The Effect of Bevacizumab on Tumour Growth of Malignant Fibrous Histiocytoma in an Animal Model

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Abstract. *Background: Bevacizumab is a specific inhibitor of angiogenesis and a neutralising antibody against vascular endothelial growth factor (VEGF). The effect of bevacizumab was evaluated on malignant fibrous histiocytoma (MFH) in vivo using an animal model. Materials and Methods: MFH cell line, NaraH, was implanted to athymic nude mice which were randomly divided into a treatment and a control group. The change in body weight and tumour volume were evaluated and immunohistochemical analysis was performed of microvessel density (MVD) and VEGF expression in the tumour tissue. Results: Bevacizumab significantly induced inhibition of tumour growth, reducing tumour volume to 48% at the end of experiment. Intratumoural MVD was significantly decreased in the bevacizumab treatment group compared to the control group. A positive correlation was found between tumour volume and MVD. Conclusion: Bevacizumab suppressed MFH tumour growth by inhibiting tumoural angiogenesis. The current study suggests that bevacizumab may be a novel therapeutic agent for MFH.*

Angiogenesis, the development and formation of new blood vessels, is important in various physiological processes, but particularly in tumourigenesis and metastasis (1,2). Vascular endothelial growth factor (VEGF) is one of the most potent positive regulators of angiogenesis (3). VEGF is a potent mitogen and survival factor for endothelial cells, which regulates normal and pathological angiogenesis. Increased expression of VEGF has been reported in a variety of malignant human tumours (4-8). It was previously reported that VEGF is often expressed in bone and soft tissue tumours (9) as well as in various solid tumours. Bevacizumab is an anti-VEGF recombinant humanised monoclonal antibody, developed to target VEGF. Recently it has been shown that bevacizumab prolongs survival and delays tumour progression in patients with metastatic colorectal cancer (10).

In this study, the effect of bevacizumab on tumour growth was investigated in the xenograft model of malignant fibrous histiocytoma (MFH). It was found that bevacizumab potently reduced MFH growth most likely through inhibition of angiogenesis.

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Key Words: VEGF, bevacizumab, MFH.

Materials and Methods

Cell cultures. The human MFH cell line, NaraH (ScienStuff Co., Nara, Japan) (11), was grown in a culture medium consisting of minimum essential Eagle's medium (Sigma-Aldrich Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich Co., Tokyo, Japan), penicillin G (100 U/ml) and streptomycin (100 μg/ml). The cell line was routinely maintained at 37˚C in a humidified 5% CO_2 atmosphere. For *in vivo* experiments, tumour cells were harvested by brief exposure to 0.25% trypsine.

Animal models and treatment. Male athymic BALB/c nude mice, aged 6 to 8 weeks, obtained from CLEA Japan were maintained in pathogen-free conditions and in accordance with institutional principals. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the host institution and were approved by the institutional animal committee. The human MFH cell line, Nara H, was used in this study. Nara H cells (1.2×10^7 cells in 0.1 ml medium) were injected subcutaneously to the dorsal area of mice. For this study of the anti-tumour activity, bevacizumab (Avastin®; Roche Co, Basel, Switzerland) was purchased.

Effect of bevacizumab on tumour growth. Whether bevacizumab affects tumour volume and survival rate was examined. Fifty mice were randomly divided into two groups, treatment group (n=25) and control group (n=25). After allowing 2 days for implantation, intraperitoneal injections of bevacizumab were started. The volume of intraperitoneal injection was 0.1 ml and the mice were injected slowly twice a week for 8 weeks (2 mg/kg of bevacizumab to treatment group or PBS only

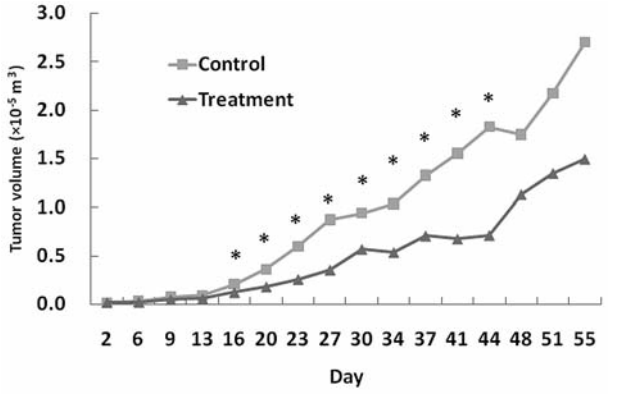

Figure 1. *Tumourigenesis of MFH xenograft models. Tumour growth was significantly inhibited in the bevacizumab treatment group when compared with the control group (*p<0.05).*

