Cancer Chemother Pharmacol (2010) 66:745–753
DOI 10.1007/s00280-009-1219-y

ORIGINAL ARTICLE

Biodistribution of humanized anti-VEGF monoclonal antibody/ bevacizumab on peritoneal metastatic models with subcutaneous xenograft of gastric cancer in mice

Yasumichi Yagi · Sachio Fushida · Shinichi Harada · Tomoya Tsukada · Jun Kinoshita · Katsunobu Oyama · Hideto Fujita · Itasu Ninomiya · Takashi Fujimura · Masato Kayahara · Seigo Kinuya · Masakazu Yashiro · Kousei Hirakawa · Tetsuo Ohta

Received: 10 October 2009 / Accepted: 9 December 2009 / Published online: 24 December 2009

Abstract
Purpose Vascular endothelial growth factor (VEGF) is correlated with peritoneal metastasis of gastric cancer, increasing vascular permeability accompanied by accumulation of ascites. The aim of the current study is to investigate the biodistribution of bevacizumab in a peritoneal metastatic model of gastric cancer and to clarify which is more suited to treatment of peritoneal metastasis, systemic or regional therapy.
Methods A highly peritoneal-seeding cell line of gastric cancer, OCUM-2MD3, which exhibited high production and release of VEGF was used in this study. The biodistribution of bevacizumab was investigated using peritoneal metastatic models together with subcutaneous xenografts, and ^{125}I-radiolabelled bevacizumab was administrated to these models subcutaneously (s.c.) or intraperitoneally (i.p.), respectively. In addition, the anti-tumor response of bevacizumab and paclitaxel was assessed as single agents or in combination using peritoneal metastatic models.
Results In the analysis of biodistribution, ^{125}I-bevacizumab administrated i.p. indicated low peritoneal clearance. On the other hand, s.c. administration of ^{125}I-bevacizumab showed preferential accumulation in subcutaneous tumors and peritoneal nodules, with a high blood concentration. In peritoneal metastatic models, the effects of bevacizumab were found for both the growth inhibition of peritoneal nodules ($P < 0.01$) and the reduction of ascites ($P < 0.05$). These effects were more prominent by s.c. administration compared with i.p. administration and were increased in combination with i.p. paclitaxel.
Conclusion Bevacizumab should be administrated systemically compared to regionally, and the combination with i.p. paclitaxel has a potential to be useful for patients with peritoneal metastasis of gastric cancer.

Keywords Peritoneal metastasis · Bevacizumab · Biodistribution · Iodine-125 label · Molecular-targeted therapy

Y. Yagi (✉) · S. Fushida · T. Tsukada · J. Kinoshita · K. Oyama · H. Fujita · I. Ninomiya · T. Fujimura · M. Kayahara · T. Ohta
Department of Gastroenterologic Surgery, Division of Cancer Medicine, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan
e-mail: y-yagi@live.jp

S. Harada
Center for Biomedical Research and Education, School of Medicine, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan

S. Kinuya
Department of Biotracer Medicine, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan

M. Yashiro · K. Hirakawa
Department of Surgical Oncology, Graduate School of Medicine, Osaka City University, 1-4-3 Asahi-machi, Abenoku, Osaka 545-8585, Japan

Introduction

Peritoneal metastasis is the most frequent metastatic pattern after surgery of gastric cancer. It is regarded at diagnosis as a terminal disease with a poor prognosis and is characteristic of re-accumulation of ascites, obstruction of digestive organs, hydronephrosis, and poor delivery of drugs. In recent years, anti-cancer agents for gastric cancer

have been developed, but the outcomes for patients with peritoneal metastasis have not improved sufficiently.

Vascular endothelial growth factor (VEGF), which is well-known as an angiogenic factor, plays a critical role in both stimulating capillary mitogenesis and ascites formation, which lead to tumor growth and often massive ascites [1]. There are some pathological features accounting for the mechanisms of ascites formation. At first, a reduced lymphatic recovery system was proposed from the evidence that tumor cells obstruct the draining of lymphatics [2, 3]. Secondly, angiogenesis is associated with the accumulation of malignant ascites based on the observations that peritoneal neovascularization accompanied some ascites, and angiogenesis inhibitor significantly reduces ascites accumulation after the inhibition of vessel proliferation [4, 5]. More recently, increased vascular permeability of the peritoneal wall is thought to be the most essential factor of ascites formation associated with malignant disease [6–8]. VEGF is 50,000 times more potent than histamine in inducing vascular permeability [9] and also is a potent mitogen for micro and macro vascular endothelial cells derived from arteries, veins, and lymphatic vessels [10, 11]. Previous studies indicate that VEGF acts on the adjacent vascular endothelium through paracrine mechanisms [12], and VEGF production is correlated directly with fluid accumulation in experimental models of malignant ascites [13]. Furthermore, clinical reports indicate that VEGF is related to ascites formation because of its ability to increase vascular permeability [14, 15], and the expression of VEGF in patients with gastric cancer is correlated with recurrence and poor prognosis [16, 17]. Although the value of quantifying serum levels of VEGF as a prognostic factor of gastric cancer is unclear, previous reports have shown high serum levels in patients with malignant ascites and pleural perfusion [18, 19]. These relations define novel therapeutic targets of anti-VEGF agents and provide mechanistic insights into the action of clinically available anti-VEGF agents. In animal models of peritoneal metastasis, VEGF blockade dramatically reduced ascites formation [20–22]. However, less specific tyrosine kinase inhibitors have been shown to reduce both ascites and peritoneal tumor burden [23], raising the question whether VEGF-specific blocking agents lack the potency to block tumor growth. When considering the effect of anti-VEGF therapy in preclinical models of peritoneal metastasis, it is important to distinguish whether VEGF therapy alone has a potential to reduce both ascites formation and tumor burden or whether it will block only ascites formation.

Bevacizumab is a molecular-targeted agent for neutralizing VEGF. Recent clinical studies indicated that combining molecular-targeted therapy with conventional chemotherapy enhances the inhibition of tumor growth and metastasis in gastric cancer [24, 25]. In patients with peritoneal metastasis, drug distribution is varied because of peritoneal hyperpermeability accompanied by accumulation of ascites, compared with patients without peritoneal metastasis.

