Bevacizumab improves the delivery and efficacy of paclitaxel

Mieko Yanagisawa[a], Keigo Yorozu[a], Mitsue Kurasawa[a], Kohnosuke Nakano[b], Koh Furugaki[a], Yoriko Yamashita[a], Kazushige Mori[a] and Kaori Fujimoto-Ouchi[a]

It has been reported that bevacizumab in combination with paclitaxel significantly prolongs progression-free survival compared with paclitaxel alone in the initial treatment for metastatic breast cancer. To understand how bevacizumab enhances the efficacy of paclitaxel, we investigated the mechanism in a MX-1 human breast cancer xenograft model. The antitumor activity of bevacizumab at 5 mg/kg in combination with paclitaxel at 20 or 30 mg/kg was significantly higher than that of either agent alone. First, we measured the paclitaxel concentration in tumor to see whether bevacizumab enhances the activity by increasing the tumor concentration of paclitaxel. When given in combination with bevacizumab, the levels of paclitaxel in the tumor increased. Paclitaxel at 30 mg/kg with bevacizumab showed a similar tumor concentration as paclitaxel alone at either 60 or 100 mg/kg, with a similar degree of tumor growth inhibition. In contrast, no remarkable differences in paclitaxel concentration in the plasma or liver were observed between the paclitaxel monotherapy group and the paclitaxel plus bevacizumab group. An increase in paclitaxel concentration by bevacizumab was also found in another model, A549. In the same MX-1 model, vascular permeability in the tumor was significantly decreased by treatment with bevacizumab. There was no difference in microvessel density between the bevacizumab alone group and the combination group. Results suggest that the synergistic antitumor activity of paclitaxel and bevacizumab in combination may be a result of the increase in paclitaxel concentration in tumor resulting from the downregulation of vascular permeability when co-administered with bevacizumab. *Anti-Cancer Drugs* 21:687–694 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2010, **21**:687–694

Keywords: bevacizumab, breast cancer, combination therapy, drug delivery to tumor, lung cancer, paclitaxel, vascular permeability, xenograft model

Departments of [a]Product Research and [b]Pre-clinical Research, Chugai Pharmaceutical Co., Ltd., Kajiwara, Kamakura, Japan

Correspondence to Kaori Fujimoto-Ouchi, PhD, Department of Product Research, Chugai Pharmaceutical Co., Ltd., 200 Kajiwara, Kamakura 247-8530 Japan
Tel: +81 467 45 7692; fax: +81 467 45 7643;
e-mail: ohuchikor@chugai-pharm.co.jp

Received 19 January 2010 **Revised form accepted** 28 April 2010

Introduction

Bevacizumab (Avastin) is a genetically engineered humanized monoclonal antibody derived from murine antihuman vascular endothelial growth factor (VEGF) monoclonal antibody, A4.6.1 [1,2]. It binds specifically to human VEGF, thereby blocking the binding of VEGF to VEGF receptors expressed on vascular endothelial cells. By blocking the biological activity of VEGF [3], bevacizumab or its murine equivalent, A4.6.1, inhibits neovascularization in tumor tissues and thus suppresses tumor growth [1,4,5].

Paclitaxel binds to β-tubulin and stabilizes microtubules, repressing the dynamic instability of spindle microtubules, and thus results in blocking the cell cycle at the metaphase-to-anaphase transition [6]. Bevacizumab in combination with paclitaxel significantly prolonged progression-free survival as compared with paclitaxel alone in the first-line treatment of metastatic breast and lung cancers [7,8].

In this study, we tried to demonstrate the synergistic antitumor activity of combination therapy with bevacizumab plus paclitaxel and investigated the mechanisms of the combination in a human breast cancer xenograft model. As it has been hypothesized that bevacizumab may enhance the delivery of chemotherapeutic agents to tumors as a result of the normalization of tumor vessels resulting from the downregulation of vascular permeability [9–11], we compared the concentration of paclitaxel in tumor tissues treated with paclitaxel in combination with bevacizumab and in those treated with paclitaxel alone.

Materials and methods

Animals

Five-week-old female BALB-nu/nu (CAnN.Cg-Foxn1 < nu > /CrlCrlj nu/nu) mice for the MX-1 xenograft model and male BALB-nu/nu mice for the A549 xenograft model were obtained from the Charles River Laboratories, Inc. (Kanagawa, Japan). The mice were acclimatized for at least 1 week in our animal facility before use. All the animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee in Chugai Pharmaceutical Co., Ltd.

0959-4973 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e32833b7598

Human cancer xenograft model

The MX-1 human breast cancer cell line was provided by Dr T. Tashiro (Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan). A piece of minced MX-1 tumor tissue (2×2 mm) was inoculated subcutaneously into the right flank region of each mouse. The A549 human lung cancer cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA) and maintained in F-12K nutrient mixture supplemented with 10% (v/v) fetal bovine serum at 37°C under 5% CO_2. 5×10^6 cells of A549 were inoculated at the same site as the MX-1.

Antitumor agents

Bevacizumab was obtained from F. Hoffman-La Roche Ltd. (Basle, Switzerland). Human IgG (HuIgG) was purchased from MP Biomedicals, Inc. (Solon, Ohio, USA). Bevacizumab and HuIgG were diluted with saline. Paclitaxel was obtained from Wako Pure Chemical Industries (Osaka, Japan). Paclitaxel was dissolved in Cremophor EL-ethanol solution (1 : 1) and diluted 1 : 10 with saline just before administration. Cremophor EL-ethanol solution (1 : 1) diluted 1 : 10 with saline was administered as the paclitaxel vehicle. Cremophor EL was purchased from Sigma-Aldrich Japan Co., Ltd. (Tokyo, Japan).

