular permeability while also mediating the development of a pleural effusion and ascites (8, 9). VEGF, also known as VEGF-A, is a member of the VEGF/platelet-derived growth factor gene family. It consists of at least six isoforms (VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206), which are regulated by splicing at the mRNA level, and VEGF165 is the most abundant and biologically potent isoform. VEGF binds with high affinity to two tyrosine kinase receptors, VEGF receptor (VEGFR)-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), on the endothelial cells, thus inducing receptor dimerization, autophosphorylation, and signal transduction. Many studies have shown VEGF to be overexpressed in most human tumors (10). Antiangiogenic strategies targeting the VEGF pathway, such as the monoclonal antibody directed against VEGF or its receptors VEGFR-1 and VEGFR-2 and the small-molecule tyrosine kinase inhibitors, have been developed and found to reveal a promising antitumor effect.

Bevacizumab, a humanized anti-VEGF monoclonal neutralizing antibody, blocks the binding of VEGF to its receptor and neutralizes all of the isoforms of human VEGF (11). Some preclinical and clinical trials of bevacizumab have shown its antitumor and chemotherapy synergic effects in a variety of malignancies, including lung, colorectal, prostate, and renal cell cancer (12–15).

We established recently a patient-like severe combined immunodeficient (SCID) mouse model by the orthotopic implantation of MPM cells (16). Using this model, we herein investigated whether bevacizumab could inhibit the production of thoracic tumors and pleural effusion produced by human MPM cell lines and further assessed the therapeutic efficacy of bevacizumab in combination with pemetrexed.

Materials and Methods

Cell lines. Human MPM cell lines, EHME-10, were kindly provided by Dr. H. Hamada (Ehime University, Ehime, Japan), and MSTO-211H was purchased from American Type Culture Collection. The tumor cell culture was maintained in MEM or RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and gentamicin at 37°C in a humidified atmosphere of 5% CO₂ in air. The human dermal microvascular endothelial cells (HMVEC) were maintained in HuMedia-MvG with growth supplements (Kurabo) and used for *in vitro* assay at passages 2 to 5.

Reagents. Bevacizumab was purchased from Genentech and Co. Pemetrexed was kindly provided by Eli Lilly and Co. Recombinant human VEGF165, recombinant human basic fibroblast growth factor (bFGF), and control human IgG were purchased from R&D Systems.

Determination of VEGF protein level. Tumor cells (2×10^5) were cultured in 2 mL MEM (EHME-10) or RPMI 1640 (MSTO-211H) with 10% fetal bovine serum for 48 h. The culture supernatants were harvested and the level of VEGF protein was determined using an ELISA kit according to the manufacturer's instructions (R&D Systems).

Expression of VEGF isoforms and VEGFRs. Expression of the VEGF isoforms and VEGFRs was determined by reverse transcription-PCR. Total cellular RNA was isolated using the RNeasy Mini kit and RNase-free DNase kits (Qiagen) according to the manufacturer's protocols. Total RNAs were reversely transcribed using an Omniscript RT kit (Qiagen). The primers for VEGF, VEGFR, and β -actin were as follows: VEGF antisense (5'-TCCAGGAGTACCCTGATGAG-3') and antisense (5'-CTTCCTGGTGAGAGATCTGG-3') immediately flanking the region of the VEGF open reading frame involved in the alternative splicing of several exons (17); VEGFR-1 sense (5'-ATTGTGATTTGGCCTTC-

3') and antisense (5'-CAGGCTCATGAAGTGAAGC-3'); VEGFR-2 sense (5'-GTGACCAACATGGAGTCGTG-3') and antisense (5'-CCAGACATTC-CATGCCACTT-3'); VEGFR-3 sense (5'-CCCACGACAGATCAAGACG-3') and antisense (5'-TGCAGAACTCCACGATCACC-3'); and β -actin sense (5'-AAGAGAGGCATCCTCACCT-3') and antisense (5'-TACATGGCTGGGTGTTGAA-3'). PCR was done using the Ex Taq Hot Start Version (Takara). The bands were visualized by ethidium bromide staining.

Proliferation of tumor cells and endothelial cells. Cell proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction method (18). Briefly, the tumor cells (2×10^3 /200 μ L/well) that had been plated in triplicate in 96-well plates were incubated in MEM containing 5% fetal bovine serum for 24 h. The HMVECs (5×10^3 /200 μ L/well) plated in triplicate in 96-well plates precoated with 1.5% gelatin were incubated in a supplemented M131 medium for 24 h. Next, the cells (200 μ L/well) were washed and incubated for 72 h with bevacizumab in a fresh MEM containing 5% fetal bovine serum in the presence or absence of VEGF, bFGF, or culture supernatants of MPM cells. Then, 50 μ L of stock MTT solution (2 mg/mL; Sigma) were added to all of the wells and the cells were incubated for 2 h at 37°C. The media containing the MTT solution were removed and the dark blue crystals were dissolved by adding 100 μ L DMSO. Absorbance was measured with an MTP-120 microplate reader (Corona Electric) at test and reference wavelengths of 550 and 630 nm, respectively.