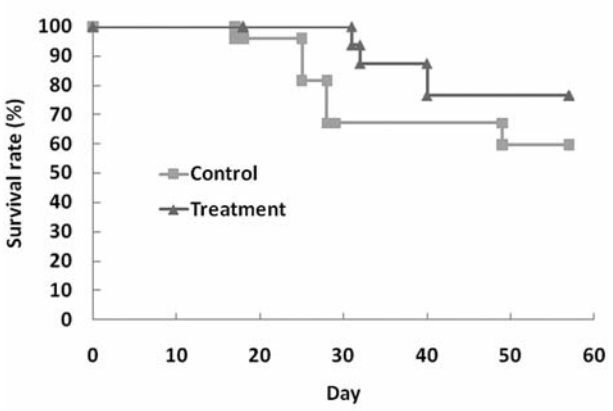

Figure 2. *At the end of the experiment, the bevacizumab treatment group (76.6%) had a higher survival rate than the control group (59.9%), but there was no significant difference.*

to control group) throughout the experimental period. Mice were followed for body weight and tumour size. After implantation, body weight and tumour dimensions were measured twice a week. Tumour volume was calculated according to the formula $V=\pi/6\times a^2\times b$, where a and b represent the shorter and the longer dimension of the tumour.

Immunohistochemical analysis. The excised tumours were embedded in OCT compound (Sakura Finetek Co, Tokyo, Japan) then frozen in liquid nitrogen. Sections 10 μm-thick were prepared on a cryostat and stored frozen at –70˚C. Sections were air-dried, fixed in 4% paraformaldehyde for 10 minutes (room temperature), and washed with PBS. After incubation with primary antibody overnight at room temperature, the sections were washed in PBS, then treated with secondary antibody for an additional 30 minutes. Subsequently, the sections were washed again in PBS. Red colour was developed using AEC stain sets followed by counterstain with haematoxylin.

To evaluate intratumoural angiogenesis of tumour, immunohistochemical staining of both VEGF and factor VIII was performed. Nineteen mice received intraperitoneal injection with bevacizumab or PBS twice a week (treatment group (n=10) and control group (n=9)). After 18 days of implantation, all tumours were excised from the dorsal area of the mice and processed for immunohistochemical staining. Anti-VEGF antibody (A20; Santa Cruz Biotechnology, Santa Cruz, USA) and anti-factor VIII–related antigen antibody (Nichirei Biosciences, Tokyo, Japan) were used as primary antibodies.

To estimate the expression of VEGF, the immunohistochemistry (IHC) score, which is a semi-quantitative evaluation system for evaluating the level of antigen expression, was used. The IHC score was defined as the sum of the two scores below. Immunoreactivity was scored as either negative (0), focal (1+, less than 25% of positive cells), moderate (2+, 25-50% of positive cells), or diffuse (3+, more than 50% of positive cells). The intensity of immunostaining was rated as follow: none (0), weak (+1), moderate (2+) and intense (+3). The specimens were evaluated by two observers (YO, TA) and finally scored by consensus of the observers.

Microvessel density (MDV) was evaluated as follows. At low power (×100), the tumourous tissue sections were screened and the three areas with the most intense neovascularization (hot spot) were selected. Microvessl counts of these areas were performed at high power field (×400). Any factor VIII-positive endothelial cells or endothelial cell clusters clearly separated from adjacent microvessels, tumour cells and connective tissue elements were considered as single countable microvessels; branching structures were counted as one, unless there was a break in the continuity of the vessel, in which case it was counted as two distinct vessels. Three fields per tumour section were counted in the areas that appeared to contain the greatest number of microvessels on scanning at low magnification. Microvessel density (MVD) was defined as the mean score from all three fields.

