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Antitumor Effect on Human Gastric Cancer and Induction of Apoptosis by Vascular Endothelial Growth Factor Neutralizing Antibody

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Induction of apoptosis by antiangiogenic therapy has been suggested as a new anticancer strategy. To clarify the mechanism of the antitumor effect achieved by inhibition of vascular endothelial growth factor (VEGF), which is a major mediator of angiogenesis, we used an orthotopic transplantation model of human gastric carcinoma line (MT2) treated with a monoclonal VEGF neutralizing antibody (VEGF Ab). We histologically examined the microvessel density (MVD) and the apoptotic index (AI) in this model. Transplanted tumor growth was significantly inhibited by the VEGF Ab ($P=0.03$), and there was a significant decrease in the number of mice with liver metastasis ($P=0.004$). The MVD detected by immunohistochemical staining with ER-MP12 antibody was 33.6±8.0 in the control group and 21.1±5.4 in the treated group, and the difference was significant ($P<0.0001$). The AI values of the control and treated groups were 4.73±1.11 and 7.26±1.62, respectively, and this difference is also significant ($P<0.0001$). However, the expression of VEGF mRNA in transplanted tumors did not show a significant difference between the control and treated groups. These results suggest that the antitumor effect of the VEGF Ab on human gastric carcinoma is exerted by inducing mild hypoxia followed by apoptosis, which does not influence VEGF mRNA expression in the carcinoma.

Key words: Antiangiogenic therapy — Apoptosis — Vascular endothelial growth factor — Microvessel density

Tumor growth is regulated by the balance between cell proliferation and cell death.[1)] There are two mechanisms of cell death, necrosis and apoptosis, and both of them influence tumor regression. Recently, it was demonstrated that most of the cell death caused by anticancer therapy was due to apoptosis,[2–4)] and thus induction of apoptosis has received a great deal of attention in the development of new anticancer strategies.

In 1995, Holmgren *et al.* showed that tumor growth could be restricted when apoptosis of tumor cells was elicited by inhibition of angiogenesis in a transplantable Lewis lung carcinoma model.[5)] They suggested that systemic suppression of angiogenesis maintained Lewis lung carcinoma micrometastases in a dormant state because rapid cell proliferation was balanced by a high rate of apoptosis, and they proposed the concept of "tumor dormancy." In addition, O'Reilly *et al.* demonstrated the induction of apoptosis *in vivo* using angiostatin[6)] and endostatin.[7)] Our previous studies also demonstrated that induction of apoptosis by TNP-470 was important for prolonging the survival of experimental animals.[8, 9)]

Tumor angiogenesis is mediated by various angiogenic factors.[10)] Among them, vascular endothelial growth factor (VEGF) is a highly potent angiogenic factor and a selective mitogen for endothelial cells,[11)] and it is secreted by various human tumors. Recently, the VEGF protein level has been studied in the primary tumors or serum of patients with some types of cancer, and some of these studies have shown a correlation between VEGF expression and prognosis.[12, 13)] This raised the possibility of a new type of anticancer therapy targeting the VEGF-ligand system.

Kim *et al.* reported that specific monoclonal antibodies could inhibit VEGF-induced angiogenesis *in vivo* and *in vitro*.[14)] It was also reported that a monoclonal VEGF neutralizing antibody (VEGF Ab) exerted a potent inhibitory effect on the growth of several human tumor cell lines.[15, 16)] Asano *et al.* developed an anti-human VEGF Ab (MV833), which showed a potent immunoneutralizing activity.[17)] MV833 inhibited the growth of human umbilical vein endothelial cells induced by $VEGF_{121}$ and the growth of human fibrosarcoma HT-1080 xenografts in nude mice.[17)] We have previously reported that administration of MV833 not only inhibited the growth of transplanted tumors, but also metastasis in spontaneous

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metastatic models of colon and gastric cancer.[18] However, it is still unclear whether blockade of the VEGF system induces apoptosis. In the present study, we used an orthotopic transplantation model of human gastric cancer treated with MV833, and examined the microvessel density (MVD) and the apoptotic index (AI) of the transplanted tumors to clarify whether apoptosis was induced by administration of MV833. To evaluate the influence of MV833 on the level of VEGF mRNA expression, we also performed northern blot analysis of VEGF mRNA in this model.