Orthotopic implantation model. The cultured EHME-10 cells or MSTO-211H cells were harvested by pipetting. The cells were washed twice and resuspended in PBS. Thereafter, the tumor cells (1×10^6 /100 μ L PBS) were injected into the thoracic cavity of the SCID mice (16). The mice were treated with bevacizumab alone or in combination with pemetrexed, and 3 weeks (MSTO-211H) or 4 weeks (EHME-10) after tumor cell inoculation, the mice were sacrificed. The thoracic tumors were then carefully removed and weighed; the pleural effusion was harvested using a 1-mL syringe, and the volume of the pleural effusion was measured.

Immunohistochemical determination of endothelial cells, proliferating cells, and VEGF production. For bromodeoxyuridine (BrdUrd) staining, the mice were injected i.p. with BrdUrd solution (200 μ L; Zymed BrdUrd staining kit, Zymed Lab). Two hours later, the mice were killed and the thoracic tumors were collected. The thoracic tumors were cut into 5-mm fragments and placed into either a buffered 10% formalin solution or an optimum cutting temperature compound (Miles Laboratories) and snap frozen in liquid nitrogen for immunohistochemical analysis. The paraffin-embedded tissues (4 μ m thick) were used to quantify the *in vivo* VEGF production using mouse anti-human VEGF monoclonal antibody (1:100 dilution; PharMingen). The frozen tissue sections (8 μ m thick) were used for identification of the endothelial cells using rat anti-mouse CD31/platelet/endothelial cell adhesion molecule-1 monoclonal antibody (1:100 dilution; PharMingen) and the proliferating cells using a BrdUrd kit (Zymed BrdUrd staining kit).

Double staining for CD31 with proliferating cells. The frozen tissue sections (8 μ m thick) were fixed with cold acetone and washed with PBS. After blocking with 5% normal horse serum, the slides were incubated with mouse anti-BrdUrd monoclonal antibody (Zymed BrdUrd staining kit) and an anti-CD31 monoclonal antibody (PharMingen) at 4°C overnight. After washing with PBS, the slides were stained with matched secondary antibodies conjugated with Alexa Fluor 594 (red) or Alexa Fluor 488 (green; 1:200 dilution; Molecular Probes). The localized green fluorescence and red fluorescence were detected by fluorescence microscope (19).

Quantification of immunohistochemistry and immunofluorescence. The five areas containing the highest numbers of staining within a section were selected for histologic quantification under light microscopy or fluorescent microscopy with a 40-fold magnification (17). The results were evaluated by two authors (Q. L. and H. U.).

Statistical analysis. The statistical significance of the difference between the *in vitro* and *in vivo* data was analyzed by Student's *t* test and one-way ANOVA with Dunnett's post test, respectively.

Results

Characteristics of human MPM cell lines in vitro. In the first set of experiments, we determined the expression of VEGF and its receptor in the MPM cell lines by ELISA and reverse transcription-PCR. The EHMES-10 cells expressed high levels of VEGF mRNA and secreted a large amount of VEGF (Fig. 1A and B). On the other hand, the MSTO-211H cells expressed very low levels of VEGF at both the protein and mRNA levels. Neither EHMES-10 nor the MSTO-211H cells expressed discernible levels of VEGFR-1 and VEGFR-3 mRNA. Both cell lines expressed VEGFR-2 mRNA, but the expression in EHMES-10 cells was very weak (Fig. 1A and B). None of VEGFR-1, VEGFR-2, or VEGFR-3 was detected at the protein level by means of Western blotting (data not shown).

We next examined the biological activity of the VEGF produced by the EHMES-10 cells. Addition to the recombinant human VEGF or recombinant human bFGF stimulating the proliferation of HMVEC, the bevacizumab inhibited the proliferation induced by VEGF, but not by bFGF, thus indicating the specificity of the anti-VEGF antibody to VEGF

(Fig. 1C). Under these experimental conditions, the proliferation of the HMVECs was significantly stimulated by the addition of the culture supernatants of the EHMES-10 cells (final concentration of VEGF in the supernatant was 50 ng/mL), and pretreatment of the supernatants with bevacizumab (10 µg/mL), but not control IgG (10 µg/mL), effectively inhibited the growth of HMVECs (Fig. 1D). These results indicate that the VEGF secreted by the EHMES-10 cells was VEGF biologically active.

We further examined whether the VEGF acted as a growth factor for the MPM cells. Neither the recombinant human VEGF nor recombinant human bFGF affected the proliferation of the MPM cells. Moreover, the bevacizumab did not affect the proliferation of the MPM cells (Fig. 1E), indicating that VEGF is not the growth factor of EHMES-10 or MSTO-211H cells, at least in our experimental condition.