Intraperitoneal chemotherapy (IPC) is a method for the management of peritoneal metastasis, which is often refractory to conventional chemotherapy. In ovarian cancer, IPC has been shown to have a survival benefit compared with intravenous chemotherapy [26–28]. As for bevacizumab, several studies showed the effectiveness of intraperitoneal administration of bevacizumab (i.p. bevacizumab) in peritoneal metastatic model [29, 30]. However, few reports about the pharmacokinetics including systemic administration of bevacizumab (systemic bevacizumab) have been described. In this study, we established a peritoneal metastatic model of gastric cancer together with subcutaneous xenograft and examined the biodistribution of bevacizumab to clarify the differences in effectiveness by route of administration.

Materials and methods

Materials

Bevacizumab (Avastin) was purchased from Roche, Inc. (Basel, Switzerland), and paclitaxel was kindly provided by Bristol-Myers Squibb Company (Japan). Bevacizumab, a recombinant humanized monoclonal antibody, blocks the binding of VEGF to its receptor. Previous studies have shown that bevacizumab binds to human VEGF with a lower affinity to rabbit VEGF and none to rat or murine VEGF [31]. Although bevacizumab blocks human VEGF derived from a human cancer cell line, both murine VEGF and host-derived VEGF stimulated by tumor cells does not bind with bevacizumab in our models. This study used bevacizumab as a potent inhibitor of tumor-derived VEGF in nude mouse models. Peak serum concentrations of bevacizumab after subcutaneous (s.c.) administration in mice were approximately half of those noted after the intravenous (i.v.) administration of a similar dose [32]. Almost bioavailability of bevacizumab in mice was equivalent between i.v. and s.c. administration [32]. Moreover, repeated i.v. injection on mice was difficult and unstable. Subsequently, s.c. injection was used as a systemic administration and i.p. injection as regional administration.

Cell lines and cell culture

OCUM-2MD3, a highly peritoneal-seeding cell line of human scirrhous gastric cancer was kindly provided by the

Department of Surgical Oncology of Osaka City University of Medicine [33]. Other human gastric cancer cell lines (MKN28, TMK-1) were obtained from the American Type Culture Collection (Rockville, MD, USA). These were seeded in a 75-cm^2 dish (Becton–Dickinson, Japan) and cultured in 10 ml of medium at 37°C in a humidified atmosphere of 5% CO_2 in air. OCUM-2MD3 cells were grown in DMEM medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Nichirei Bioscience Inc., Japan), 100 IU/ml penicillin, 100 mg/ml streptomycin (Gibco), 2 mM glutamine (Nissui Pharmaceutical Co., Ltd., Japan), and 0.5 mM sodium pyruvate. The culture medium for MKN28 and TMK-1 cells was RPMI (Nissui Pharmaceutical Co., Ltd., Japan) with the same additives as mentioned earlier. Cells were grown to confluence and harvested by trypsinization with 0.25 mg/ml trypsin/EDTA (Gibco) and suspended in culture medium before use. To confirm that the OCUM-2MD3 cells released high levels of VEGF, samples of the cell culture supernatants in serum-free conditions were collected individually from day 0–4, centrifuged, and stored at −80°C until VEGF was measured using ELISA.

MTT assay

The viability of OCUM-2MD3 cells treated with bevacizumab was determined by a standard 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. OCUM-2MD3 cells were plated (5×10^3 per well) in 96-well plates and incubated overnight at 37°C. After incubation, the supernatant was discarded and replaced with fresh serum-free culture medium. Bevacizumab was dissolved in phosphate-buffered saline (PBS) and added to the cell culture medium at various concentrations. After 72 h of exposure to bevacizumab at the indicated concentration, the supernatant was discarded and MTT solution was added to each well (500 μg/mL final concentration) and incubated at 37°C for 3 h. Then, the supernatant was removed, and 150 μL of DMSO was added. The absorbance of the solution was read at a wavelength of 540 nm using a microplate reader (BIO-RAD550, USA). The percentage inhibition was determined by comparing the cell density of the drug-treated cells with that of the untreated cells. All experiments were repeated at least three times.

ELISA

A quantitative sandwich enzyme-linked immunosorbent assay technique with a Quantikine human VEGF immunoassay kit (R&D Systems, Minneapolis, NM, USA) was used in accordance with the manufacturer's instructions to measure VEGF in cell culture supernatants from OCUM-2MD3 cells and ascites from the peritoneal metastatic model. All experiments were performed in triplicate.

Animals and xenograft model treated with bevacizumab

BALB/c *nu/nu* mice (female, 4–6 weeks old, Charles River Laboratories, Japan, Inc) were used as a xenograft model. They were housed in specific-pathogen-free conditions and fed standard chow pellets and water ad libitum. Experiments were performed according to the standard guidelines for animal experiments of Kanazawa University. The effect of bevacizumab on the xenograft model was examined as follows: OCUM-2MD3 cells (2×10^6 cells) were inoculated s.c. in the dorsal side of the mouse. The mice were divided into two groups: a control group (PBS s.c., $n = 5$) and a bevacizumab-treated group (10 mg/kg s.c., twice weekly, $n = 5$). The treatment was started on day 7 after xenografting and discontinued after 5 weeks. Tumors were measured twice weekly with vernier calipers. Tumor volume was calculated using the following formula: $volume = length \times width \times height \times 0.5236$. At the end of the experiment, tumor specimens were collected for immunohistochemical examination.

In vivo treatment with bevacizumab in a peritoneal metastatic model with OCUM-2MD3 cells

The effects of bevacizumab and paclitaxel were examined in peritoneal metastatic model. OCUM-2MD3 cells (1×10^7 cells) were inoculated i.p. via the left flank of a BALB/c *nu/nu* mouse (female, 4–6 weeks old). The mice were split into five groups on day 7 and then administered medication for 4 weeks as follows: control group (PBS, i.p.), paclitaxel i.p. group (2.5 mg/kg, weekly), bevacizumab i.p group (5 mg/kg, twice weekly), bevacizumab s.c group (5 mg/kg, twice weekly), bevacizumab s.c. and paclitaxel i.p. group (same dose as mentioned earlier). Each group was composed of five mice. The dose of paclitaxel was chosen based on preliminary dose–response studies. At 4 weeks after inoculation, the mice were killed. Samples of ascites and peritoneal nodules were collected, and the weights of ascites fluid and total peritoneal tumor burden were counted for each individual mouse.