MATERIALS AND METHODS

Materials An anti-human VEGF Ab (MV833) was established using hybridomas obtained by fusing spleen cells obtained from $VEGF_{121}$-immunized mice with mouse myeloma cells (Sp2/O-Ag14). The activity and characteristics of this antibody have already been reported.[17]

Human gastric carcinoma MT2 human gastric carcinoma, which is a poorly differentiated adenocarcinoma,[19] was used in this study. Xenografts were established from a surgical specimen at our department and were maintained by serial subcutaneous transplantation in nude mice.

Experimental design Five-week-old male BALB/c-*nu*/*nu* mice were obtained from Clea Japan, Inc. (Tokyo). The method of tumor transplantation was reported previously.[20, 21] In brief, the stomach wall was carefully exposed and the serosa on the middle of the greater curvature was removed, and a piece of tumor tissue (200 mg) was fixed to the site with a transmural suture of 6-0-coated Vicryl (Ethicon, Somerville, NJ). Then the mice were randomly divided into a control group (n=15) and a treated group (n=15) on day 11 after transplantation. Mice in the treated group were given MV833 intraperitoneally at a dose of 100 μg/2 ml on alternate days (12 times in total). The same volume of phosphate-buffered saline (PBS) was given to mice in the control group. In a previous experiment, we confirmed that there was no difference between using normal IgG or PBS for the control group.[18] All mice were weighed and killed on day 42 after transplantation, and autopsy was performed immediately.

Assay of tumor growth and hepatic metastasis The transplanted tumors growing on the gastric wall and the livers were removed from each animal at autopsy. The gastric tumors were weighed, and macroscopic metastases visible on the liver surface were evaluated carefully. Specimens were examined histologically in the usual manner.

Immunohistochemical staining and determination of the MVD Microvessels in the transplanted tumors were stained immunohistochemically using a rat monoclonal antibody (ER-MP12) by the avidin-biotin-peroxidase complex technique. For determination of the MVD in transplanted tumors, the three most vascular areas within a section were chosen and the stained vessels were counted under a light microscope at 200-fold magnification. The average count was recorded as the MVD for each animal.

Determination of the AI Apoptotic cells were detected in hematoxylin-eosin (HE)-stained sections under a light microscope with high-power (400-fold) magnification, according to the criteria of Kerr *et al.*[22] and Walker *et al.*[23] The AI was expressed as the percentage of apoptotic cells and apoptotic bodies relative to all tumor cells, and was calculated after counting at least 2000 tumor cells in each animal.

Northern blot analysis of VEGF mRNA Specimens resected from the transplanted tumors were frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted by the acid guanidinium-phenol-chloroform method using TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The quantity of RNA was determined spectrophotometrically. Thirty micrograms of total RNA per lane was fractionated on 1.0% agarose gel containing 2.2 *M* formaldehyde, transferred to a nylon membrane (Hybond-N, Amersham, Tokyo), and cross-linked by exposure to ultraviolet light. Then the membrane was hybridized with ^{32}P-labeled cDNA probes for human VEGF and human β-actin in hybridization buffer at 42°C for 16 h and washed three times. Blots were exposed to X-ray film (MS-1, Kodak, Rochester, NY) at −80°C using an intensifying screen. The signal intensity was quantified using a GT-9000 image scanner (Epson, Tokyo) and image analysis software (NIH Image, ver 1.55, Bethesda, MD). For each sample measured, the ratio of the intensity of the VEGF signal to that of β-actin was calculated.

A VEGF cDNA fragment (573 bp) corresponding to the amino acid-coding region of $VEGF_{165}$ was obtained by the reverse transcription-polymerase chain reaction method of Sharkey *et al.*[24] Human β-actin cDNA (β-actin DNA Probe Solution) was obtained from Nippon Gene (Tokyo). For ^{32}P labeling, the random priming method was employed using a Megaprime DNA labeling system (Amersham).

Statistical analysis The tumor weight, MVD, and AI data are given as the mean±standard deviation and the significance of differences was evaluated by using Student's t test. The χ^2 test and Fisher's exact test were used to compare the numbers of mice with liver metastasis. A P value <0.05 was considered significant for all analyses.