Therapeutic effect of bevacizumab on the production of the thoracic tumor and pleural effusion by VEGF-high-producing EHMES-10 cells. We have shown previously that an intrathoracic injection of EHMES-10 cells into SCID mice produced thoracic tumors and a pleural effusion within 30 days of the

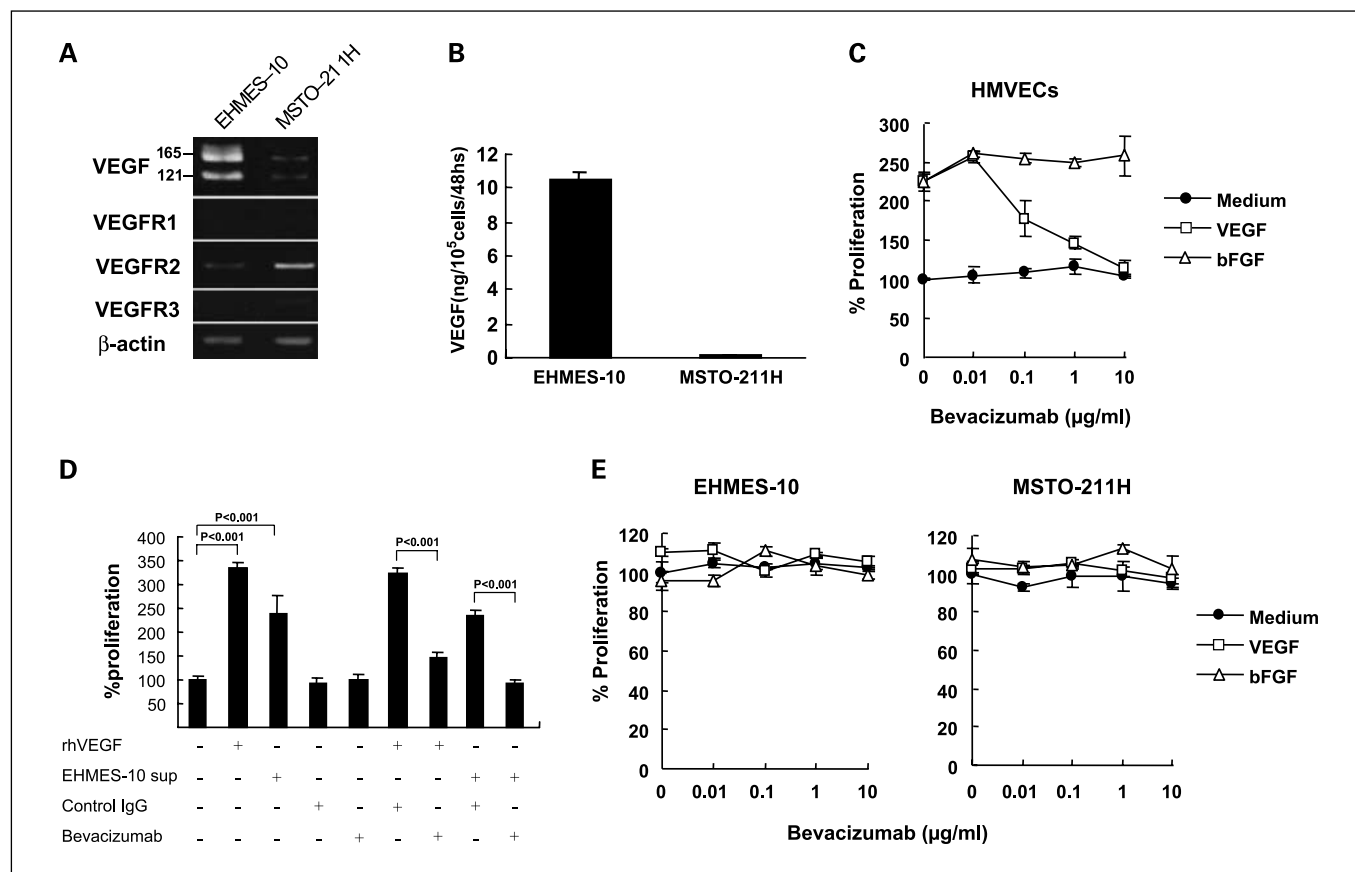


Fig. 1. Characteristics of human MPM cell lines *in vitro*. *A*, expression of the VEGF isoforms and VEGFRs was determined by reverse transcription-PCR. The EHMES-10 cells expressed high levels of VEGF and the MSTO-211H cells expressed very low levels of VEGF. The MSTO-211H cells, but not the EHMES-10 cells, expressed VEGFR-2 mRNA. *B*, VEGF protein secretion by the MPM cell lines was determined by ELISA. Only the EHMES-10 cells secreted a high level of the VEGF protein. *C*, specificity of bevacizumab to VEGF was determined by MTT assay. *D*, the biological activity of the VEGF expressed by the EHMES-10 cells was determined by MTT assay. The HMVECs (5×10^3 /200 µL/well) were incubated for 72 h with bevacizumab (10 µg/mL) or control IgG (10 µg/mL) in the presence or absence of recombinant human VEGF (rhVEGF; 50 ng/mL) or recombinant human bFGF (50 ng/mL; *C*) or culture supernatants of EHMES-10 (final concentration of VEGF in the supernatant was 50 ng/mL; *D*). Next, MTT assay was done. Bevacizumab inhibited the proliferation of the endothelial cells stimulated by VEGF in dose-dependent manner, and the VEGF secreted by the EHMES-10 cell was biologically active. *E*, the effect of bevacizumab on the *in vitro* proliferation of the MPM cells determined by MTT assay. Bevacizumab did not affect the proliferation of the MPM cells *in vitro*. Data are representative of three independent experiments with similar results. Columns and points, mean of triplicate cultures; bars, SD.