Evaluation of antitumor activity

After the tumors were sufficiently established in the mice, treatments with the antitumor agents were started (day 1). Bevacizumab or HuIgG was administered intraperitoneally and paclitaxel was administered intravenously once a week for 3 weeks for the evaluation of antitumor activity. The tumor volume was estimated by using the equation $V = ab^2/2$, in which a and b are the tumor length and width, respectively. Tumor volume was measured twice a week, and the degree of tumor growth inhibition was evaluated on day 22 (21 days after the initiation of the treatment).

Quantification of vascular permeability and microvessel density in tumor tissues

Seventeen days after the MX-1 inoculation, bevacizumab or HuIgG at 5 mg/kg and paclitaxel at 30 mg/kg were administered once (day 1). Tumor tissues were collected on day 2, day 5, and day 8. Microvessel density (MVD) and vascular permeability in the tumor were evaluated immunohistochemically. Immunohistochemical staining was performed using avidin–biotin–peroxidase complex on 5-μm thick sections from freshly frozen tissues. MVD (%) was calculated from the ratio of the CD31 stained area to the total area observed in three to six regions (0.4977 mm^2 each). Vascular permeability in the tumor was determined from the difference between the area with CD31 positive staining and the area showing fluorescein isothiocyanate (FITC)–lectin positive staining in adjacent tissue sections [10]. FITC-labeled lectin (molecular weight 117K) was injected 1 h before collecting the tissues. Calculation of MVD and vascular permeability was performed automatically using the imaging analysis software Win ROOF (Mitani Corporation, Fukui, Japan). Rat anti-mouse CD31 monoclonal antibody clone MEC 13.3 (BD Biosciences, New Jersey, USA) and goat anti-FITC polyclonal antibody (Bethyl Laboratories, Inc., Texas, USA) were used in the assay. The MVD for each group was evaluated in four tumor samples.

Measurement of paclitaxel concentration in plasma and tissue samples by high-performance liquid chromatography

Sixteen days after the tumor inoculation, 30, 60, or 100 mg/kg of paclitaxel was administered 1 h after the administration of 5 mg/kg of bevacizumab or HuIgG in the MX-1 model. Mice were killed 48 h after the paclitaxel administration. Blood, tumor, and liver were collected. Plasma was obtained from the blood collected in a tube with sodium heparin by centrifugation at 10 000 rpm for 10 min. Sixty microliters of plasma was mixed vigorously with 1 ml hexane–ethylacetate (1 : 1) in a reciprocal shaker for 30 min and then centrifuged at 2500 rpm for 10 min; 700 μl of the supernatant was evaporated to dryness. Tumor and liver were homogenized in a 19- or 4-fold volume of distilled water, respectively; 200 μl of the homogenate was mixed vigorously with 2 ml hexane–ethylacetate (1 : 1) in a reciprocal shaker for 30 min and then centrifuged at 2500 rpm for 10 min. Next, 1.5 ml of the supernatant was evaporated to dryness. The extraction residue was reconstituted in 500 μl of 90% acetonitrile solution, and aliquots of 35 μl were injected into a Lachrom D-7000 series high-performance liquid chromatography (HPLC) system consisting of a degasser (L-7610), pump (L-7100), programmable autosampler (L-7250), column oven (L-7300), and UV-vis detector (L-7420) (Hitachi High-Technologies Corporation, Tokyo, Japan). Chromatographic separation was achieved using a Capcell Pak UG120, S5, column (4.6 mm i.d. × 250 mm) with a Guard cartridge Capsell UG120, S5 (4.0 mm i.d. × 20 mm) (5 μm particle size, C_{18}; Shiseido Company Limited, Tokyo, Japan). Elution was performed using a 15-min linear gradient from 40 to 95% (v/v) of acetonitrile. The mobile phase consisted of 95% acetonitrile held for 5 min, and then 40% acetonitrile held for 15 min. UV detection was performed at 227 nm. Ethanol, ethyl acetate, hexane, and acetonitrile were purchased from Wako Pure Chemical Industries. For pharmacokinetics calculations, 30 mg/kg of paclitaxel was administered 1 h after the administration of 5 mg/kg of HuIgG or bevacizumab in the MX-1 model. Mice were killed 5 min, 1, 2, 4, 12, 18, 24, or 48 h after the paclitaxel administration. Blood, tumor, and liver were treated and paclitaxel concentration was measured by HPLC as described above. The mean concentration at each time point was used because of the nonserial blood sampling in this study. Pharmacokinetic parameters were

calculated with the Watson software (Thermo Fisher Scientific Inc., Massachusetts, USA) using the model-independent calculation method. The apparent terminal half-life ($T_{1/2}$) was calculated as $0.693/k$.

Twenty-two days after the A549 tumor inoculation, 20 mg/kg of paclitaxel was administered 1 h after the administration of 5 mg/kg of HuIgG or bevacizumab. Mice were killed 48 h after the paclitaxel administration. Paclitaxel concentration in the blood, tumor, and liver was analyzed by HPLC.

Statistical analysis

Statistical analysis for the evaluation of the antitumor activity and concentration of paclitaxel was performed using the Wilcoxon test (SAS preclinical package, SAS Institute, Inc., Tokyo, Japan). Differences were considered to be significant at $P \leq 0.05$. A two-way analysis of variance was used to determine the differences in vascular permeability.