RESULTS

Tumor growth and liver metastasis As reported previously,[18] MT2 showed a high rate of metastasis to the liver, and administration of MV833 significantly inhibited the growth of both transplanted MT2 tumors and liver metastases. A transplanted tumor growing on the gastric

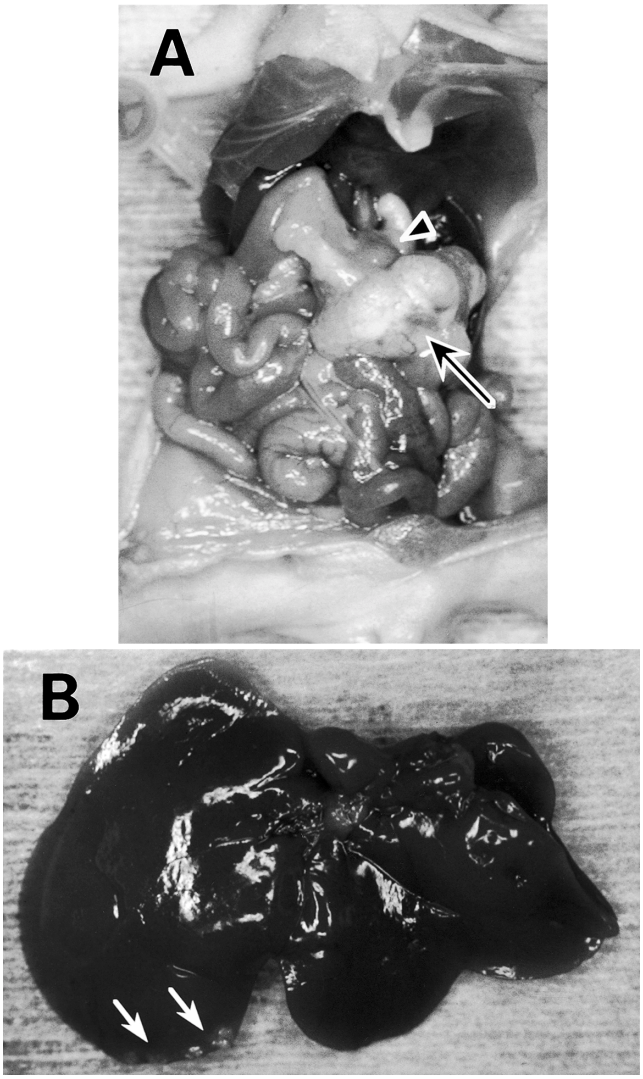

Fig. 1. Macroscopic findings of transplanted tumor and liver metastasis. A: Transplanted tumor growing on the gastric wall 42 days after transplantation (arrowhead, gastric wall; arrow, transplanted tumor). B: Arrows show macroscopic metastases on the liver surface.

wall and liver metastases on day 42 after transplantation are shown in Fig. 1, A and B. Transplanted tumor growth was significantly inhibited in the MV833-treated group ($P=0.03$) (Fig. 2). Concerning liver metastasis, there was a significant decrease in the number of mice with liver metastasis ($P=0.004$), but there was no significant difference in the number of metastatic foci (Table I).

Immunohistochemical staining and assessment of the MVD In the control group, the transplanted tumors contained well-developed microvessels, while the microvessels were underdeveloped in the treated group and only a few vessels were found (Fig. 3, A and B). The MVD ranged from 14.3 to 48.3, with a median value of 33.6±8.0 in the control group and 21.1±5.4 in the MV833-treated group, and the difference was significant ($P<0.0001$) (Fig. 3C).

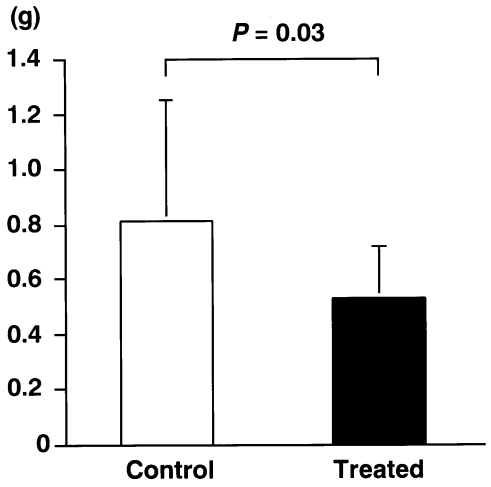

Fig. 2. Inhibition of transplanted tumor growth by MV833. MV833 inhibited the transplanted tumor growth (g) significantly (0.81±0.11 in the control group versus 0.53±0.50 in the treated group). Error bars, SD.