Table 1. Effect of bevacizumab on thoracic tumor and pleural effusion produced by MPM cells in SCID mice

Experiment	Cell line	Treatment	Dose ($\mu\text{g}/\text{mouse}$)	Schedule (day)	Thoracic tumor		Pleural effusion	
					Incidence	Weight (mg), median (range)	Incidence	Volume (μL), median (range)
1	EHMES-10	PBS			5/5	180 (160-470)	4/5	500 (0-700)
		Control IgG	30	7, 10, 14, 17	4/4	190 (70-220)	3/4	750 (0-1,200)
		Bevacizumab	3	7, 10, 14, 17	4/5	160 (0-370)	3/5	250 (0-900)
			10	7, 10, 14, 17	4/5	50* (0-120)	0/5	0 [†] (0)
			30	7, 10, 14, 17	5/5	30* (20-50)	0/5	0 [†] (0)
2	EHMES-10	PBS			5/5	120 (60-230)	3/5	750 (0-900)
		Bevacizumab	10	7, 10, 14, 17	5/5	40* (20-60)	0/5	0 [†] (0)
			10	7, 10, 14, 17, 21, 25	3/4	55* (0-60)	0/5	0 [†] (0)
			10	14, 17, 21, 25	3/5	90 (0-190)	1/5	0 [†] (0-100)
3	MSTO-211H	PBS			5/5	400 (360-570)	1/5	0 (0-50)
		Bevacizumab	10	7, 10, 14, 17	4/4	400 (370-420)	0/4	0 (0)
			30	7, 10, 14, 17	5/5	380 (360-580)	1/5	0 (0-40)
			30	4, 7, 10, 14	4/4	190 (60-430)	1/4	0 (0-70)

NOTE: EHMES-10 cells or MSTO-211H cells (1×10^6) were inoculated into thoracic cavity of SCID mice on day 0. Mice inoculated with EHMES-10 were treated with bevacizumab at different dose on days 7, 10, 14, and 17 (experiment 1) or treated with bevacizumab 10 $\mu\text{g}/\text{mouse}$ i.p. on different days (experiment 2). All of the mice were sacrificed on day 28. Mice inoculated with MSTO-211H were treated with bevacizumab at different dose or on different days (experiment 3), and then the mice were sacrificed on day 21. The thoracic tumor and pleural effusion were evaluated as described in Materials and Method. Values are the median (minimum-maximum).

* $P < 0.05$ compared with control group.

[†] $P < 0.01$ compared with control group (one-way ANOVA with Dunnett's post test).

inoculation of the cells. The administration of bevacizumab or control IgG (twice weekly continued for 2 weeks) commenced 7 days after the tumor cell inoculation (because at this time, the EHMES-10 cells progressed to a primary tumor more than

2 mm in the thoracic cavity), and 4 weeks later, the mice were killed and the formation of the thoracic tumor and pleural effusion were evaluated. The EHMES-10 cells developed a thoracic tumor and large volume bloody pleural effusion in the control mice (Table 1, experiment 1; Fig. 2), as reported previously. Treatment with the control IgG or a low dose (3 $\mu\text{g}/\text{mouse}$) of bevacizumab had no effect on the thoracic tumor or the pleural effusion, whereas treatment with bevacizumab (10 $\mu\text{g}/\text{mouse}$ or 30 $\mu\text{g}/\text{mouse}$) significantly prevented the growth of the thoracic tumors and dramatically inhibited the production of the pleural effusion (Table 1, experiment 1; Fig. 2). Moreover, bevacizumab did not show any adverse events *in vivo* (data not shown). Based on these results, we chose a dose of 10 μg bevacizumab/mouse for further experiments.

To evaluate the effect of timing of initiation of bevacizumab treatment on the production of thoracic tumors and pleural effusion by the EHMES-10 cells, we injected the tumor cells into the thoracic cavity of the SCID mice on day 0 and then treated the mice with bevacizumab 10 $\mu\text{g}/\text{mouse}$ twice weekly starting on day 7 or 14 for 2 or 3 weeks. Both treatments with bevacizumab remarkably inhibited the pleural effusion production and the early treatment effectively prevented the thoracic tumor growth; however, the late treatment did not (Table 1, experiment 2).

We also examined the effect of bevacizumab on production of thoracic tumor and pleural effusion produced by the VEGF-low-producing MSTO-211H cells. The MSTO-211H cells produced a thoracic tumor and small volume pleural effusion (Table 1, experiment 3). Treatment with bevacizumab, at 10 μg or 30 $\mu\text{g}/\text{mouse}$ started on day 7, had no significant effect on the production of thoracic tumors and pleural effusion (Table 1, experiment 3). Whereas treatment with bevacizumab at 30 $\mu\text{g}/\text{mouse}$ started on day 4 tended to inhibit the weight of