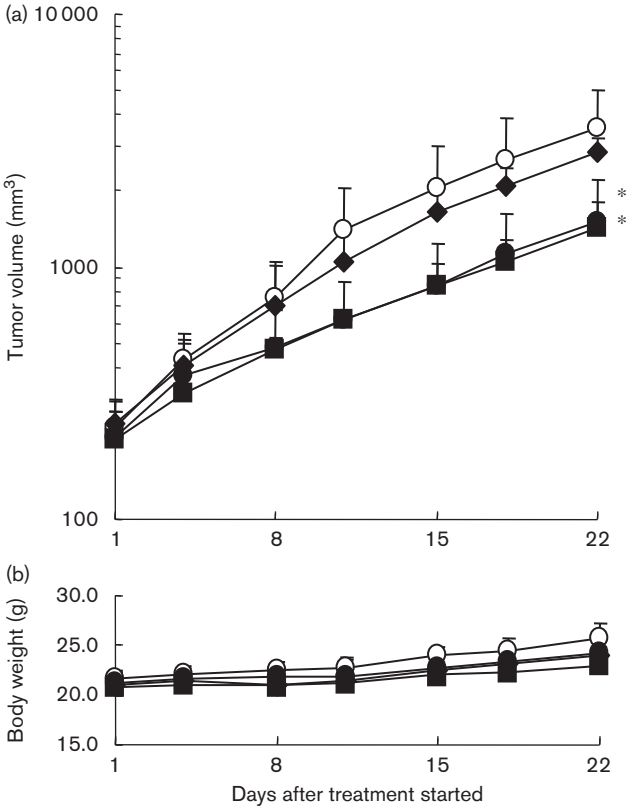
Results

Antitumor activity of bevacizumab as monotherapy and in combination therapy with paclitaxel

We examined the antitumor activity of bevacizumab as monotherapy in an MX-1 human breast cancer xenograft model. Bevacizumab at doses of 1.25, 5, or 20 mg/kg or HuIgG at 20 mg/kg as the control was administered. Bevacizumab showed significant antitumor activity at doses of 5 and 20 mg/kg ($P \leq 0.05$, Fig. 1a). There was no significant decrease in body weight – an indicator of toxicity – during the treatment (Fig. 1b). The percentage of tumor growth inhibition after 3 weeks of administration of bevacizumab at 1.25, 5, or 20 mg/kg was 22, 64, and 61%, respectively.

We also evaluated the antitumor activity of bevacizumab in combination with paclitaxel in the same MX-1 tumor xenograft model. Paclitaxel and bevacizumab at the doses of 20 and 5 mg/kg, respectively were administered once a week for 3 weeks. On day 22, there were statistically significant differences in tumor volume between the control and the groups treated with paclitaxel, bevacizumab, and paclitaxel plus bevacizumab. Both paclitaxel and bevacizumab significantly inhibited tumor growth (Fig. 2a). In the MX-1 model, tumor volume percent change against the control in the paclitaxel and bevacizumab groups was 9.02 and 37.5%, respectively. Using these values to calculate percent change in the combination results in a change of 3.38%. However, in practice the tumor volume percent change in the combination group was –4.32%, and thus 7.70% lower than the calculated change. Hence, paclitaxel and bevacizumab in combination showed potent activity, more than merely additive and with no reduction in body weight. There were no significant decreases in body weight on day 22 compared with day 1 in any of the groups (Fig. 2b). We also examined the antitumor activity of bevacizumab 5 mg/kg in combination with paclitaxel 30 mg/kg. Tumor growth (Fig. 2c) was delayed because the potent efficacy of the combination resulted in tumor volume regression. The time required for tumor volume to reach 1000 mm^3 in the control, bevacizumab 5 mg/kg alone, paclitaxel 30 mg/kg alone, and paclitaxel plus bevacizumab combination group was 11.3, 17.0, 53.1, and 84.9 days, respectively. The difference in time between bevacizumab alone and paclitaxel plus bevacizumab combination was 67.9 days, longer than that between the control group and paclitaxel alone (41.8 days). The results indicate that bevacizumab inhibited tumor growth in combination with paclitaxel at 20 mg/kg and 30 mg/kg.