Statistical analysis. The statistical significance of the individual findings and their association indices were evaluated by the Mann-Whitney *U*-test, Student's *t*-test or Spearman's rank-order correlation co-efficient. Overall survival duration was calculated from the start of treatment using the Kaplan-Meier method. Probability, *p*-values less than 0.05 were considered significant.

Results

Effect of bevacizumab on tumour growth. The anti-tumour activity of bevacizumab in nude mice bearing MFH xenografts was investigated. Implantation of 1.2×10^7 cells into the dorsal area of nude mice resulted in the development of tumours in 100% of animals. From day 16 to day 44, tumour growth in the treatment group was significantly inhibited compared with that in the control group. Although there was no significant difference between the two groups after day 44, at the end of the experimental period, the mean tumour volume of the treatment group and control group were 2.7×10^{-5} m^3 and 1.4×10^{-5} m^3, respectively (Figure 1). There was no significant difference in survival rate between the two groups, however the survival rate of the treatment group was higher than that of the control group (76.6% in treatment group and 59.7% in control group) (Figure 2). During this experimental period, no side-effects such as loss of body weight were observed in the treatment group.

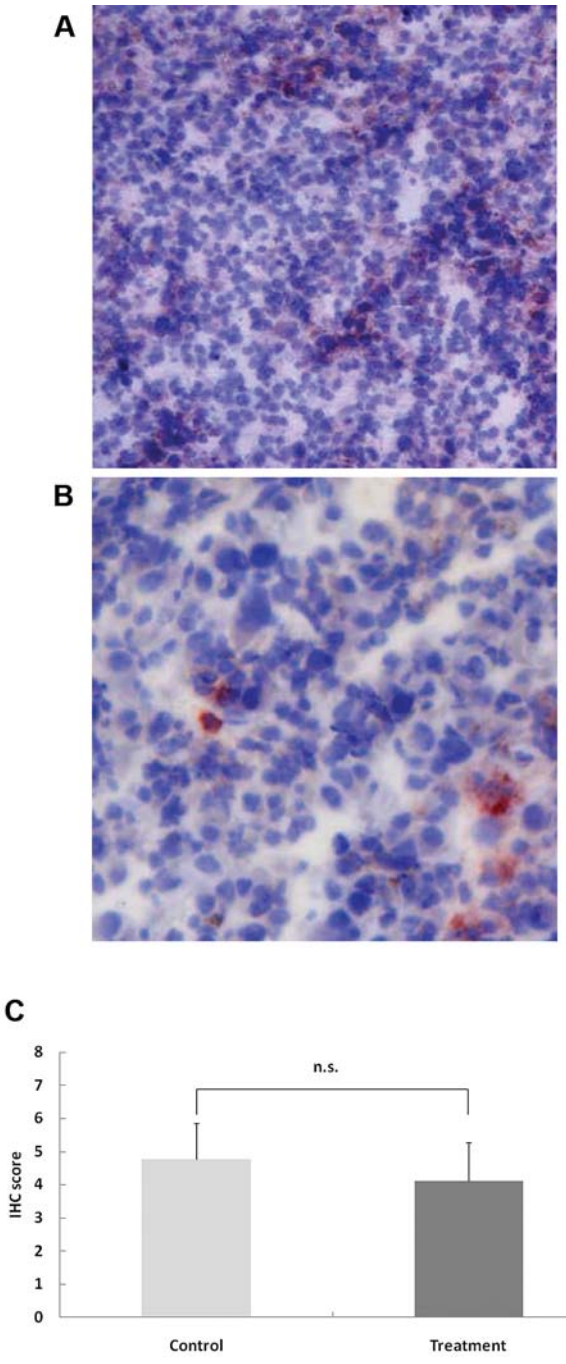

Figure 3. *Immunohistochemical analysis of the expressions of VEGF in the control group (A) and bevacizumab treatment group (B). There was no significant difference in IHC score indicating the level of VEGF expression (C).*

