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# Combination Therapy with Vascular Endothelial Growth Factor Neutralizing Antibody and Mitomycin C on Human Gastric Cancer Xenograft

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**Antiangiogenic therapy has been proposed as a new strategy for the treatment of solid tumors. To enhance the therapeutic effect of antiangiogenic agents, combination with conventional anticancer therapy should be investigated. In the present study, we investigated the therapeutic effect of the combination of vascular endothelial growth factor neutralizing antibody (VEGF Ab) and mitomycin C (MMC) on MT-2, a human gastric cancer xenograft. When small pieces of MT-2 were transplanted orthotopically into 62 nude mice, liver metastasis developed 6 weeks after transplantation. The VEGF Ab (100 $\mu$g/mouse) was administered i.p. in the VEGF Ab group ($n$=14) and the combination group ($n$=16) twice a week from day 10 after transplantation. MMC (2 mg/kg) was administered in the MMC group ($n$=16) and the combination group ($n$=16) on days 10, 17 and 24 after transplantation. Compared with the control group, in which saline solution was administered i.p., all three treatments inhibited tumor growth significantly and the effects of MMC and combination therapy were potent. Liver metastases were also inhibited significantly by the administration of VEGF Ab alone, MMC alone or combination therapy. Liver metastasis developed in 9 mice of the control group, 3 of the VEGF Ab group, and 4 of the MMC group, but no mice had liver metastasis in the combination therapy group. However a significant body weight loss and a decrease in spleen weight were observed in the MMC and combination groups, with no significant difference between the two groups. These results suggest that combination therapy with VEGF Ab and MMC may be a potent therapy for human gastric cancer.**

Key words: Gastric cancer—VEGF neutralizing antibody—Mitomycin C—Liver metastasis—Combination

Tumor angiogenesis is essential for the growth and metastasis of solid tumors,[1)] and its induction is mediated by various angiogenic factors. Several angiogenic factors have been identified, such as acidic and basic fibroblast growth factor, VEGF, tumor necrosis factor-$\alpha$ and -$\beta$, angiogenin, angiotropin, and platelet-derived endothelial cell growth factor. VEGF is a selective mitogen for endothelial cells and a potent angiogenic factor *in vivo*.[2)] It has attracted much interest, because VEGF expression has been demonstrated in a wide variety of human cancer cell lines as well as in surgical tumor specimens.[3–7)] Expression of VEGF has been suggested to be associated with the progression and prognosis of colon and gastric carcinoma.[6, 7)] Inhibition of VEGF activity by an immunoneutralizing antibody has been reported to suppress both primary tumor growth and metastasis.[3, 8–10)] In previous studies, we demonstrated a therapeutic effect on colon and gastric carcinoma, which are representative malignant tumors of digestive organs.[11)]

However, the effect of combination therapy with VEGF Ab and conventional antineoplastic modalities has not been investigated satisfactorily. Among the conventional anticancer therapies, chemotherapy is the most common and effective modality for gastrointestinal malignancies. In the present study, the inhibitory effect of VEGF Ab and the antineoplastic agent Mitomycin C (MMC) on human gastric cancer xenograft was investigated.

## MATERIALS AND METHODS

**Preparation of VEGF neutralizing antibody** An anti-human VEGF monoclonal antibody was established from hybridomas of spleen cells from mouse immunized with $VEGF_{121}$ and mouse myeloma Sp2/O-Ag14 cells. The activity and characteristics of MV833 have been described elsewhere.[12)] MMC was purchased from Kyowa Hakko Co., Ltd. (Tokyo).

**Preparation of human cancer xenografts** Human gastric cancer xenograft MT-2, a poorly differentiated adenocarcinoma, was used. It had been established from surgical specimens at our department and maintained by serial s.c. transplantation in nude mice.[13)]

Expression of VEGF mRNA and production of VEGF protein were confirmed by northern blot analysis and enzyme-linked immunoassay, respectively, as previously reported.[11, 14)]

**Experimental design** Five-week-old male BALB/c *nu*/

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*nu* mice were purchased from Clea Japan Inc. (Tokyo) for use in this experiment. The method of tumor transplantation was based upon that previously reported.[13, 15] Each tumor tissue of 200 mg was transplanted into the orthotopic site. In each experiment, mice were divided into 4 groups, consisting of a control group ($n$=16), a MMC group ($n$=16), a VEGF Ab group ($n$=14) and a combination group ($n$=16), on day 10 after transplantation. Mice in the VEGF Ab and combination groups were given 100 $\mu$g of the anti-VEGF antibody i.p. twice a week from day 10 to 42. Those in the MMC and combination groups were administered 2 mg/kg of MMC on days 10, 17 and 24 after transplantation. Saline was administered i.p. to mice of the control group. On day 42 after transplantation, all mice were weighed and killed to evaluate primary tumor growth and macroscopic liver metastasis, and the specimens were examined histologically. The liver was processed for routine histological examination to detect metastases after careful macroscopic examination.