Table I. Inhibitory Effect of MV833 on Liver Metastasis

Group	Number of mice with liver metastasis	Number of metastatic foci
Control group	10/15 (66.7%)	4.00±2.87
Treated group	2/14 (14.3%)[a)]	2.50±0.71[b)]

a) $P<0.05$.
b) NS.

Histological findings and the AI A photomicrograph of an HE-stained transplanted tumor from the MV833-treated group is shown in Fig. 4A. Apoptotic cells were present randomly and singly among the tumor cells. The AI values of the control group and the treated group were 4.73±1.11 and 7.26±1.62, respectively, showing a significant difference ($P<0.0001$) (Fig. 4B).

Northern blot analysis Expression of VEGF mRNA was confirmed in both the control and treated groups, with no significant difference between them (Fig. 5).

DISCUSSION

It has been shown that apoptosis plays an important role in tumor regression,[25)] and the mechanism of its induction by anticancer treatments, such as chemotherapy and radia-

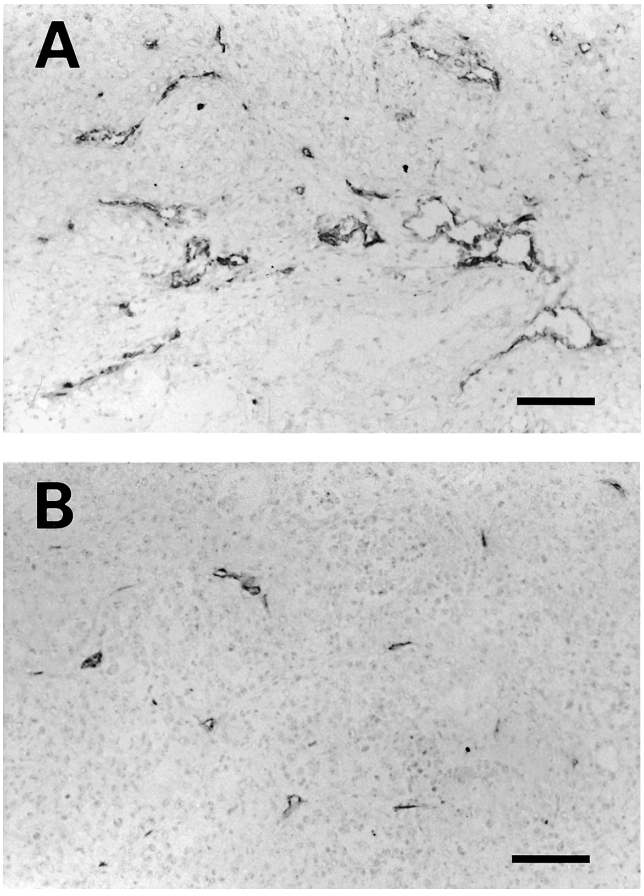

Fig. 3. Immunohistochemical staining with ER-MP12 antibody and the MVD. A: Immunohistochemical staining with ER-MP12 antibody with a weak hematoxylin counterstaining in the control group. Well-developed microvessels of the transplanted tumor were observed. B: In the treated group, the microvessels were underdeveloped and only a few microvessels were seen. Scale bars, 100 μm. C: MVD (counts/×200 fields). Error bars, SD.