Iodine-125-radiolabelled bevacizumab and biodistribution in a peritoneal metastatic model with subcutaneous xenografts

Bevacizumab was radiolabelled with iodine-125(^{125}I) (PerkinElmer Co. Ltd. Japan) by the chloramine-T method and subsequently purified on a PD-10 column (GE Healthcare UK Ltd.) that was equilibrated with 1% bovine

serum albumin in PBS to separate it from free ^{125}I and to evaluate the labeling efficiency [34–36]. The specific radioactivity of the purified ^{125}I-bevacizumab was 142 MBq/mg and the immunoreactivity was assessed as 1% in antigen excess conditions with 1×10^7 OCUM-2MD3 cells, indicating no binding ability to intracellular VEGF. Then, a peritoneal metastatic model with subcutaneous xenograft was established. BALB/c *nu/nu* mice (female, 4–6 weeks old) were inoculated i.p. with 1×10^7 OCUM-2MD3 cells and were xenografted s.c. with 2×10^6 OCUM-2MD3 cells at the same time (Fig. 1). These mice were divided into four groups as follows: the mice were injected with 74 kBq (2 mCi) of ^{125}I-bevacizumab s.c. or i.p. and each mouse was killed at 6 or 24 h after the injection. In addition, normal mice were injected ^{125}I-bevacizumab s.c. or i.p. and each mouse was killed at 6 h after the injection. Each group was composed of three mice. Blood, ascites, peritoneal nodule, and other major organs were removed and weighed. The samples were then subjected to radioactivity counting using a gamma counter. The biodistribution was expressed as a percentage of the injected dose per gram of tissue (%ID/g). Animal studies using the radioisotope were performed in compliance with the regulations of our institutions, and the principles of laboratory animal care were followed.

The following abbreviation is used for clarity; the group of mice treated subcutaneously was the s.c. group, and the group of mice treated intraperitoneally was the i.p. group.

Histology and immunohistochemical examination

Tumor specimens obtained from subcutaneous tumors and peritoneal nodules were fixed in 10% neutral buffered formalin and embedded in paraffin. The sections were stained with H&E and immunostained with a anti-VEGF antibody (A-20, rabbit polyclonal IgG, Santa Cruz; 1:200 dilution) at 4°C overnight. The sections were reacted with EnVision reagent (Dako Co. Japan) for visualization.

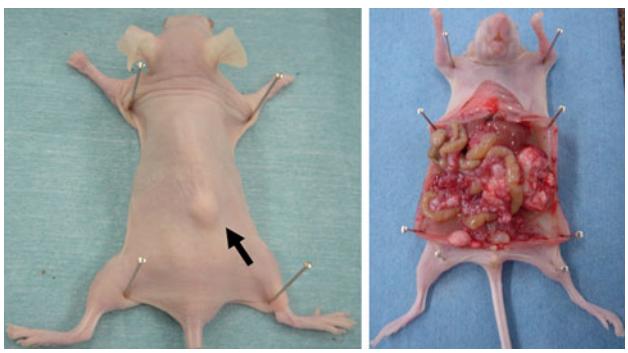

Fig. 1 A peritoneal metastatic model together with subcutaneous tumors. Xenografted subcutaneous tumors were produced by inoculating 1×10^6 OCUM-2MD3 cells in the dorsal side of a nude mouse (*arrow*). In the same mouse, 1×10^7 OCUM-2MD3 cells were inoculated into the peritoneal cavity

Western blotting

Intracellular VEGF levels in gastric cancer cell lines were analyzed in cell lysates by western blotting using an anti-VEGF antibody (A-20, rabbit polyclonal IgG, Santa Cruz; diluted 1:1,000). Whole-cell lysates were prepared in denaturing SDS sample buffer and subjected to SDS–PAGE (ATTO Co. Ltd. Japan). The immunoblots were visualized using an ECL Plus kit (GE Healthcare UK Ltd.).

Statistical analysis

Comparisons among the data sets were made by ANOVA followed by Student's *t*-test using the computer software package SPSS10.0. Differences were considered to be statistically significant when the P value was less than 0.05.

Results

Expression and secretion of VEGF by OCUM-2MD3 cells

The expression of VEGF in OCUM-2MD3 cells was observed in the cytoplasm (Fig. 2a). OCUM-2MD3 cells showed high levels of intracellular VEGF compared with the other human gastric cancer cell lines (Fig. 2b). The VEGF concentration of the culture medium increased gradually from 1,101 to 7,763 pg/mL over the course of 4 days (Fig. 2c). The VEGF level of ascites in the peritoneal metastatic model was also high (6,553 ± 751 pg/mL, $n = 3$). In vivo expression of VEGF was observed in both subcutaneous tumors and peritoneal nodules obtained from the peritoneal metastatic model with subcutaneous xenografts (Fig. 3).

Effect of bevacizumab on the growth of OCUM-2MD3 cells in vitro

As shown in Fig. 4a, bevacizumab at a high dose (more than 500 μg/mL) significantly inhibited the growth of OCUM-2MD3 cells ($P < 0.05$). There was no degeneration of cancer cells observed by phase-contrast microscopy (data not shown). This result showed slightly dose-dependent inhibition by blocking VEGF and the effect seemed to be cytostatic, not cytotoxic.