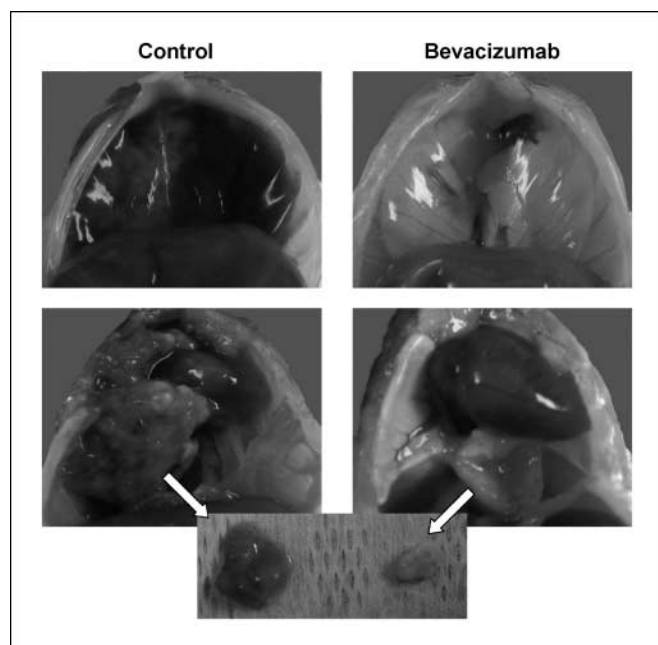


Fig. 2. Formation of thoracic tumor and pleural effusion by EHMES-10 cells with or without bevacizumab treatment. The SCID mice inoculated with EHMES-10 cells (1×10^6) were treated with or without bevacizumab (10 $\mu\text{g}/\text{mouse}$ i.p. on days 7, 10, 14, and 17). Four weeks after tumor cell inoculation, the mice were sacrificed and the formations of the pleural effusion and thoracic tumor were evaluated as described in Materials and Methods. The EHMES-10 cells produced a large volume of bloody pleural effusion and thoracic tumors. Treatment with bevacizumab inhibited the production of the thoracic tumors and pleural effusion.