**Statistical analysis** The data on tumor weight, spleen weight and body weight are given as the mean±standard deviation. The data on tumor weight, number of metastatic foci, spleen weight and body weight were analyzed for significance by using Student's $t$ test. The $\chi^2$ test was used to compare the number of mice with liver metastasis and $P<0.05$ was considered significant.

## RESULTS

**Inhibition of tumor growth** The transplanted tumors grew on the gastric wall and liver metastases were identified macroscopically. The macroscopic appearance was previously reported[11] and the microscopical findings are illustrated in Fig. 1. Administration of VEGF Ab and/or MMC significantly inhibited tumor growth in all three treated groups. Fig. 2 shows the actual tumor weight at the end of the experiment. Combination therapy inhibited tumor growth to 1% of that in the control mice; this may have been mainly caused by MMC, because MMC inhibited the tumor growth to 3% of that in control mice.

**Liver metastasis** Liver metastasis was also inhibited significantly by VEGF Ab alone, MMC alone, or combination therapy. Liver metastasis developed in 9 mice of the control group, 3 of the VEGF Ab group, and 4 of the MMC group, but no animal had liver metastasis in the combination group (Table I). A significant inhibitory effect was also observed with respect to the number of metastatic foci. In this model, the metastatic foci were a few millimeters in diameter when evaluated.

**Body weight and spleen weight** Body weight and spleen weight are shown in Fig. 3. The VEGF Ab group did not

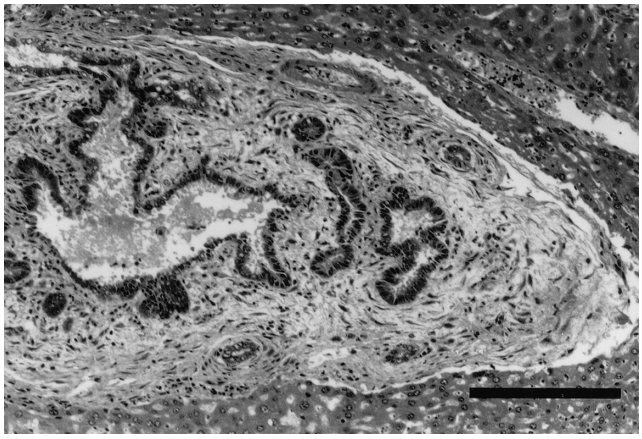

Fig. 1. HE staining of liver metastasis. Capsulated adenocarcinoma was observed in the liver. Scale bar=200 $\mu$m.

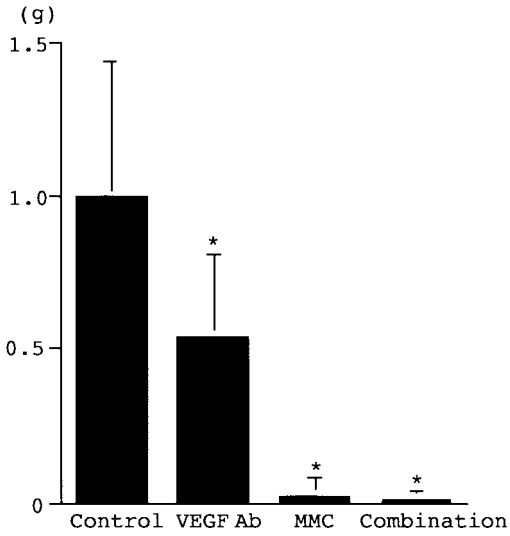


Fig. 2. Primary tumor weight at sacrifice. * Significant difference from the control group ($P<0.001$). Error bars, SD.

Table I. Inhibitory Effect of Combination Therapy on Liver Metastasis

| Group | Number of mice with liver metastasis (%) | Number of foci of liver metastasis |
|---|---|---|
| Control | 9/16 (56) | 2.88±3.24[b] |
| VEGF Ab | 3/14 (21) | 0.57±0.26[c] |
| MMC | 4/16 (25) | 0.31±0.60[d] |
| Combination | 0/16 (0)[a] | 0[d] |

*a*) $P<0.001$ vs. control.
*b*) Mean±SD.
*c*) $P<0.05$ vs. control.
*d*) $P<0.01$ vs. control.