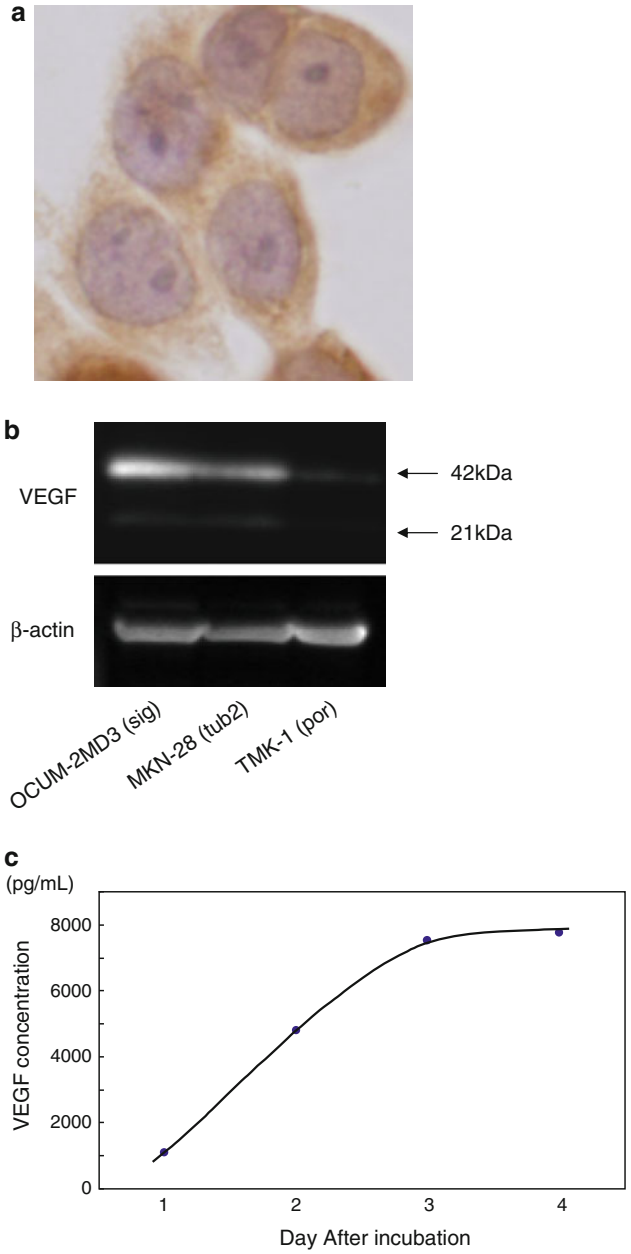

Fig. 2 **a** Immunohistochemical examination of VEGF expression in a human gastric cancer cell line, OCUM-2MD3, cells shows strong expression of VEGF in the cytoplasm. Original magnification ×400. **b** Western blotting analysis of VEGF expression in human gastric cancer cell lines. Relatively high expression of VEGF in OCUM-2MD3 cells was observed compared with the other cell lines. 21 kDa (monomer). 42 kDa (dimer). **c** Time course of VEGF levels of OCUM-2MD3 cells in culture medium (serum-free conditions). The concentration of medium was evaluated by ELISA

Effect of bevacizumab in subcutaneous xenograft models in vivo

The time course of subcutaneous tumor volumes is shown in Fig. 4b. The mean tumor volume in the bevacizumab-treated group (90.6 ± 6.7 mm^3) was significantly reduced by 54.5%, compared with the control group (166.1 ± 22.0 mm^3) at 4 weeks after treatment ($P < 0.01$).

Biodistribution of ^{125}I-radiolabelled bevacizumab in peritoneal metastatic models with subcutaneous xenografts

In normal mice, the biodistribution of ^{125}I-bevacizumab was almost equivalent between the s.c group and the i.p group at 6 h (Fig. 5a). In the peritoneal metastatic model with subcutaneous xenografts, strong localization of VEGF was observed in both subcutaneous tumors and peritoneal nodules (Fig. 5b). The blood uptake in the s.c. group at 24 h after injection was as high as 15%ID/g, which was 1.7-fold higher compared with that of the i.p. group ($P < 0.05$). The uptake in small peritoneal nodules of the s.c. group at 24 h after injection was as high as 4.9%ID/g, which was 1.5-fold higher compared with that of the i.p. group ($P = 0.116$), and that of large peritoneal nodules in the s.c. group at 24 h after injection was as high as 3.7%ID/g, which was 1.8-fold higher compared with that of the i.p. group ($P = 0.144$). The uptake of bevacizumab by subcutaneous tumors in the s.c. group at 24 h after injection was as high as 3.9%ID/g, which was 2.5-fold higher compared with that of the i.p. group ($P = 0.070$).

On the other hand, the uptake of bevacizumab in ascites in the i.p group at 24 h after injection was as high as 15%ID/g, which was 2.1-fold higher compared with that of the s.c group ($P < 0.05$).

Effect of bevacizumab in the peritoneal metastatic model

The mean weights of ascites in the paclitaxel alone group (2.78 ± 0.39 g), the i.p. group (2.04 ± 0.76 g), and the s.c. group (1.1 ± 0.34 g) were significantly reduced by 8.5, 32.8, and 63.8%, respectively, compared with the control group (3.04 ± 0.49 g). Moreover, little ascites was detectable in the bevacizumab s.c plus paclitaxel-treated group (Fig. 6a). The mean peritoneal tumor burdens in the i.p. group (1.04 ± 0.18 g) and the s.c. group (0.6 ± 0.09 g) were significantly reduced by 58.4 and 76%, respectively, compared with the control group (2.5 ± 0.25 g). The mean peritoneal tumor burden in the paclitaxel-alone (2.12 ± 0.16 g) and bevacizumab s.c. plus paclitaxel-treated (0.38 ± 0.04 g) groups was reduced by 15.2 and 84.8%, respectively, compared with the control group (Fig. 6a). The peritoneal tumor of the s.c. group was effectively inhibited compared with that of the i.p. group ($P < 0.05$). The combination of subcutaneous bevacizumab and i.p. paclitaxel gave favorable results compared with the group receiving only i.p. bevacizumab. In the control group, there was an extensive increase in