Fig. 1

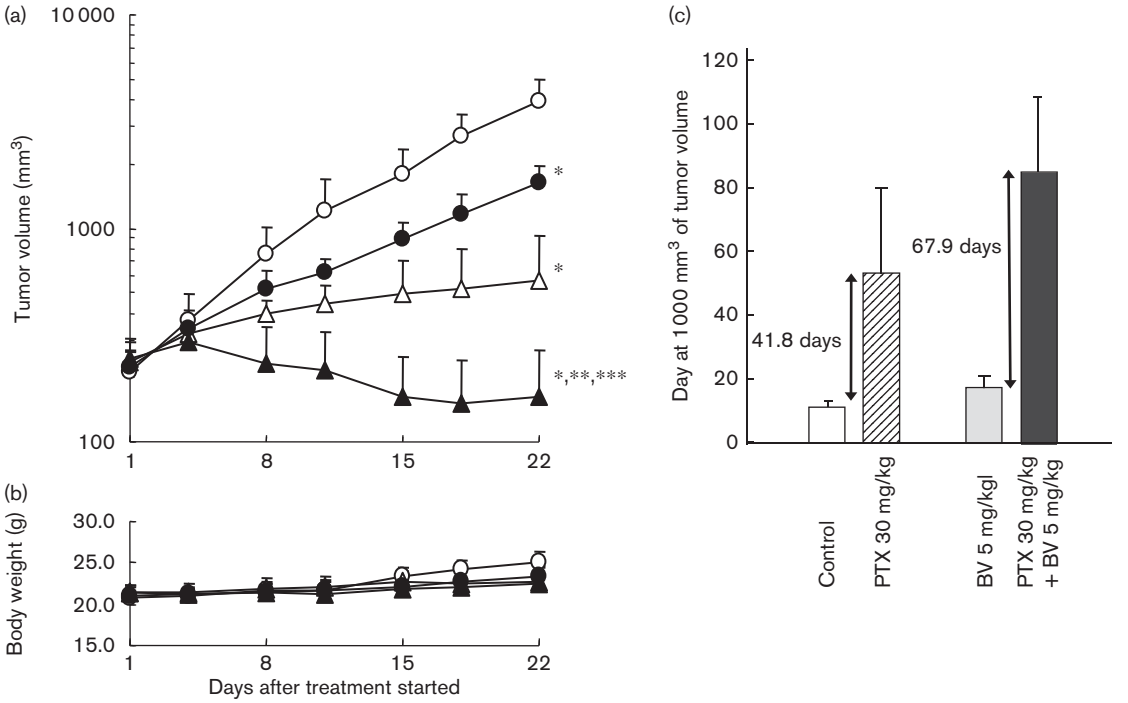


Antitumor activity of bevacizumab (BV) in the MX-1 human breast cancer xenograft model. Tumor volume change (a) and body weight change (b) were shown. Treatment was started 14 days after the tumor inoculation. Mice were randomly divided into four groups of six mice. Data points are mean plus standard deviation of tumor volume (mm^3). ○: control (HulgG 20 mg/kg), ◆: BV 1.25 mg/kg, ■: BV 5 mg/kg, ●: BV 20 mg/kg. Asterisks indicate statistically significant differences. *$P \leq 0.05$ versus control group by Wilcoxon test.

Increase of paclitaxel concentration in tumor tissues by bevacizumab

To investigate the mechanism of the synergistic activity of paclitaxel plus bevacizumab combination therapy, we measured paclitaxel concentration in the tumor by HPLC. First, we compared the antitumor activity of

Fig. 2



Antitumor activity of bevacizumab (BV) in combination with paclitaxel (PTX) in the MX-1 human breast cancer xenograft model. (a and b) Tumor volume and body weight in combination therapy with BV plus PTX. Treatment was started 14 days after the inoculation of the tumor cells. Mice were randomly divided into four groups of six mice for the PTX and BV combination study. Data points: mean plus standard deviation (SD) of tumor volume (mm^3, a) or body weight (gram, b). ○: control group (HulgG 5 mg/kg + PTX vehicle), ●: BV group (BV 5 mg/kg + PTX vehicle), △: PTX group (HulgG 5 mg/kg + PTX 20 mg/kg), ▲: combination group (HulgG 5 mg/kg + PTX 20 mg/kg). Statistically significant differences are shown. $*P \leq 0.05$ versus control group, $**P \leq 0.05$ versus PTX 20 group, $***P \leq 0.05$ versus BV group by Wilcoxon test. (c) Tumor growth delay by BV treatment. Treatment was started 17 days after the inoculation of the tumor cells. Mice were randomly divided into four groups of six mice for the PTX and BV combination study. Bars: mean + SD of the day at 1000 mm^3 of tumor volume.

paclitaxel alone with that of the combination therapy of paclitaxel and bevacizumab in the MX-1 model (Fig. 3a). Tumor volume in the groups treated with paclitaxel alone at 10, 30, 60, and 100 mg/kg on day 22 showed dose-dependent tumor growth inhibition. The antitumor activity of 30 mg/kg paclitaxel in combination with 5 mg/kg bevacizumab was comparable with that of paclitaxel alone at both 60 and 100 mg/kg.

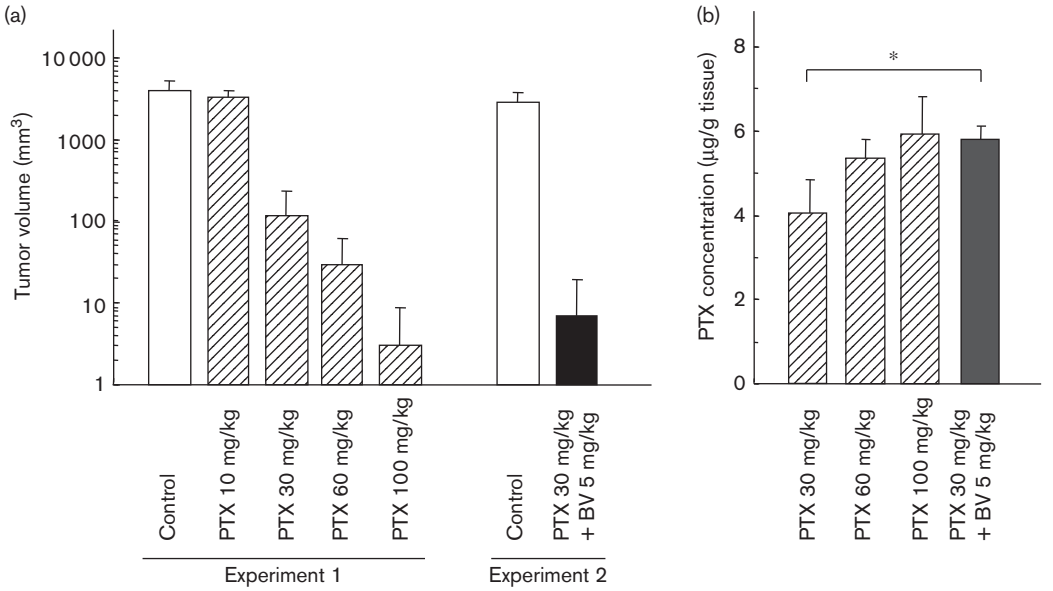
The concentration of paclitaxel in the tumor in the mice treated with paclitaxel 30 mg/kg plus bevacizumab 5 mg/kg was 5.75 ± 0.31 μg/g of tissue and was significantly higher than in the tumor treated with paclitaxel 30 mg/kg alone (4.00 ± 0.85 μg/g of tissue) 48 h after the paclitaxel injection in the MX-1 model ($P = 0.0022$, Fig. 3b). The paclitaxel concentration in the tumor treated with paclitaxel 30 mg/kg plus bevacizumab 5 mg/kg was equivalent to that in the tumor treated with 100 mg/kg of paclitaxel. The paclitaxel levels correspond to the degree of antitumor activity of paclitaxel alone and paclitaxel plus bevacizumab. In contrast to the tumor, no remarkable differences were observed for paclitaxel concentration in the plasma or liver between the paclitaxel 30 mg/kg plus bevacizumab 5 mg/kg group and the paclitaxel 30 mg/kg group. Maximum drug concentration (C_{max}) and $T_{1/2}$ values were calculated from the mean values of paclitaxel concentration in each group (Table 1). The maximum drug concentration time (T_{max}) of paclitaxel in the plasma, tumor, and liver were 5 min, 2 h and 5 min, respectively. After paclitaxel injection, the paclitaxel concentration was at the lower limit of quantitation in the plasma at 12 h or later and in the liver at 18 h or later.