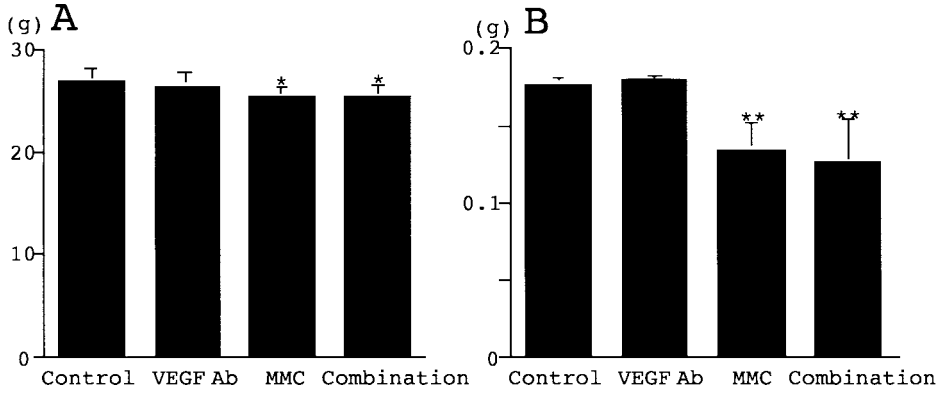


Fig. 3. A, body weight at sacrifice. B, spleen weight at sacrifice. A significant loss of body weight and a decrease in spleen weight were observed in the MMC and combination groups. * $P<0.01$ vs. control group. ** $P<0.001$ vs. control group. Error bars, SD.

show any significant decrease of either body weight or spleen weight compared with the control group. A significant loss of body weight and a decrease of spleen weight compared with the control group were observed in the MMC group and combination group. This adverse effect was probably mainly induced by MMC, because there was no significant difference between the two groups.

## DISCUSSION

Many studies concerning the efficacy of chemotherapy for gastric cancer have been reported, in which the effect was assessed in terms of tumor shrinkage. However, tumor shrinkage does not necessarily improve survival. It is well known that MMC shows a strong cytotoxic effect against a wide variety of tumor cells *in vitro* and *in vivo* by inducing G2 arrest. We selected MMC as the chemotherapeutic agent for the present study because it is regarded as a representative treatment for gastric cancer.

Antiangiogenic therapy has been suggested to have great potential for the treatment of solid tumors, because angiogenesis is essential for the growth and metastasis of such tumors.[1)] Various antiangiogenic therapies have been evaluated clinically. Blockade of the VEGF ligand system is regarded as a useful antiangiogenic therapy for solid tumors, because this system seems to be the most potent factor promoting tumor angiogenesis.[3, 8–10, 16, 17)] Signal transduction proceeds via VEGFR1 (Flt-1) or VEGFR2 (flk-1) located on endothelial cells of the tumor vasculature. The epitope recognized by the neutralizing antibody used in the present study has not been identified, but presumably includes the binding site of VEGF to VEGFR2, because mitotic signals are transduced through the VEGF/VEGFR2 system.[12, 17)]

The present study clearly demonstrated an enhanced therapeutic effect with combination therapy. We reported previously on the difference of antitumor effect between an angiogenesis inhibitor, TNP-470, and MMC.[18)] MMC demonstrated a significant inhibitory effect on transplanted tumor growth, but had only a marginal effect on liver metastasis. Thus, chemotherapy was effective for tumor shrinkage but was not so effective in preventing metastasis. This finding is broadly compatible with the present results, because the inhibitory effect on liver metastasis did not differ between the VEGF Ab group and the MMC group (21% versus 25%; compared with the control group), although that on transplanted tumor growth was remarkably different (54% versus 3%). Angiogenesis is thought to be essential for tumor growth beyond the size of a few cubic millimeters,[1)] so the micrometastases in our model would have been largely angiogenesis-dependent. It is also possible that endothelial cells have a high vulnerability to antiangiogenic agents in the early stage of liver metastasis. Borgstrom *et al.* reported that an anti-VEGF antibody caused inhibition of angiogenesis and blocked the growth of micrometastasis from a human rhabdomyosarcoma cell line, but dormant microcolonies remained viable.[19)] These results suggested that anti-VEGF Ab may induce tumor dormancy but not tumor eradication, so we examined combination therapy with MMC. The present study demonstrated an enhanced antitumor effect of combination therapy with VEGF Ab and MMC. In particular, the complete inhibition of liver metastasis by the combined therapy suggests that the combined therapy may be able to prolong the survival of patients with gastric cancer.

VEGF Ab administration did not induce body weight loss, whereas MMC induced significant weight loss and splenic involution. The combination therapy of VEGF Ab with MMC did not have a greater effect on body weight or spleen weight than that of MMC alone.

Kakeji *et al.* reported the induction of VEGF expression after chemotherapy and suggested that hypoxia induced by

chemotherapy contributed to overexpression of VEGF.[20] Thus, combination therapy with a VEGF Ab and chemotherapy may be a reasonable strategy for treating gastrointestinal carcinoma.

We also observed an increase of the apoptotic index of gastric cancer xenografts in the VEGF Ab group, suggesting that the therapeutic effect of VEGF Ab was related to induction of apoptosis.[14]

Tumor neovascularization also provides a route for cancer cells to metastasize spontaneously to distant organs from the primary lesion. From about day 10 after transplantation, cancer cells have been reported to enter the circulation.[21] The discrepancy in the inhibitory effect of MMC between transplanted tumor and liver metastases suggests that inhibition of growth at the primary site is not sufficient to prevent distant metastasis.

In conclusion, combined therapy with an antiangiogenic agent and a chemotherapeutic agent(s) may be a potent new strategy for treating advanced gastric cancer.

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