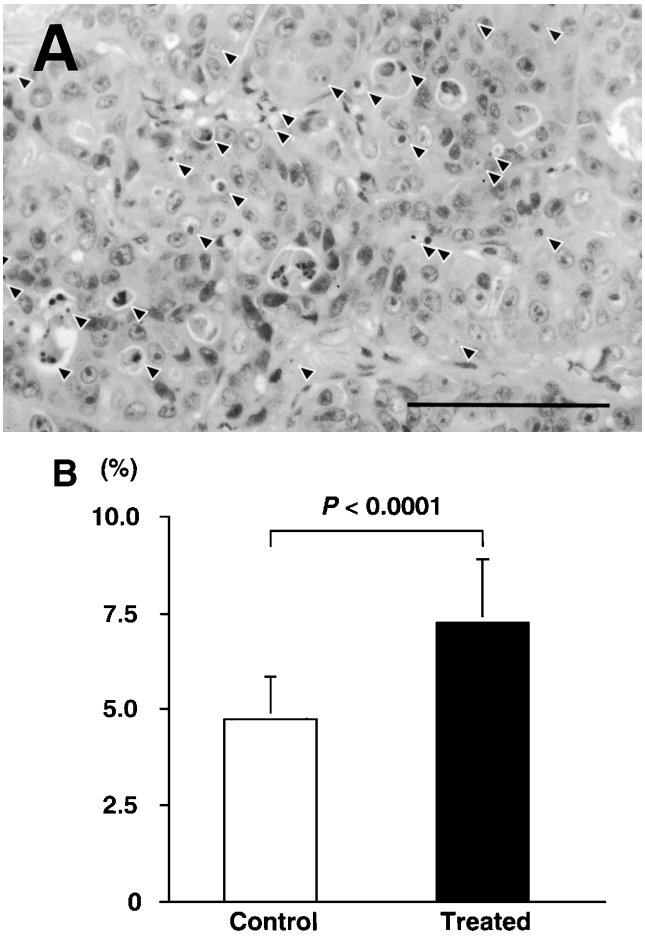

Fig. 4. Histological findings of the transplanted tumor and the AI. A: Arrowheads show the apoptotic cells and apoptotic bodies in the treated group (HE stain). Scale bar, 50 μm. B: AI (%). Error bars, SD.

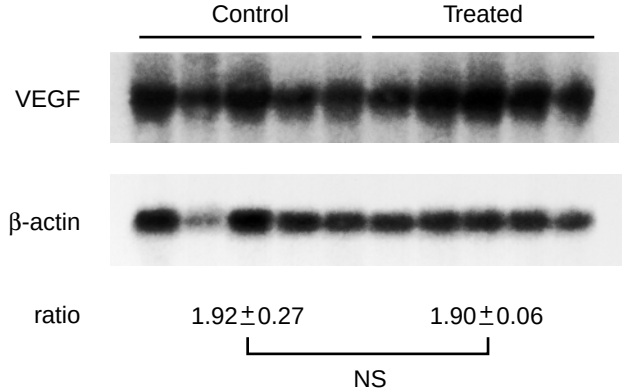

Fig. 5. Northern blot analysis of VEGF and β-actin expression. Total RNA (30 μg) was loaded onto each lane. Expression of VEGF mRNA in the treated group was not significantly different from that in the control group.

tion therapy, has gradually been elucidated by recent studies.[26, 27] It is also well known that tumor angiogenesis plays a pivotal role in the progression of solid neoplasms,[28] and so the adoption of antiangiogenic therapy for the inhibition of tumor angiogenesis has been suggested as a new anticancer strategy. Although the mechanism of the antitumor effect of antiangiogenic agents is not well understood, the fact that antiangiogenic agents, including angiostatin,[6] endostatin[7] and TNP-470,[9] induce tumor cell death by apoptosis suggests that induction of apoptosis is one of the most important steps in antiangiogenic therapy. The target of these antiangiogenic agents is not the tumor itself, but the vascular endothelial cells, and these agents inhibit endothelial cell proliferation.[7, 29, 30] By targeting the endothelial cells of the tumor vasculature, it is considered that the tumor can be destroyed without the induction of tumor drug resistance. However, it has remained unclear how the inhibition of endothelial cell proliferation induces apoptosis. In this study, we demonstrated that apoptosis of transplanted tumor cells was induced by a VEGF Ab, and we examined the significance of apoptosis in tumor regression.

We previously reported that MT2 cells express VEGF protein.[18] VEGF released from tumor cells and stromal cells activates the vascular endothelium via the VEGF/VEGF receptor system, and promotes proliferation and tube formation of endothelial cells. The expression of VEGF is up-regulated by hypoxia, p53 mutation, and cytokines such as IL-1, IL-6, and TGF-β.[31] VEGF is overexpressed in response to these stimuli, and its inhibition suppresses the development of tumor neovasculature. Although several animal experiments have already demonstrated the antiangiogenic effect of VEGF Ab using chicken embryos or HT-1080 cells,[14, 32] there have been no reports about the antiangiogenic effect of this Ab on solid tumors in an orthotopic transplantation model.