We also examined the effect of bevacizumab on paclitaxel concentration in the human lung cancer xenograft model, A549, and again found an increase in paclitaxel concentration, further showing the potent antitumor activity of the combination of paclitaxel and bevacizumab (Fig. 4a). Paclitaxel concentrations in the tumor treated with paclitaxel 20 mg/kg plus bevacizumab 5 mg/kg were significantly higher than those treated with paclitaxel alone, as in the MX-1 model (Fig. 4b).

Decrease in the vascular permeability of tumor tissues caused by bevacizumab

Next, we examined the decrease in the vascular permeability of the tumor caused by bevacizumab. A tumor treated with bevacizumab was collected on days

Fig. 3



Comparison of paclitaxel (PTX) concentration in the tumor in MX-1 model. (a) Antitumor activity of PTX alone or PTX plus bevacizumab (BV) in MX-1. Mice were randomly allocated to groups ($n=6$/group). Bars: mean plus standard deviation of tumor volume (mm^3) on day 22. Tumor volume of the control group on day 1 in Experiments 1 and 2 was 185 ± 50 and $192 \pm 77\ mm^3$, respectively. Tumor volume of the treatment group on day 1 was equivalent to the volumes for the control groups. (b) Concentration of PTX in the tumor. Mice were randomly allocated to four groups of six mice. PTX was administered intravenously 1 h after HuIgG or BV was administered intraperitoneally. Mice were killed 48 h after the administration of PTX. The tumor was collected from each mouse and the PTX concentration was analyzed by high-performance liquid chromatography. $*P \leq 0.05$ versus PTX alone by the Wilcoxon test.

Table 1 C_{max} **and** $T_{1/2}$ **of paclitaxel in paclitaxel alone or paclitaxel plus bevacizumab groups in a MX-1 xenograft model**

	C_{max} (μg/ml)	C_{max} (μg/g tissue)		$T_{1/2}$ (h)		
Administration	Plasma	Tumor	Liver	Plasma	Tumor	Liver
Paclitaxel 30 mg/kg + HuIgG 5 mg/kg	60.4	12.9	201	0.952	39.0	1.66
Paclitaxel 30 mg/kg + bevacizumab 5 mg/kg	70.0	12.9	226	0.834	68.6	1.46
Δ%	16	0	12	−12	76	−12

Paclitaxel was administered intravenously 1 h after HuIgG or bevacizumab was administered intraperitoneally. Mice were killed within 5 min to 48 h after the administration of paclitaxel. Blood, tumor, and liver were collected from each mouse and paclitaxel concentration was analyzed by high-performance liquid chromatography. C_{max} or $T_{1/2}$ was calculated from the average of each group ($n=6$/group).
HuIgG, human immunoglobulin G.

2, 5, and 8. The vascular permeability in the tumor was evaluated by the difference between the area with CD31-positive staining and the area with FITC–lectin-positive staining in adjacent tissue sections. Typical images from immunohistochemical staining and automated image processing using the analysis software Win ROOF are shown in Fig. 5. Bevacizumab showed a significant decrease in the vascular permeability of tumor (Table 2). The ratio of the vascular permeability of the tumor treated with bevacizumab to the control tumor reached 50% by day 5.

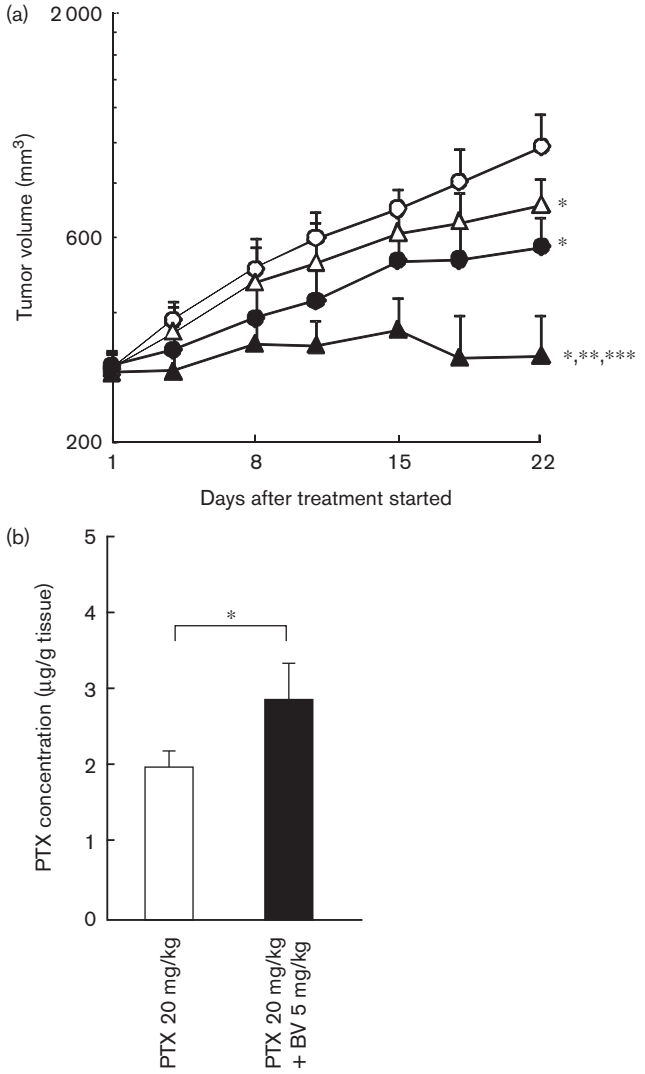
Effect of bevacizumab or paclitaxel plus bevacizumab on microvessel density