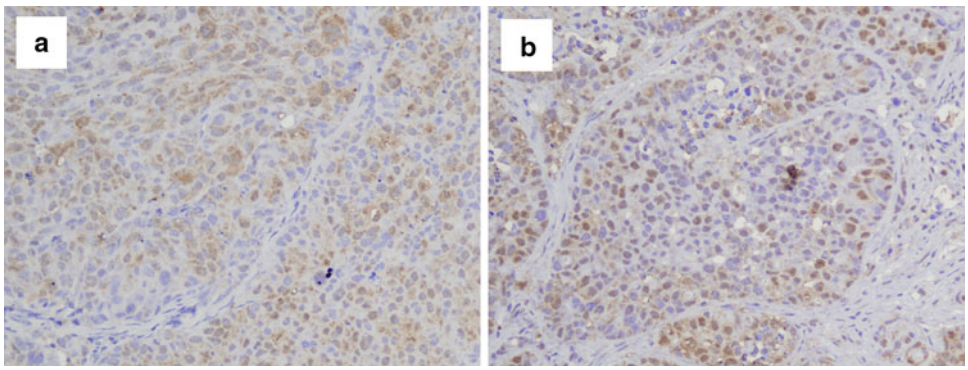

Fig. 3 Immunohistochemical examination of VEGF expression in subcutaneous xenografts and peritoneal tumors in a peritoneal metastatic model with subcutaneous xenografts. Strong expression of VEGF was observed in both subcutaneous xenografts **a** and peritoneal tumors **b**. Original magnification ×200 (**a**, **b**)

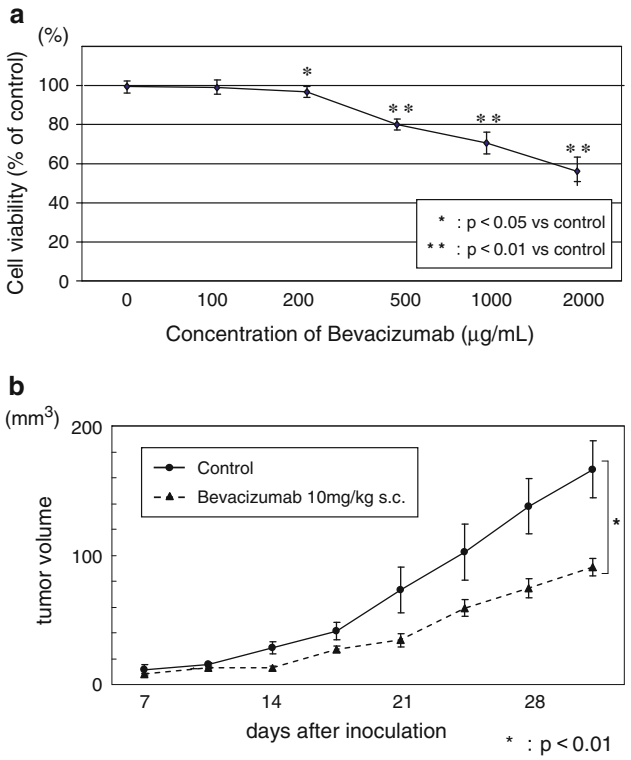

Fig. 4 **a** Effect of bevacizumab on the growth of OCUM-2MD3 cells in vitro. Cell viability was assessed using an MTT assay. OCUM-2MD3 cells were treated with the indicated doses of bevacizumab (0–2,000 μg/mL) in serum-free medium. Results are expressed as means ± SE ($n = 3$). **b** Effect of bevacizumab in a subcutaneous xenograft model in vivo. Bevacizumab (10 mg/kg) or PBS (control) was administered subcutaneously twice weekly. Tumor volumes were measured twice weekly and calculated as the product of the length, width, and height of each tumor (see "Materials and methods". Results are expressed as means ± SE ($n = 5$)

abdominal girth, indicative of ascites, tumor burden, and the extent of peritoneal metastasis. In contrast, bevacizumab reduced both abdominal girth and extent of peritoneal metastasis in both the s.c. group and the i.p. group (Fig. 6b) and gave a marked decrease in girth and tumor burden in the bevacizumab s.c. plus paclitaxel-treated mice.

Discussion

The gastric cancer cell line OCUM-2MD3 showed high expression and high release of VEGF, leading to strong location of ^{125}I-bevacizumab in tumor and ascites in the present model. Then, using this cell line, a double xenografted model was established; peritoneal metastasis and subcutaneous tumors in the same mouse, to evaluate the effect for hematogenous metastasis and peritoneal metastasis simultaneously. The biodistribution of ^{125}I-bevacizumab in normal mice showed the equivalence between s.c. and i.p. administration, and this result indicated that i.p. bevacizumab gave an effect equivalent to systemic bevacizumab in the circumstance without peritoneal metastasis. In the double xenografted model, the s.c. group maintained high blood levels and showed a high uptake of ^{125}I-bevacizumab in subcutaneous tumors and peritoneal nodules compared with the i.p. group. These differences between the s.c. group and the i.p. group in these models might result from hyperpermeability of peritoneum and a large amount of VEGF in ascites. According to the previous reports investigating the biodistribution of the peritoneal metastatic model using radiolabelled antibodies recognizing surface antigen of cancer cell, intraperitoneal injection of the MAb resulted in higher accumulation in peritoneal nodule than after i.v. injection [37, 38]. In other words, a complete tumor-specific antibody is more effective by i.p. administration in the peritoneal metastatic model. One of the differences from the present study is that bevacizumab recognizes only soluble VEGF. If bevacizumab is administrated i.p. in the peritoneal metastatic model, most of the antibody will be quenched in malignant ascites containing a large amount of VEGF resulting in low blood concentration.

In the present study, bevacizumab alone had an antitumor effect in vivo by blocking only tumor-derived VEGF, though the influence of VEGF produced by human peritoneal mesothelial cells and stromal cells [31, 39, 40] must be considered. In clinical application, bevacizumab could block stromal-derived VEGF as well as tumor-derived VEGF and would be more available. In contrast,

