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A novel synergistic effect of iron depletion on antiangiogenic cancer therapy

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Iron is an essential element for both normal and cancer cells in humans. Treatment to reduce iron levels has been shown to suppress tumor growth *in vivo*. However, iron depletion monotherapy by iron decreased treatment has not been thought to be superior to ordinary chemotherapy and is not part of the standard therapeutic strategy for the treatment of cancer. Iron depletion is also known to reduce serum hemoglobin and oxygen supply to the tissue, which indicates that iron depletion may induce angiogenesis. Therefore, we hypothesized that iron depletion with antiangiogenic therapy can have a novel therapeutic effect in the treatment of cancer. Human nonsmall cell carcinoma cell lines A549 and H1299 were used in our study. An iron-deficient diet and an iron chelator were used to simulate an iron-depleted condition. The antitumor effects of iron depletion and antiangiogenic therapy were determined on A549 xenograft mice. The iron-depleted condition produced by an iron-deficient diet suppressed tumor growth. Tumor tissue from the iron-deficient diet group showed that cancer cell proliferation was suppressed and hypoxia was induced. Microvessel density of this group was increased which suggested that the iron-depleted condition induced angiogenesis. Bevacizumab administration had a synergetic effect on inhibiting the tumor growth on Day 39. An iron-depleted condition inhibited cancer cell proliferation and reciprocally induced angiogenesis. Bevacizumab synergistically enhanced the iron-depleted antitumor effect. Treatment to deplete iron levels combined with anti-angiogenic therapy could induce a novel therapeutic effect in the treatment of cancer.

Introduction

Chemotherapy is continually evolving and various anticancer drugs have been produced during the past decades. Recently, therapies targeting molecules in cancer cells have been developed and used in the clinical setting. Moreover, new drugs targeting the tumor stroma have been developed. Bevacizumab, an antibody against vascular endothelial growth factor (VEGF), is the first tumor stroma molecular-targeting drug.[1] Many kinds of cancer cells are known to become hypoxic during progression of the tumor. However, the cells survive with angiogenesis through activation of VEGF signaling *via* hypoxia-induced factor 1α (HIF-1α).[2] Bevacizumab has been used to treat many different cancers all over the world and some clinical studies revealed that bevacizumab prolonged survival.[3–5]

Iron metabolism and its relationship with cancer cells have been studied for a long time. Iron is an essential element for both human normal and cancer cells. Iron overload is known to induce some kinds of cancer, which suggests that the prevention of iron overload may become a therapeutic strategy for cancer prevention.[6,7] In fact, reduction of serum iron with phlebotomy lowered the risk of developing hepatocellular carcinoma in patients with chronic hepatitis C.[8] Iron-depletion treatment is also known to suppress tumor growth *in vivo*.[9] However, iron-depletion monotherapy has generally been thought to not be superior to ordinary chemotherapy and a standard therapeutic strategy in the treatment of cancer.

In human biology, iron depletion was known to reduce serum hemoglobin and oxygen supply to the tissue.[10,11] Therefore, cancer cells could respond to iron depletion and induce angiogenesis to compensate for the reduced oxygen supply. Subsequently, their iron-decreased status could make the cancer cell more dependent on angiogenesis so that the effectiveness of antiangiogenic therapy would be increased in an iron-depleted condition.

Key words: angiogenesis, bevacizumab, chelator, iron

Abbreviations: CD31: cluster of differentiation 31; HIF-1α: hypoxia-induced factor 1α; MVD: microvessel density; NSCLC: non-small cell lung cancer; RECIST: response evaluation criteria in solid tumors; TfR-1: transferrin receptor 1; VEGF: vascular endothelial factor

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What's new?

Withholding iron from tissues reduces their access to oxygen, and can suppress tumor cell proliferation. If reducing the availability of iron could weaken tumors, making them less able to withstand traditional therapies, that would be an inexpensive and simple way to boost outcomes. The current study investigated whether limiting the iron supply enhanced the effectiveness of anti-angiogenic therapy. In mice fed an iron-deficient diet, tumor growth slowed. When an iron-chelating agent was used in conjunction with the anti-angiogenesis drug bevacizumab, the anti-tumor treatment worked significantly better, suggesting that iron reduction could be a very promising way to enhance cancer therapy.

In our study, we investigated whether iron-depletion and anti-angiogenic therapy can have a novel therapeutic effect for the treatment of cancer.