ER-MP12 is the mouse hematopoietic progenitor cell antigen, and it was recently identified as platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31).[33] PECAM-1, a 130 kD member of the immunoglobulin gene superfamily, is expressed on endothelial cells of adult mice. In the present study, the vascular endothelial cells of the transplanted tumors were stained specifically by a monoclonal antibody (ER-MP12), and this was extremely useful for detecting the neovasculature in the tumors. We demonstrated that the administration of MV833 inhibited angiogenesis in the transplanted tumors by measuring the MVD. This finding may help to provide a theoretical basis for antiangiogenic therapy.

It is quite natural that inhibition of tumor angiogenesis should induce ischemic changes of the tumor. Cell death caused by hypoxia had been regarded as necrosis, and it was considered that the rapid growth or acute occlusion of the feeding artery of a tumor would result in severe ischemia and necrotic cell death. However, recent studies have shown that hypoxia-induced apoptosis can occur[34, 35] and that the induction of apoptosis by hypoxia is regulated by ceramide[36] and CD95 (APO-1/Fas) ligand.[37] These findings suggest that the ischemia induced by inhibition of angiogenesis is mild, and that the cell death in tumors affected by mild hypoxia caused by antiangiogenic agents may be due to apoptosis. Although the mechanism of induction of apoptosis by antiangiogenic agents is still obscure, hypoxia may be a link between angiogenesis and apoptosis. The mechanism of antiangiogenic therapy-induced apoptosis remains to be solved. In our previous study using a rat hepatoma cell line (AH-130) liver metastasis model,[9] we demonstrated that the percentage of tumors with necrosis in the control group was significantly higher than that in the TNP-470-treated group. AH-130 is characterized by rapid growth, and it is considered that the untreated tumors developed ischemia because of excessive growth and this led to necrosis. The percentage of tumors containing apoptotic cells in the TNP-470-treated group was significantly higher than that in the control group and metastatic liver tumors were reduced by TNP-470.[9] The growth of MT2, the tumor used in this study, was not as rapid as that of AH-130, and the level of necrosis was low in the transplanted tumor specimens. We suggest that MV833 induced a mild ischemic state that triggered apoptosis in the transplanted tumors, and that the difference in AI between the control and treated groups led to the antitumor and antimetastatic effects of MV833.

Although MV833 has an immunoneutralizing activity against human VEGF, the region recognized by this Ab on the VEGF molecule remains unclear. Keyt *et al.* demonstrated that the recognition site of anti-human VEGF Ab (A4.6.1) overlapped with the KDR-binding region.[38] Although the mechanism of the antitumor effect of MV833 is still unclear, the following sequence can be suggested: (a) Blockade of the VEGF/KDR system by MV833 inhibits angiogenesis; (b) inhibition of angiogenesis exposes the tumor to mild hypoxia; and (c) the mild hypoxia triggers apoptosis and results in tumor dormancy. Thus, this study has suggested the significance of apoptosis with respect to the antitumor effect of antiangiogenic agents. It seems possible that the evaluation of apoptosis caused by antiangiogenic therapy may be necessary for estimation of its clinical usefulness.

As mentioned above, blockade of the VEGF system seems to cause mild tumor hypoxia, leading to apoptosis and dormancy. However, the possibility also exists that VEGF expression is up-regulated by mild hypoxia.[38] Before antiangiogenic therapy with a VEGF Ab is applied clinically, whether VEGF expression is influenced by administration of VEGF Ab must be considered, so we assessed VEGF mRNA expression by northern blot analysis in this study. VEGF mRNA expression was demon-

strated in both the control and treated groups, and there was no significant difference between them. This result suggests that if mild hypoxia is induced by antiangiogenic therapy with VEGF Ab, it does not lead to the stimulation of VEGF expression.

Moreover, we should consider the effect of blocking the physiological function of VEGF. The safety of antiangiogenic therapy with VEGF Ab has not yet been established. In the present study, no toxicity, including body-weight loss, was observed (data not shown). The vasculature is quiescent except at menstruation, pregnancy and wound healing in a normal adult, so it is tempting to speculate that the inhibition of the angiogenic response by VEGF Ab would not cause severe toxicity. Further studies are required to clarify the toxicity of the antibody.

The present study demonstrated that neutralization of VEGF activity by MV833 inhibited angiogenesis and induced apoptosis in an orthotopically transplanted human gastric cancer model, and this should lead to tumor regression and inhibition of metastasis. Other antiangiogenic agents may similarly induce apoptosis. Tumor dormancy-inducing therapy targeting the VEGF/VEGF receptor system may have the potential to prolong the survival of patients with gastric cancer.

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