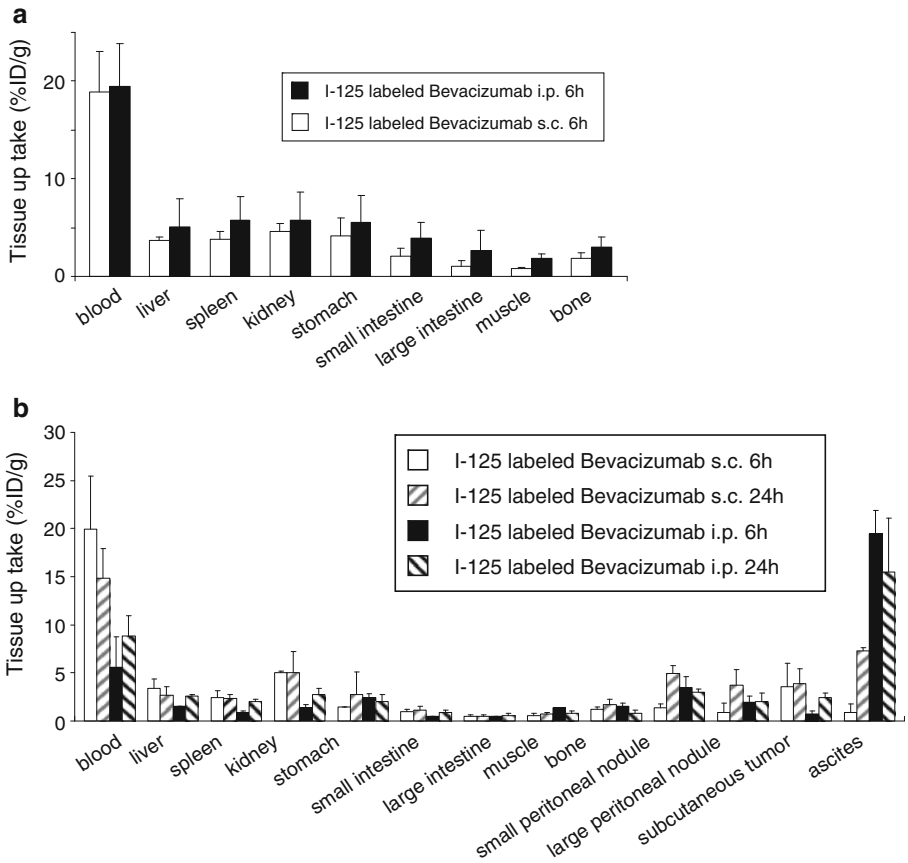

Fig. 5 **a** Biodistribution of ^{125}I-radiolabelled bevacizumab in normal mice. **b** Biodistribution of ^{125}I-radiolabelled bevacizumab in a peritoneal metastatic model with subcutaneous xenografts. Expressed as a percentage of the injected dose per gram of tissue. (Mean ± SE, $n = 3$)*; $P < 0.05$

the inhibition in in vitro experiments exhibited cytostatic effects even at extra high doses as most of molecular-targeted agents [30]. Therefore, bevacizumab should be used in combination with another cytotoxic chemotherapeutic agent [41]. In ovarian cancer, the combination of another anti-VEGF agent and paclitaxel inhibits tumor growth and metastasis without observable side effects [20, 21]. As paclitaxel, a cytotoxic chemotherapeutic agent, is commonly used to treat patients with gastric cancer, we combined it with bevacizumab in anticipation of the synergistic effect. It was shown that bevacizumab combined with paclitaxel also revealed a striking reduction of peritoneal tumors and a notable inhibition of ascites formation in the present models. The potency of bevacizumab with low dose of paclitaxel is likely to contribute the success of this drug combination. One of the reasons is that by normalizing the vascular permeability, bevacizumab allows better tumor perfusion, and thus paclitaxel is delivered to the tumor more successfully. A second possibility may be that paclitaxel has also anti-angiogenic activity as well as its anti-tumor effects, because the dividing, genetically stable endothelial cells of newly forming tumor vessels are believed to be sensitive to chemotherapeutic drugs [42–44], and blockade of VEGF that promotes endothelial cell survival may enhance this sensitivity. In combination with bevacizumab, weekly paclitaxel would be more tolerated due to a low toxicity and could give anti-tumor and anti-angiogenic responses in patients with gastric cancer.

In most of studies concerning usefulness of bevacizumab in peritoneal metastatic model, bevacizumab has been injected i.p. as systemic administration, because i.p. injection is an easy method to treat mice and is regarded as equivalent to i.v. injection. However, considering special condition of peritoneal metastasis with peritoneal hyperpermeability and ascites, i.p. bevacizumab is regarded to be neither suitable method nor systemic administration.

Although a phase III clinical study of bevacizumab added to systemic chemotherapy in advanced gastric cancer (AVAGAST study) is ongoing, the efficacy of bevacizumab added to regional chemotherapy has not been evaluated. In peritoneal metastasis of ovarian cancer, i.p. paclitaxel as regional treatment has been recognized as effective. As for gastric cancer, Ishigami et al. [45] also described that i.p. paclitaxel is well tolerated and active in patient with peritoneal metastasis.

Understanding the tissue distribution of administrated bevacizumab is helpful for treating peritoneal metastasis. The present data of the biodistribution in a peritoneal metastatic model support the utility of systemic bevacizumab due to the high blood concentration and high intra-tumoral density of VEGF. It is suggested that systemic bevacizumab treatment in combination with i.p.