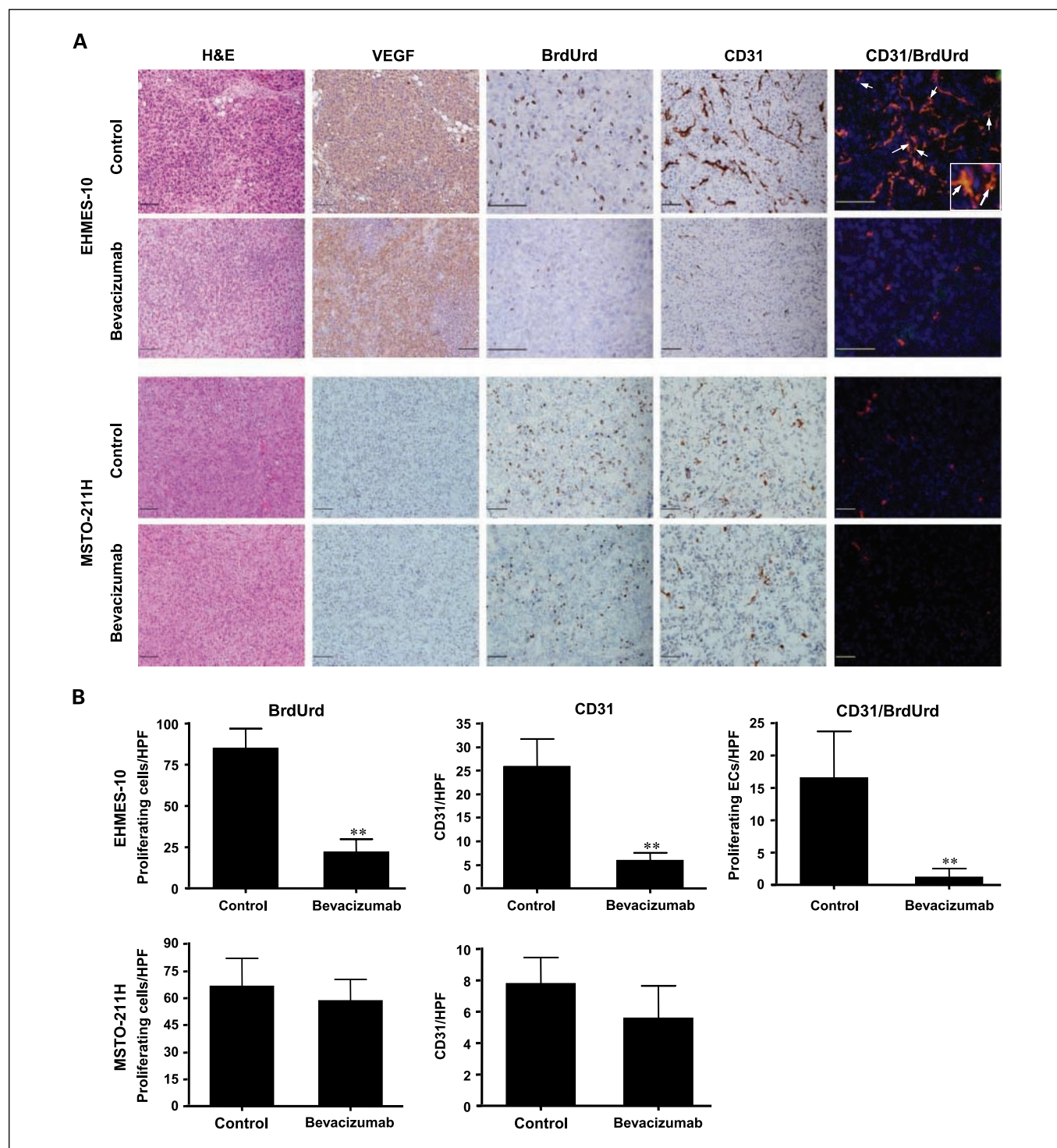


Fig. 3. Histologic analysis of thoracic tumor produced by MPM cells. The MPM cell – bearing SCID mice were treated with or without bevacizumab (10 μ g/mouse i.p. on days 7, 10, 14, and 17) and killed on day 21 (MSTO-211H) or 28 (EHMES-10). The thoracic tumors were analyzed by H&E, immunohistochemistry, and immunofluorescence. **A**, treatment with bevacizumab inhibited the number of the proliferating tumor cells (*BrdUrd*), tumor-associated endothelial cells (*CD31*), and proliferating endothelial cells (double positive for *CD31* and *BrdUrd*). Bar, 100 μ m. Arrows, double-positive cells. Magnification, \times 400. **B**, quantitative immunohistochemistry and immunofluorescence analyses. Columns, mean number from five independent areas; bars, SD. **, $P < 0.01$ compared with the control. HPF, high-power field.

thoracic tumors, the difference was not statistically significant. These results are consistent with the effect of bevacizumab being associated with the level of VEGF production by the MPM cells.

Immunohistochemical and immunofluorescence staining to clarify the antitumor mechanisms of bevacizumab. Treatment with bevacizumab 10 μ g/mouse on days 7, 10, 14, and 17 reduced the number of BrdUrd-positive proliferating tumor

cells and CD31/BrdUrd double-positive proliferating endothelial cells and decreased the microvessel density in the thoracic tumor produced by the EHMS-10 cells but did not affect the VEGF expression (Fig. 3). In the MSTO-211H tumors, the CD31 and CD31/BrdUrd double staining also showed a low proliferation of endothelial cells and only slight vascularization (Fig. 3). Similar results were obtained when the vascularization was determined using CD34 staining (data not shown).

Effect of bevacizumab in combination with chemotherapeutic agent against EHMS-10 cells in vitro and in vivo. In the final set of experiments, we evaluated the effect of bevacizumab in combination with the chemotherapeutic agent (pemetrexed) on the proliferation of HMVECs and MPM cells *in vitro* and the production of thoracic tumors and pleural effusion in the EHMS-10 cell-bearing SCID mice. *In vitro*, the bevacizumab in combination with pemetrexed did not show a significant difference in the proliferation of HMVEC, EHMS-10 (Fig. 4A), or MSTO-211H cells (data not shown) compared with the pemetrexed single therapy. *In vivo*, the SCID mice bearing EHMS-10 or MSTO-211H cells were treated with bevacizumab (10 µg/mouse or 30 µg/mouse twice weekly for 2 weeks starting on day 7) alone or in combination with pemetrexed (100 mg/kg thrice weekly for 2 weeks starting on day 7). The combination therapy more effectively inhibited the tumor growth than the single therapy in SCID mice bearing EHMS-10 cells but not MSTO-211H cells (Table 2). Furthermore, the combina-

tion therapy significantly prolonged the survival time of EHMS-10 cell-bearing SCID mice compared with the single therapy (Fig. 4B).

Discussion

Neovascularization has been strongly suggested to play a critical role in tumor growth and the progression in various malignant diseases, including MPM, and VEGF is one of the major molecular targets for antiangiogenic therapy. In the present study, we evaluated the therapeutic efficacy of the anti-VEGF antibody, bevacizumab, in patient-like orthotopic implantation models using two MPM cell lines with different VEGF-producing potentials. As expected, the VEGF-low-producing MSTO-211H cells produced thoracic tumors with a small amount of pleural effusion, and bevacizumab had no significant therapeutic effect on this cell line. In contrast, the VEGF-high-producing EHMS-10 cells produced thoracic tumors with a large amount of bloody pleural effusion, as reported previously (16). The bevacizumab potently inhibited the production of the thoracic tumors and shut off the pleural effusion formation, thus prolonging the survival of the mice bearing the VEGF-high-producing EHMS-10 cells. We further showed that these effects were augmented by the combined use of pemetrexed, which is a new anticancer drug approved recently for the treatment of MPM. Although several lines of evidence have shown the involvement of VEGF in the