We examined the effect of bevacizumab on MVD in the tumor of the MX-1 xenograft model by immunohistochemical staining for CD31. MVD on day 5 in the tumor of the bevacizumab alone group was significantly lower than in the control group (Fig. 6), but a further MVD decrease was shown by the paclitaxel plus bevacizumab group. MVD in the paclitaxel alone group also did not change significantly compared with that of the control group (data not shown).

Discussion

In a phase III trial (E2100), bevacizumab in combination with paclitaxel significantly prolonged progression-free survival and increased the objective response rate compared with paclitaxel alone in patients with metastatic breast cancer [7]. In this preclinical study, we also showed the synergistic antitumor activity and tumor growth delay effect of bevacizumab in combination with paclitaxel in an MX-1 breast cancer xenograft model and investigated the mechanisms of this synergism.

Fig. 4

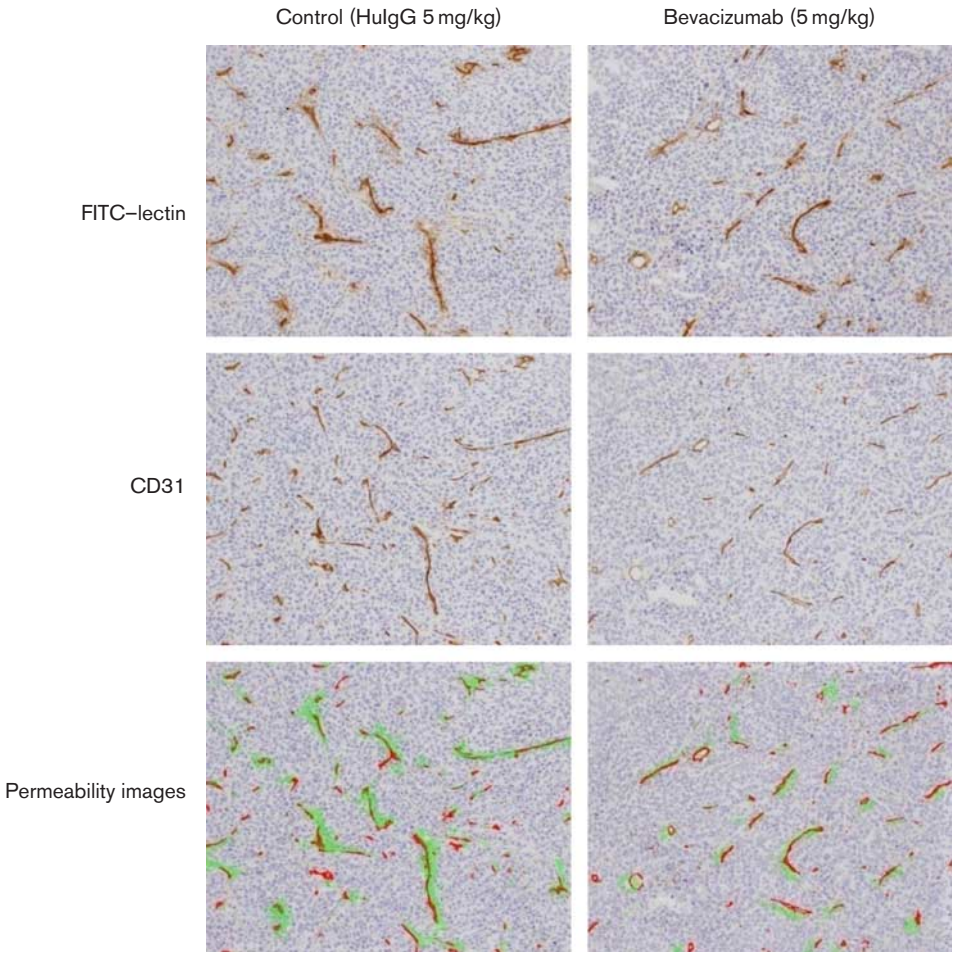


Combination of bevacizumab (BV) with paclitaxel (PTX) and increased PTX in the tumor in the A549 model. (a) Antitumor activity of PTX plus BV in A549. Mice were randomly allocated to groups ($n=8$/group) 32 days after inoculation. Data points: mean plus standard deviation of tumor volume (mm^3). ○: control group (HulgG 5 mg/kg + PTX vehicle), ●: BV group (BV 5 mg/kg + PTX vehicle), △: PTX group (HulgG 5 mg/kg + PTX 20 mg/kg), ▲: combination group (BV 5 mg/kg + PTX 20 mg/kg). Statistically significant differences are shown. *$P \leq 0.05$ versus control group, **$P \leq 0.05$ versus PTX 20 group,***$P \leq 0.05$ versus BV group by Wilcoxon test. (b) Concentration of PTX in the tumor. Mice were randomly allocated to two groups of six mice. PTX was administered intravenously 1 h after HulgG or BV was administered intraperitoneally. Mice were killed 48 h after the administration of PTX. The tumor was collected from each mouse and the PTX concentration was analyzed by high-performance liquid chromatography. *$P \leq 0.05$ versus PTX alone by the Wilcoxon test.