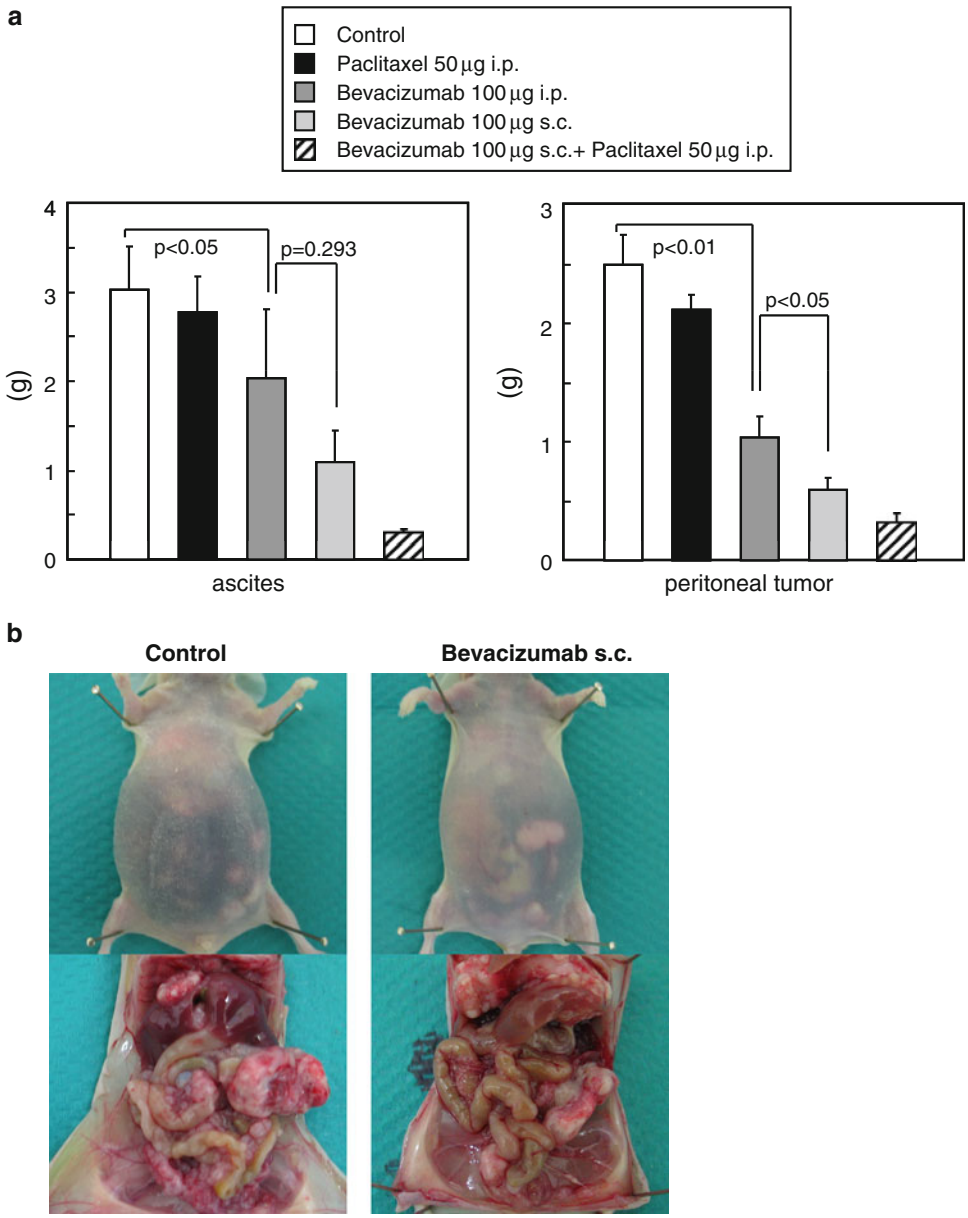

Fig. 6 **a** Mean ascites volume and mean tumor burden of peritoneal nodules treated with bevacizumab and paclitaxel for 4 weeks, alone and in combination (Mean ± SE, $n = 5$). **b** Effect of bevacizumab on the peritoneal metastatic model. Four weeks following the day of treatment, a representative mouse from the control and bevacizumab-treated groups is shown

paclitaxel may have novel clinical applications for inhibiting peritoneal tumor growth and reducing ascites in gastric cancer.

Acknowledgments The authors declare that there are no conflicts of interest and no support in this study.

References

1. Holm-Nielsen P (1953) Pathogenesis of ascites in peritoneal carcinomatosis. Acta Palhol Microbiol Scand 33:10–21
2. Feldman GB, Knapp RC, Order SE, Hellman S (1972) The role of lymphatic obstruction in the formation of ascites in a murine ovarian carcinoma. Cancer Res 32:1663–1666
3. Feldman GB, Knapp RC (1974) Lymphatic drainage of the peritoneal cavity and its significance in ovarian cancer. Am J Obstet Gynecol 119:991–994
4. Brown HR (1976) Kinetics of angiogenesis in small vessels related to mouse parietal peritoneum. Anal Ree 184:364
5. Heuser LS, Taylor SH, Folkman J (1984) Prevention of carcinomatosis and bloody malignant ascites in the rat by an inhibitor of angiogenesis. J Surg Res 36:244–250
6. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985
7. Garrison RN, Kaelin LD, Heuser LS, Galloway RH (1986) Malignant ascites clinical and experimental observations. Ann Surg 203:644–649
8. Garrison RN, Galloway RH, Heuser LS (1987) Mechanisms of malignant ascites production. J Surg Res 42:126–132