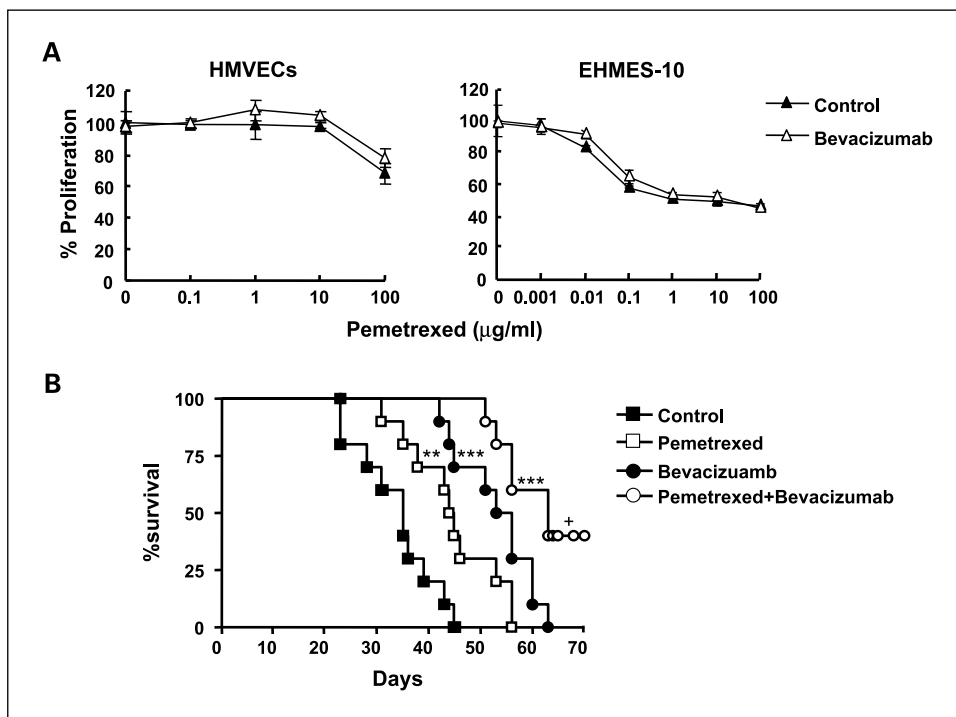


Fig. 4. Effect of bevacizumab in combination with pemetrexed on *in vitro* proliferation of HMVECs and EHMS-10 cells and survival time of EHMS-10 cell-bearing SCID mice. **A**, the effect of pemetrexed alone or in combination with bevacizumab on proliferation of the EHMS-10 cells and endothelial cells. The MPM cells (2×10^3 per well) or HMVECs (5×10^3 per well) were incubated with pemetrexed alone or in combination with bevacizumab (1 µg/mL). Next, MTT assay was done. Pemetrexed inhibited the proliferation of the EHMS-10 cells and the IC_{50} arrived at 1 µg/mL but not HMVECs even at a dose as high as 100 µg/mL. Bevacizumab in combination with pemetrexed also did not show an additional or synergistic effect on the proliferation of the EHMS-10 or endothelial cells *in vitro*. Data are representative of three independent experiments with similar results. Points, mean of triplicate cultures; bars, SD. **B**, the EHMS-10 cell-bearing SCID mice were treated with bevacizumab (10 µg/mouse i.p. on days 7, 10, 14, and 17) or in combination with pemetrexed (100 mg/kg i.p. on days 7, 8, 9, 14, 15, and 16). Bevacizumab in combination with the chemotherapeutic agent more effectively prolonged the survival time compared with monotherapy. **, $P < 0.01$ compared with the control group; ***, $P < 0.0001$ compared with the control group; +, $P < 0.05$ compared with the bevacizumab monotherapy group.

Table 2. Therapeutic efficacy of bevacizumab in combination with chemotherapeutic agent on production of thoracic tumor and pleural effusion produced by MPM cells in SCID mice

Cell line	Treatment	Thoracic tumor		Pleural effusion	
		Incidence	Weight (mg), median (range)	Incidence	Volume (μ L), median (range)
EHMES-10	PBS	5/5	110 (70-160)	5/5	600 (100-900)
	Bevacizumab	5/5	10* (10-20)	0/5	0 [†] (0)
	Pemetrexed	4/5	30* (0-120)	2/5	0 (0-900)
	Combination	1/5	0* [‡] (0-10)	0/5	0 [†] (0)
MSTO-211H	PBS	5/5	400 (360-570)	1/5	0 (0-50)
	Bevacizumab	5/5	380 (360-580)	1/5	0 (0-40)
	Pemetrexed	4/4	345 (150-390)	3/4	60 (0-200)
	Combination	4/4	330 (250-430)	1/5	0 (0-50)

NOTE: EHMES-10 or MSTO-211H (1×10^6) cells were inoculated into thoracic cavity of SCID mice on day 0, and the mice were treated with bevacizumab 10 μ g/mouse (EHMES-10) or 30 μ g/mouse (MSTO-211H) i.p. on days 7, 10, 14, and 17 or in combination with pemetrexed (100 mg/kg i.p. on days 7, 8, 9, 14, 15, and 16). The mice were sacrificed on day 28 (EHMES-10) or day 21 (MSTO-211H) and thoracic tumor and pleural effusion were evaluated as described in Materials and Method. Values are the median (minimum-maximum).

* $P < 0.01$ compared with control group (one-way ANOVA with Dunnett's post test).

[†] $P < 0.05$ compared with control group.

[‡] $P < 0.05$ compared with bevacizumab group (Mann-Whitney test).

progression of MPM, this is the first preclinical report, to our best knowledge, showing the therapeutic efficacy of bevacizumab against the progression of MPM cells.

VEGF is a multifunctional cytokine, which seems to have two major roles on the progression of MPM. First, VEGF can act as an endothelial cell mitogen stimulating angiogenesis and thus tumor growth. Second, it acts as a vascular permeability factor facilitating the production of pleural effusion, as we reported previously using the VEGF-high-producing lung adenocarcinoma PC14PE6 cells. Our findings in the present study confirmed them because the VEGF-high-producing EHMES-10 cells, but not the VEGF-low-producing MSTO-211H cells, developed highly angiogenic thoracic tumors with proliferating endothelial cells and a large amount of pleural effusions. In addition, treatment with bevacizumab dramatically inhibited the endothelial cell proliferation, production of thoracic tumors, and formation of pleural effusion by the EHMES-10 cells but not the MSTO-211H cells.