Using the model, we examined tumor levels of paclitaxel after the treatment with this agent to investigate the effect of bevacizumab on tumor in combination chemotherapy. VEGF has been reported to increase vascular permeability and to elevate the interstitial fluid pressure (IFP) in tumor. Theoretically, the delivery rate of low-molecular weight chemotherapeutic agents is reduced in tumor as a result of an increase in IFP, and as bevacizumab reduces the IFP, the delivery of the chemotherapeutic agents into the tumor is increased [5]. Indeed, Wildiers *et al.* [11] has reported that A4.6.1 showed a tendency to increase the tumor levels of CPT-11 in the human colorectal cancer xenograft model. Dickson *et al.* [12] also showed that bevacizumab induced increases in the tumor levels of topotecan and etoposide in the human neuroblastoma xenograft models. In this study, bevacizumab treatment significantly increased the concentration of paclitaxel in the tumor compared with the tumors treated with human IgG. According to the elevation of paclitaxel concentration in the tumor, the degree of antitumor activity of paclitaxel at 30 mg/kg administered in combination with bevacizumab was equivalent to that of 60 or 100 mg/kg monotherapy. Therefore, in the MX-1 model, the synergistic antitumor activity of paclitaxel plus bevacizumab may be explained by the improved delivery of paclitaxel into tumor. The increase in the paclitaxel concentration of the tumor with bevacizumab was also observed in A549, in which potent efficacy was observed with the combination of paclitaxel and bevacizumab, and therefore, this mechanism does not seem to occur only in the MX-1 model.

The delivery of paclitaxel into the plasma or liver did not seem to be altered by bevacizumab administration. Therefore, VEGF produced by MX-1 tumor cells would affect the vascular permeability and the delivery of paclitaxel into the tumor locally but not systemically. The following actions reported for bevacizumab may also help us to understand the mechanism of synergism between bevacizumab and chemotherapeutic agents: blood vessel normalization [9], vascular permeability downregulation [5], and interstitial pressure decrease [5,13,14]. In this study, we investigated the change in vascular permeability in tumor tissues as one of the parameters of blood vessel normalization by bevacizumab treatment and found that bevacizumab significantly decreased the permeability of the MX-1 tumor. This is first report that shows both increased paclitaxel concentration and improved permeability in the tumor by bevacizumab in the breast cancer xenograft model that also exhibits synergistic antitumor activity of bevacizumab in combination with paclitaxel. We attempted to measure the change in vascular flow by ultrasonic imaging and in IFP using the wick-in-needle technique [15] in the same MX-1 tumor. Unfortunately, at present, we have not established an appropriate evaluation system. No significant evidence was seen regarding causality between the decrease in vascular permeability and the increase in drug delivery. VEGF has been reported to block vessel maturation such as pericyte coverage [16,17], and thus examination of vessel maturity is important in the investigation of vessel normalization by bevacizumab. However, in this study, we did not explore vessel maturation to clarify the mechanism

Fig. 5



Immunohistochemical analysis of fluorescein isothiocyanate (FITC)–lectin and CD31. Mice were randomly divided into six groups of four mice. HulgG or bevacizumab was administered intraperitoneally on day 1. Mice were killed on day 2, day 5, or day 8. The tumor tissues were collected from each mouse and the permeability in the tumor was determined from the difference between the area with CD31 positive staining and the area showing FITC–lectin-positive staining in adjacent tissue sections. The calculation of permeability was performed automatically by the imaging analysis software Win ROOF. Green area is FITC-lectin-positive staining and red area is CD31-positive staining in permeability images.

Table 2 Effect of bevacizumab on vascular permeability in MX-1 tumor tissue

	Permeability (%)			
Administration	Day 2	Day 5	Day 8	*P* value
Control (HulgG 5 mg/kg)	3.05 ± 1.65	3.65 ± 1.37	2.87 ± 1.24	–
Bevacizumab 5 mg/kg	2.25 ± 1.05	1.80 ± 1.18	1.80 ± 0.94	0.0315

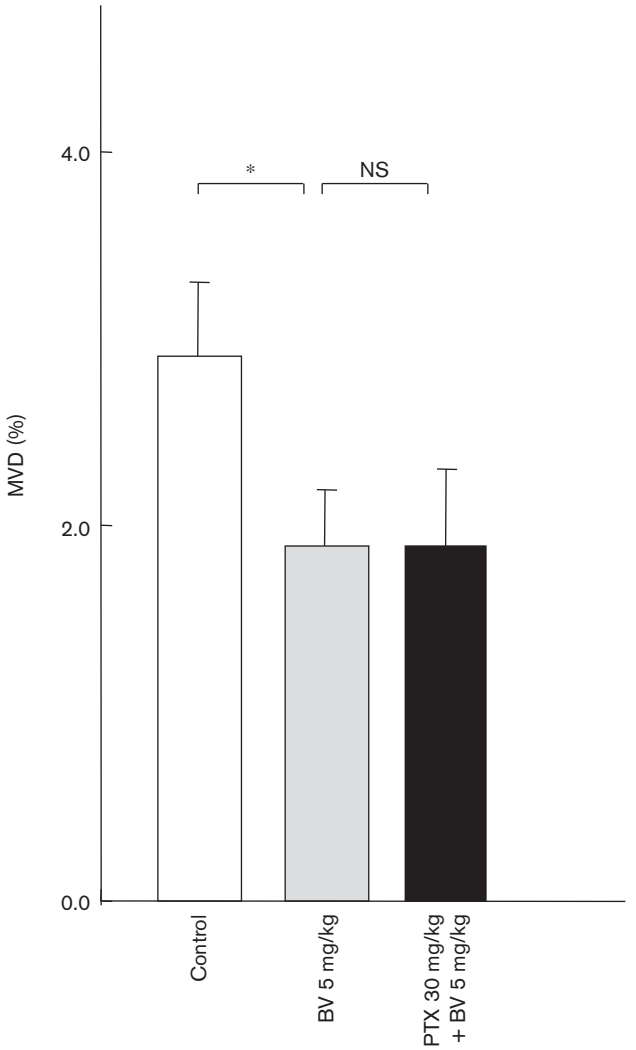
Statistical analysis of the difference between the control and the bevacizumab group was performed using a two-way analysis of variance.

of paclitaxel concentration increase in the tumor because the increase in paclitaxel concentration in the tumor was recognized 49 h after bevacizumab treatment (48 h after paclitaxel treatment), whereas we assume the time for vessel maturation is longer than 49 h. Further investigation into the mechanisms is required to clarify the synergism.