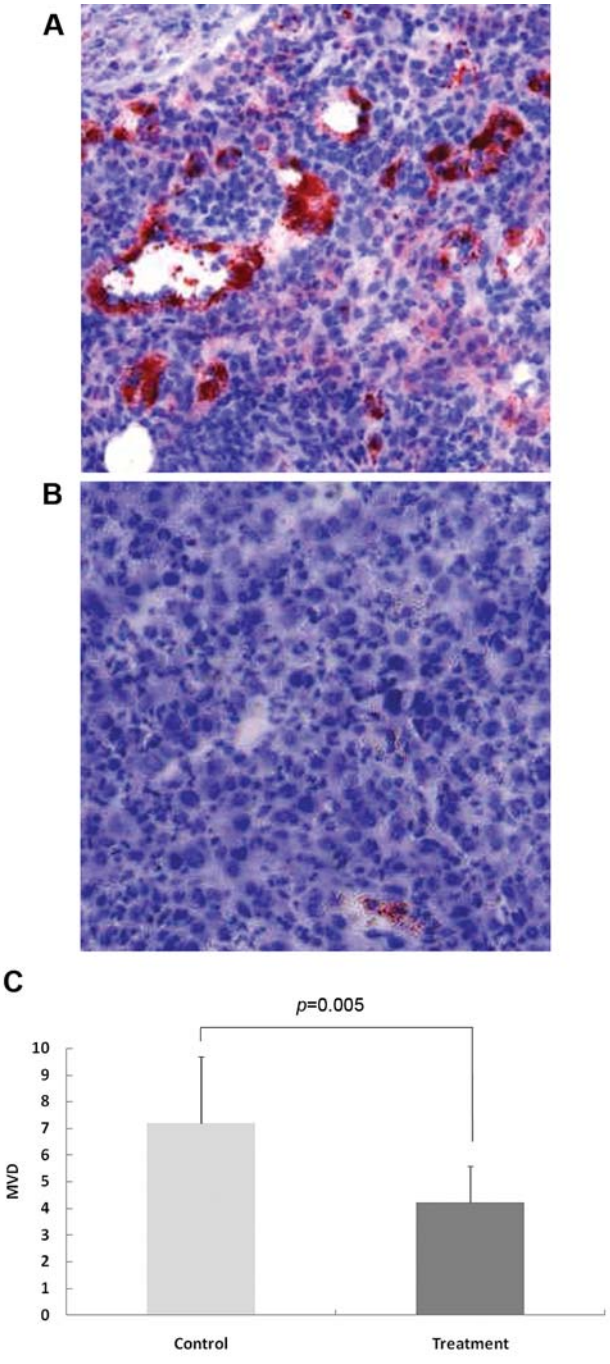

Figure 4. *Immunohistochemical analysis of the expressions of factor VIII in control group (A) and bevacizumab treatment group (B). Microvessel density in the control group was significantly higher than that in bevacizumab treatment group (C).*

Effect of bevacizumab on VEGF expression, microvascular content. There was no significant difference in IHC score of VEGF expression between the two groups. The mean IHC score (±S.D.) of the treatment group and control group were 4.8±1.1 and 4.1±1.2, respectively (Figure 3). MVD, determined by immunohistochemical staining of factor VIII, was significantly decreased in the treatment group. The mean MVD (±S.D.) value was 4.2±1.4 in the treatment group and 7.2±2.5 in the control group (p=0.005) (Figure 4). A significant correlation was found between tumour volume and

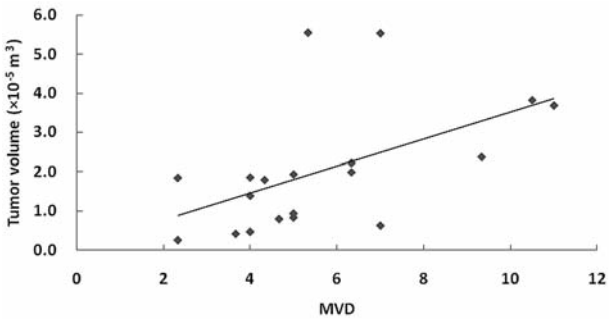

Figure 5. *A significant correlation was found between tumour volume and MVD (p=0.02, r=0.53).*

MVD (p=0.02, r=0.53) (Figure 5), whereas no correlation was found between VEGF expression and either tumour volume or MVD.