VEGF was reported recently as an autocrine or paracrine growth factor for several types of tumor cells, including MPM, expressing VEGFR-2. However, this is not the case because neither recombinant human VEGF nor bevacizumab affected the proliferation of the two MPM cell lines *in vitro* in the presence or absence of various concentrations of fetal bovine serum (Fig. 1E; data not shown). MSTO-211H expressed VEGFR-2 at the mRNA level (Fig. 1A), but we could not detect VEGFR-2 at the protein level (data not shown). Therefore, other growth factor receptors and their signaling pathways may be much more important for the proliferation of this cell line.

Malignant pleural effusion is associated with a patient being highly symptomatic, and most patients with pleural effusion present with progressive dyspnea, cough, or chest pain, which compromises their quality of life (20). Previous reports have shown that drainage followed by the instillation of sclerosing agents is useful for controlling pleural effusion and improving the quality of life in such patients. However, the efficacy of this treatment is variable and does not extend the survival of lung cancer patients (21). Clearly, a more effective therapy for malignant pleural effusion is needed. We therefore focused on

VEGF as a therapeutic molecule because of its activity inducing vascular hyperpermeability. VEGF induces interendothelial gaps and endothelial fenestration and augments vascular permeability by activating the cytoplasmic vesicular-vacuolar organelles in the endothelial cells (22–24). An increased permeability of the blood vessels facilitates the extravasation of proteins and, thus, the formation of a pleural effusion. In addition, we have shown that the malignant pleural effusion due to lung cancer and MPM contained a high level of VEGF (8, 16). Moreover, we developed an animal model for pleural effusion with human lung adenocarcinoma cell lines (PC14PE6) and showed the therapeutic potential of the VEGFR-2 tyrosine kinase inhibitors, PKI 787 and ZD6474 (25, 26). In the present study, we reported the dramatic effect of the anti-VEGF antibody against the pleural effusion formation by the VEGF-high-producing MPM cells. Collectively, a novel strategy targeting the VEGF/VEGFR signaling pathway to control pleural effusion may be useful for improving the quality of life of patients with the VEGF-high-producing MPM.

Because VEGF is reported to be essential for the development of a neovasculature in the very early stages of tumorigenesis rather than in the advanced phase of cancer (27), the timing of the initiation of the antiangiogenesis treatment may be important. Because a tumor requires a vascular blood supply to grow beyond 1 to 2 mm (28), we confirmed that the tumorigenesis (1-2 mm) of the EHMES-10 cells was produced 7 days after the tumor cell inoculation in our model. The present data showed that the treatment with bevacizumab was most effective on the inhibition of the tumor when started early (from day 7 after tumor inoculation), suggesting that an antiangiogenic treatment may be effective for controlling the clinical early stage of MPM.

Pemetrexed is the first and only chemotherapy agent that has been granted marketing approval for use in combination with cisplatin for the treatment of chemonaive patients with unresectable MPM. Although pemetrexed combined with cisplatin showed a significant survival prolongation compared with cisplatin alone, the difference was only 2.8 months (29). It is therefore necessary to augment the therapeutic effect of

pemetrexed to further improve the survival of MPM patients. On the other hand, bevacizumab has been shown to augment the therapeutic efficacy of conventional chemotherapy against solid tumors, including colon cancer and non-small cell lung cancer (12, 13). In the present study, we clearly showed the increased efficacy of pemetrexed in combination with bevacizumab against MPM in the orthotopic implantation model. We could not do a histologic examination to clarify the mechanism of the increased efficacy of bevacizumab and conventional chemotherapy because the combined therapy was so effective that we could not obtain enough tumor samples. One of the important processes with cytotoxic agents is gaining access to the interior of the tumor. However, overexpressing VEGF produces irregular, high-density, and hyperpermeability vessels, which cause increased interstitial fluid pressure in tumors. These abnormalities result in the inhibition of tumor penetration by cytotoxic agents and therefore limit the efficacy of chemotherapy (30). One of the proposed mechanisms of action of bevacizumab is the normalization of the vasculature by decreasing the macromolecular permeability (and thus the interstitial fluid pressure)

and thereby improving the blood perfusion (31). Therefore, bevacizumab might improve the delivery of pemetrexed to the interior of the tumor, thus augmenting the beneficial effect over monotherapy in SCID mice bearing VEGF-high-producing MPM cells (EHMES-10).

In summary, we showed that bevacizumab, an anti-VEGF neutralizing antibody, potently inhibited the production of thoracic tumors while also preventing pleural effusion formation and thus prolonging the survival of the mice bearing VEGF-high-producing EHMES-10 cells. We further showed these effects to be augmented by the combined use with pemetrexed, which is a new anticancer drug that has been approved recently for MPM. These results suggest that bevacizumab in combination with pemetrexed may therefore be a promising treatment modality for controlling the progression of VEGF-high-producing MPM.

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