Paclitaxel has been shown to reduce MVD in tumor tissue [18]. Therefore, we examined the possibility that paclitaxel would reduce MVD and exert synergistic effects with the anti-angiogenic activity of bevacizumab. In the MX-1 model, paclitaxel did not significantly reduce MVD in the tumor and showed no significant augmentation of the anti-angiogenic activity of bevacizumab. These findings suggest that the mechanisms of the synergistic antitumor activity observed in the MX-1 model are not related to the additional reduction of MVD by the combination therapy.

In this study, we showed the synergistic antitumor activity of bevacizumab with paclitaxel in a breast cancer xenograft model. The improved delivery of paclitaxel into the tumor helps to explain the mechanism of the synergistic effects of the combination of bevacizumab and paclitaxel. Further studies to clarify the mechanism are needed.

Fig. 6



Effect of bevacizumab (BV) or paclitaxel (PTX) plus BV on microvessel density (MVD). Mice were randomly allocated to groups ($n=4$/group). PTX vehicle or PTX was administered intravenously on day 1. HuIgG 5 mg/kg (control) or BV 5 mg/kg was administered intraperitoneally on day 1. Mice were killed on day 5. The tumor tissues were collected from each mouse and the MVD was evaluated. *$P=0.05$ versus control by the Wilcoxon test. NS, not significant.

Acknowledgements

The authors thank Haruyoshi Shirai for his excellent help in conducting experiments and discussion, and Kumiko Kondo, Masako Takahashi, and Naohiko Hayakawa for their assistance with the experiments.

References

1 Presta LG, Chen H, O'Connor SJ, Chishlm V, Meng YG, Krummen L, *et al.* Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. *Cancer Res* 1997; **57**:4593–4599.
2 Kim KJ, Li B, Houck K, Winer J, Ferrara N. The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. *Growth Factors* 1992; **7**:53–64.
3 Wang Y, Fei D, Vanderlaan M, Song A. Biological activity of bevacizumab, a humanized anti-VEGF antibody, in vitro. *Angiogenesis* 2004; **7**:335–345.
4 Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, *et al.* Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. *Nature* 1993; **362**:841–844.
5 Gerber H-P, Ferrara N. Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. *Cancer Res* 2005; **65**:671–680.
6 Horwitz SB. Mechanism of action of taxol. *Trends Pharmacol Sci* 1992; **13**:134–136.
7 Miller K, Wang M, Gralow J, Dickler M, Cobleigh M, Perez EA, *et al.* Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* 2007; **357**:2666–2676.
8 Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, *et al.* Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006; **355**:2542–2550.
9 Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science* 2005; **307**:58–62.
10 Hu L, Hofmann J, Jaffe RB. Phosphatidylinositol 3-kinase mediates angiogenesis and vascular permeability associated with ovarian carcinoma. *Clin Cancer Res* 2005; **11**:8208–8212.
11 Wildiers H, Guetens G, De Boeck G, Verbeken E, Landuyt B, Landuyt W, *et al.* Effect of antivascular endothelial growth factor treatment on the intratumoral uptake of CPT-11. *Br J Cancer* 2003; **88**:1979–1986.
12 Dickson PV, Hamner JB, Sims TL, Fraga CH, Ng CYC, Rajasekeran S, *et al.* Bevacizumab-induced transient remodeling of the vasculature in neuroblastoma xenografts results in improved delivery and efficacy of systemically administered chemotherapy. *Clin Cancer Res* 2007; **13**:3942–3950.
13 Lee CG, Heijin M, di Tomaso E, G-Etienne G, Ancukiewicz M, Koike C, *et al.* Anti-vascular endothelial growth factor treatment augments tumor radiation response under normoxic or hypoxic conditions. *Cancer Res* 2000; **60**:5565–5570.
14 Willet CG, Boucher Y, di Tomaso E, Duda DG, Munn LL, Tong RT, *et al.* Direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer. *Nat Med* 2004; **10**: 145–147.
15 Fandes HO, Reed RK, Aukland K. Interstitial fluid pressure in rats measured with a modified wick technique. *Microvasc Res* 1977; **14**:27–36.
16 Verheul HMW, Hammer H, van Erp K, Wei Y, Sanni T, Salumbides B, *et al.* Vascular endotherial growth factor trap blocks tumor growth, metastasis formation, and vascular leakage in an orthotropic murine renal cell cancer model. *Clin Cancer Res* 2007; **13**:4201–4208.
17 Lu C, Thaker PH, Lin Yvonne G, Spannuth W, Landen CN, Merritt WM, *et al.* Impact of vessel maturation on antiangiogenic therapy in ovarian cancer. *Am J Obstet Gynecol* 2008; **4**:477e1–477e10.
18 Grant DS, Williams TL, Zahaczewsky M, Dicker AP. Comparison of antiangiogenic activities using paclitaxel (Taxol) and docetaxel (Taxotere). *Int J Cancer* 2003; **104**:121–129.