Material and Methods

Cell lines and cultures

The human nonsmall cell lung cancer (NSCLC) cell lines A549 and H1299 were used in our study. A549 was cultured in Dulbecco's modified eagle medium (DMEM, Sigma-Aldrich, St. Louis, MO) and H1299 was cultured in RPMI1640 medium (Sigma-Aldrich) at 37°C in humidified air with 5% CO_2. Each medium was supplemented with 10% fetal calf serum (FCS, Hyclone, Logan, UT), 100 units/mL penicillin, and 100 mg/mL streptomycin (Sigma-Aldrich).

Reagents

Bevacizumab, commercialized as AVASTIN™ by Roche (Basel, Switzerland), was purchased from Chugai Pharmaceutical (Tokyo, Japan). Bevacizumab was diluted to the final concentration of 5 mg/kg with 0.9% sodium chloride before use *in vivo*. Deferasirox, commercialized as EXJADE™ was purchased from Novartis Pharma (Tokyo, Japan).

Cell viability assay

The proliferation of A549 and H1299 cells was evaluated using a sulfonated tetrazolium salt (WST-1). The cells were plated at a density of 1×10^3 cells/well in 96-well micro plates, in 10% FCS containing each mediu, and incubated at 37°C in a humidified atmosphere of 5% CO_2. Twenty-four hours after each treatment, the cells were incubated with 10 μL/well of WST-1/PBS solution (Roche) for 3 hr under the same conditions as indicated above. The absorbance of the samples was measured at 450 nm using a microplate reader with a background control as the blank. The cell viability ratio was expressed as a percentage of the control.

Cell-cycle analysis by flow cytometry

For the cell-cycle analysis, cancer cells were plated in six-well tissue culture plates and treated with different concentrations of deferasirox (0, 1, 10,100 or 1,000 μM). After 24 hr, the cells were harvested and stained with 20 mg/mL propidium iodide. The DNA content was analyzed with a fluorescence-activated cell sorter (FACScan, Becton Dickinson, Franklin Lakes, NJ) using Cell Quest software (BD Biosciences, San Jose, CA).

Western blotting

Whole-cell lysates and nuclear protein were extracted using M-PER buffer (Thermo Fisher Scientific, Rockford, IL) and NE-PER buffer (Thermo Fisher Scientific), respectively. Total protein extraction and nuclear protein from homogenized A549 xenograft tumor tissue samples were extracted using T-PER buffer (Thermo Fisher Scientific) and NE-PER buffer (Thermo Fisher Scientific). The collected supernatants were subjected to protein concentration and equal amounts of protein were electrophoresed under reducing conditions in gradient polyacrylamide gels (ATTO, Tokyo, Japan) and were then transferred onto polyvinylidene difluoride filter membranes (Millipore, Billerica, MA). The membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 1 hr. An Amersham chemiluminescent ECL Plus Western Blotting Detection system (GE Healthcare, Piscataway, NJ) was used for signal detection. Western blotting materials were as follows: hydroxy-HIF-1α (Pro564) (D43B5) was obtained from Cell Signaling Technology (Beverly, MA); cyclin D1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); β-actin was obtained from Sigma-Aldrich; horseradish peroxidase-conjugated rabbit anti-mouse IgG was obtained from Dako Cytomation (Glostrup, Denmark); and goat anti-rabbit IgG was obtained from American Qualex Antibodies (La Mirada, CA).

Histology and immunohistology

Surgically resected tissues from the A549 xenograft model were used for histological and immunohistochemical study. Paraffin sections were prepared from the 10% formalin-fixed tumors and stained with hematoxylin/eosin and Prussian blue. Prussian blue staining was performed by incubating fixed tissue in a mixture of 2% potassium ferrocyanide and 1% HCl for 30 min. Glass slides were rinsed in distilled water and counterstained with Nuclear Fast Red for 5 min. Immunohistochemical procedures were followed as described previously.[12] Deparaffinized tissue sections were immersed in methanol containing 3% hydrogen peroxide to block endogenous peroxide activity. An autoclave pretreatment in citrate buffer was done for antigen retrieval. A Ki-67 staining kit (Dako) and CD31 (endothelial cell adhesion molecule-1) rabbit monoclonal antibody (Santa Cruz Biotechnology) were used. After incubation with a blocking buffer, the sections were treated with Ki-67 and CD31 antibodies for 1 hr at room temperature followed by immunobridging with Avidin DH-biotinylated