Discussion

Angiogenesis, the process of new blood vessel formation, is fundamental for the growth and spread of solid tumours. Tumour angiogenesis is a complex process based on the concept that a tumour requires a vascular blood supply to grow beyond 1 to 2 mm (12). Moreover, angiogenesis not only permits further growth of the primary tumour but also provides a means for metastatic dissemination. Therefore, inhibition of specific molecules essential for tumour vascular development has become a key therapeutic antitumour strategy. Over-expression of VEGF, one of the specific molecules for angiogenesis, is responsible for the abnormal angiogenesis process in tumour (13). There are many inhibitors which target VEGF signaling, such as anti-VEGF antibody, VEGF trap and VEGF receptor tyrosine kinase inhibitor. These inhibitors, which induce different levels of suppression in various types of tumours, are also being investigated (14, 15). Bevacizumab, one of these inhibitors, is a humanised antibody against VEGF. The clinical trials of bevacizumab in various malignancies such as colorectal cancer, breast cancer, non-small cell lung cancer and renal cell cancer have been assigned and reported (10, 16). In soft tissue tumours, some studies demonstrated that anti-VEGF antibody suppressed the tumour growth of leiomyosarcoma (17), Ewing's sarcoma (18), fibrosarcoma (19) and rhabdomyosarcoma (17, 20-22) in xenograft models.

The current study, is the first to report that bevacizumab also significantly inhibited tumour growth of MFH *in vivo*. The maximum inhibition rate of tumour volume was 61%, based on tumour volumes on day 44, compared with the control group. Significant inhibition of tumour growth was not observed after day 44. The Authors believe that reflected the statistical numerical decrease of mice by tumour death in the control group. Although all potential adverse effects of bevacizumab were not specifically checked, the animals tolerated this therapy relatively well, showing normal growth without any visible serious adverse effects such as external bleeding. In addition, these results indicated that there was a significant correlation between tumour volume and immunohistochemical analysis of MVD. These findings strongly suggest that bevacizumab is able to inhibit MFH tumour growth by suppressing vascularization.

Immunohistochemical analysis of MVD and VEGF expression confirmed that the effect of bevacizumab is achieved in part by antiangiogenic mechanisms. The current results indicated that the number of tumour vessels in the bevacizumab treatment group was statistically significantly fewer than that in the control group, but did not affect VEGF expression in the MFH tumour model. In previous reports, immunohistochemical analysis of the xenograft samples confirmed that treatment with bevacizumab decreased MVD (23-27) but VEGF expression had various results (26, 27). Li *et al*. also confirmed that in malignant pleural mesothelioma xenograft samples, treatment with bevacizumab decreased MVD but did not affect VEGF expression (26). In contrast, Zhang *et al*. reported that immunohistochemical analysis of the bronchial carcinoid cancer cell xenograft samples confirmed that treatment with bevacizumab decreased MVD but increased VEGF expression. It is also suggested that there was up-regulation of VEGF transcription using bevacizumab (27).

In the current study, treatment with bevacizumab alone was effective in the MFH xenograft model. However, there is experimental and clinical evidence of the synergistic effect of anti-VEGF inhibitors in combination with chemotherapy and/or radiotherapy against several types of cancer (10, 16, 28, 29). Jain *et al*. proposed that 'normalised' tumour vasculature with anti-VEGF therapy induced the synergistic effect of combination therapy by increasing the delivery of drugs or oxygen to the tumour (30). There is another hypothesis that bevacizumab can block the cell stress response induced by chemotherapeutic agents and/or radiation, and enhance the antiangiogenic effect of chemotherapeutic agents themselves. Thus it is necessary to evaluate the effects of bevacizumab in combination therapy for MFH in the further study.

In conclusion, the current results suggest that bevacizumab may suppress MFH tumour growth by inhibiting intratumoural micro vessel formation and that bevacizumab may be a novel therapeutic agent for MFH.

Acknowledgements

The Authors express their thanks to Ms. Janina Tubby for her editorial assistance.

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Received May 6, 2010
Revised June 3, 2010
Accepted June 9, 2010