9. Brown LF, Detmar M, Claffey K et al (1997) Vascular permeability factor/vascular endothelial growth factors: a multifunctional angiogenic cytokine. EXS 79:233–269
10. Ferrera N (1996) Vascular endothelial growth factor. Eur J Cancer 32A:2413–2422
11. Pepper MS, Wasi S, Ferrera N, Orci L, Montesano R (1994) In vitro angiogenic and proteolytic properties of bovine lymphatic endothelial cells. Exp Cell Res 210:298–305
12. Bausero P, Cavaille F, Meduri G, Freitas S, Perrot-Applanat M (1998) Paracrine action of vascular endothelial growth factor in the human endometrium: production and target sites, and hormonal regulation. Angiogenesis 2:167–182
13. Yeo KT, Wang HH, Nagy JA et al (1993) Vascular permeability factor (vascular endothelial growth factor) in guinea pig and human tumor and inflammatory effusions. Cancer Res 53:2912–2918
14. Mesiano S, Ferrara N, Jaffe RB (1998) Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. Am J Pathol 153:1249–1256
15. Nagy JA, Masse EM, Herzberg KT et al (1995) Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. Cancer Res 55:360–368
16. Kakeji Y, Koga T, Sumiyoshi Y et al (2002) Clinical significance of vascular endothelial growth factor expression in gastric cancer. J Exp Clin Cancer Res 16:125–129
17. Aoyagi K, Kouhuji K, Yano S et al (2005) VEGF significance in peritoneal recurrence from gastric cancer. Gastric Cancer 8:155–163
18. Thickett DR, Armstrong L, Millar AB (1999) Vascular endothelial growth factor (VEGF) in inflammatory and malignant pleural effusions. Thorax 54:707–710
19. Kraft A, Weindel K, Ochs A et al (1999) Vascular endothelial growth factor in the sera and effusions of patients with malignant and nonmalignant disease. Cancer 85:178–187
20. Hu L, Hofmann J, Zaloudek C, Ferrara N, Hamilton T, Jaffe RB (2002) Vascular endothelial growth factor immunoneutralization plus Paclitaxel markedly reduces tumor burden and ascites in athymic mouse model of ovarian cancer. Am J Pathol 161:1917–1924
21. Hu L, Hofmann J, Holash J, Yancopoulos GD, Sood AK, Jaffe RB (2005) Vascular endothelial growth factor trap combined with paclitaxel strikingly inhibits tumor and ascites, prolonging survival in a human ovarian cancer model. Clin Cancer Res 11:6966–6971
22. Byrne AT, Ross L, Holash J et al (2003) Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin Cancer Res 9:5721–5728
23. Hu L, Hofmann J, Lu Y, Mills GB, Jaffe RB (2002) Inhibition of phosphatidylinositol 3′-kinase increases efficacy of paclitaxel in in vitro and in vivo ovarian cancer models. Cancer Res 62:1087–1092
24. Shah MA, Ramanathan RK, Ilson DH et al (2006) Multicenter phase II study of irinotecan, cisplatin, and bevacizumab in patients with metastatic gastric or gastroesophageal junction adenocarcinoma. J Clin Oncol 24:5201–5206
25. Pinto C, Di Fabio F, Siena S et al (2007) Phase II study of cetuximab in combination with FOLFIRI in patients with untreated advanced gastric or gastroesophageal junction adenocarcinoma (FOLCETUX study). Ann Oncol 18:510–517
26. Alberts DS, Liu PY, Hannigan EV et al (1996) Intraperitoneal cisplatin plus intravenous cyclophosphamide versus intravenous cisplatin plus intravenous cyclophosphamide for stage III ovarian cancer. N Engl J Med 335:1950–1955
27. Markman M, Bundy BN, Alberts DS et al (2001) Phase III trial of standard-dose intravenous cisplatin plus paclitaxel versus moderately high-dose carboplatin followed by intravenous paclitaxel and intraperitoneal cisplatin in small-volume stage III ovarian carcinoma: an intergroup study of the Gynecologic Oncology Group, Southwestern Oncology Group, and Eastern Cooperative Oncology Group. J Clin Oncol 19:1001–1007
28. Armstrong DK, Bundy B, Wenzel L et al (2006) Intraperitoneal cisplatin and paclitaxel in ovarian cancer. N Engl J Med 354:34–43
29. Ninomiya S, Inomata M, Tajima M et al (2009) Effect of bevacizumab, a humanized monoclonal antibody to vascular endothelial growth factor, on peritoneal metastasis of MNK-45P human gastric cancer in mice. J Surg Res 154:196–202
30. Mabuchi S, Terai Y, Morishige K et al (2008) Maintenance treatment with bevacizumab prolongs survival in an in vivo ovarian cancer model. Clin Cancer Res 14:7781–7789
31. Liang WC, Wu X, Peale FV et al (2006) Cross-species vascular endothelial growth factor (VEGF)-blocking antibodies completely inhibit the growth of human tumor xenografts and measure the contribution of stromal VEGF. J Biol Chem 281:951–961
32. Lin YS, Nguyen C, Mendoza JL et al (1999) Preclinical pharmacokinetics, interspecies scaling, and tissue distribution of a humanized monoclonal antibody against vascular endothelial growth factor. J Pharmacol Exp Ther 288:371–378
33. Yashiro M, Chung YS, Nishimura S, Inoue T, Sowa M (1996) Peritoneal metastatic model for human scirrhous gastric carcinoma in nude mice. Clin Exp Metastasis 14:43–54
34. Nakamura K, Kubo A (1997) Biodistribution of iodine-125 labeled monoclonal antibody/interleukin-2 immunoconjugate in athymic mice bearing human tumor xenografts. Cancer 80:2650–2655
35. Kinuya S, Yokoyama K, Kawashima A et al (2000) Pharmacologic intervention with angiotensin II and kininase inhibitor enhanced efficacy of radioimmunotherapy in human colon cancer xenografts. J Nucl Med. 41:1244–1249
36. Kinuya S, Yokoyama K, Izumo M et al (2005) Locoreginal radioimmunotherapy with 186Re-labeled monoclonal antibody in treating small peritoneal carcinomatosis of colon cancer in mice in comparison with 131I-counterpart. Cancer Lett 219:41–48
37. Kinuya S, Li XF, Yokoyama K et al (2003) Intraperitoneal radioimmunotherapy in treating peritoneal carcinomatosis of colon cancer in mice compared with systemic radioimmunotherapy. Cancer Sci 94:650–654
38. Kinuya S, Yokoyama K, Fukuoka M et al (2007) Intraperitoneal radioimmunotherapy to treat the early phase of peritoneal dissemination of human colon cancer cells in a murine model. Nucl Med Commun 28:129–133
39. Mandl-Weber S, Cohen CD, Haslinger B, Kretzler M, Sitter T (2002) Vascular endothelial growth factor production and regulation in human peritoneal mesothelial cells. Kidney Int 61: 570–578
40. Sako A, Kitayama J, Yamaguchi H et al (2003) Vascular endothelial growth factor synthesis by human omental mesothelial cells is augmented by fibroblast growth factor-2: possible role of mesothelial cell on the development of peritoneal metastasis. J Surg Res 115:113–120
41. Gerber HP, Ferrara N (2005) Pharmacology and pharmacodynamics of bevacizumab as monotherapy or in combination with cytotoxic therapy in preclinical studies. Cancer Res 65:671–680
42. Belotti D, Vergani V, Drudis T et al (1996) The microtubule affecting drug paclitaxel has antiangiogenic activity. Clin Cancer Res 2:1843–1849
43. Browder T, Butterfield CE, Kräling BM et al (2000) Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer 60:1878–1886
44. Ma J, Waxman DJ (2008) Combination of antiangiogenesis with chemotherapy for more effective cancer treatment. Mol Cancer Ther 7:3670–3784
45. Ishigami H, Kitayama J, Otani K et al (2009) Phase I pharmacokinetic study of weekly intravenous and intraperitoneal paclitaxel combined with S-1 for advanced gastric cancer. Oncology 76:311–314