Table 1. Content of control and iron-deficient diets

	Control diet	Iron-deficient diet
g/kg diet		
Corn starch	610	610
Casein	220	220
Cellulose	50	50
Soybean oil	40	40
Vitamin mixture	10	10
Mineral mixture		
Potassium	17.3	17.3
Phosphorus	15	15
Calcium	13.55	13.55
Magnesium	8	8
Corn starch	8	9.9
Sodium	6	6
Iron	1.9	
Manganese	0.154	0.154
Zinc	0.06	0.06
Iodine	0.0154	0.0154
Copper	0.0126	0.0126
Chloride	0.004	0.004

horseradish peroxide complex (Nichirei, Tokyo, Japan). Signal detection was done for 2–5 min using a solution of 3,3′-diaminobenzidine tetrahydrochloride in 50 mmol/L Tris-HCl (pH = 7.5) containing 0.001% hydrogen peroxide. The sections were counterstained with Mayer's hematoxylin for 6 hr at room temperature followed by immunobridging with Avidin DH-biotinylated horseradish peroxide complex (Nichirei). Ki-67 labeling index was calculated as the average percentage of Ki-67-positive nuclei in three high-power fields (HPFs).

Hypoxia assay

A Hypoxyprobe-1 kit (Chemicon International, Temecula, CA) was used to investigate tumor hypoxia. Mice were injected intraperitoneally (ip) with Hypoxyprobe TM-1 [pimonidazole hydrochloride 60 mg/kg] 45 min before tumor collection.[13,14] The collected tumor sections were incubated for 1 h with the Hypoxyprobe-1 primary antibody supplied with the kit.

Micro vessel density

Angiogenesis activity was determined to count microvessel density. CD31-immunostained sections were used in the previous report.[15] The highest density of blood vessels (hot spots) was selected at a low-power field and the number of blood vessels was counted per 0.20 mm^2 in five separate hot spots at a HPF.[16] All sections were scored independently by two individual experienced microscopists and no significant differences were observed between scorers.

Table 2. Blood analysis results of the normal and iron-deficient diet groups

	Fe (+)	Fe (−)	*p*-Value
RBC ($\times 10^4$)	891 ± 46	802 ± 43	0.029
WBC	4,425 ± 1,258	3,575 ± 150	0.228
Hb	14.9 ± 0.7	11.4 ± 0.6	0.001
HCT	46.2 ± 3.7	38.5 ± 3.8	0.027
MCV	52.0 ± 2.0	48.5 ± 2.4	0.065
MCHC	30.3 ± 1.3	29.8 ± 1.3	0.594
Fe	222.3 ± 20.1	115.3 ± 43.3	0.004
Ferritin	255.0 ± 108.6	113.8 ± 24.9	0.044

VEGF ELISA assay

To evaluate the supernatant VEGF secreted by A549 and H1299 cells, we used a VEGF enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). The cancer cells were plated in six-well tissue culture plates and were treated with different concentrations of deferasirox (0, 1, 10, 100 or 1,000 μM). After a 24-hr treatment, the supernatant and cells were harvested and VEGF content was assayed by ELISA according to the protocol provided by the manufacturer. The results were normalized to the concentration of total protein extraction per plate. Data were presented as mean ± SD from three independent experiments.

Animal experiments

The animal experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University, Okayama, Japan. All of the mice and their diets (normal and iron deficient) were purchased from Clea (Tokyo, Japan) (Table 1). The 6-week-old male BALB/c nu/nu mice were randomized into two groups of eight mice each; (*i*) normal diet as a control and (*ii*) iron-deficient diet. After 3 weeks, A549 subcutaneous xenografts were produced on the backs of mice by injecting 3×10^6 cells mixed with Matrigel (BD Biosciences) at a 1:1 ratio.[17,18] Water was provided and the mice were allowed to drink freely. Tumor volume was measured weekly (length × $width^2/2$). For the bevacizumab administration study, 6-week-old male BALB/c nu/nu mice were randomized into two groups of 20 mice each as above. After 3 weeks, A549 subcutaneous xenografts were produced in the same way. After a week, the mice in each diet group were randomized into two groups of four mice each (*i*) bevacizumab (5 mg/kg twice/week for 5 weeks), (*ii*) saline alone as a control.[15] The drug was administered ip and tumor volume measured twice a week. Both diets (normal and iron deficient) and water were provided *ad libitum*.

Statistical analysis

A Student's *t*-test was used to compare data between the two groups. Data represent the mean ± SD; $p < 0.05$ was considered statistically significant.

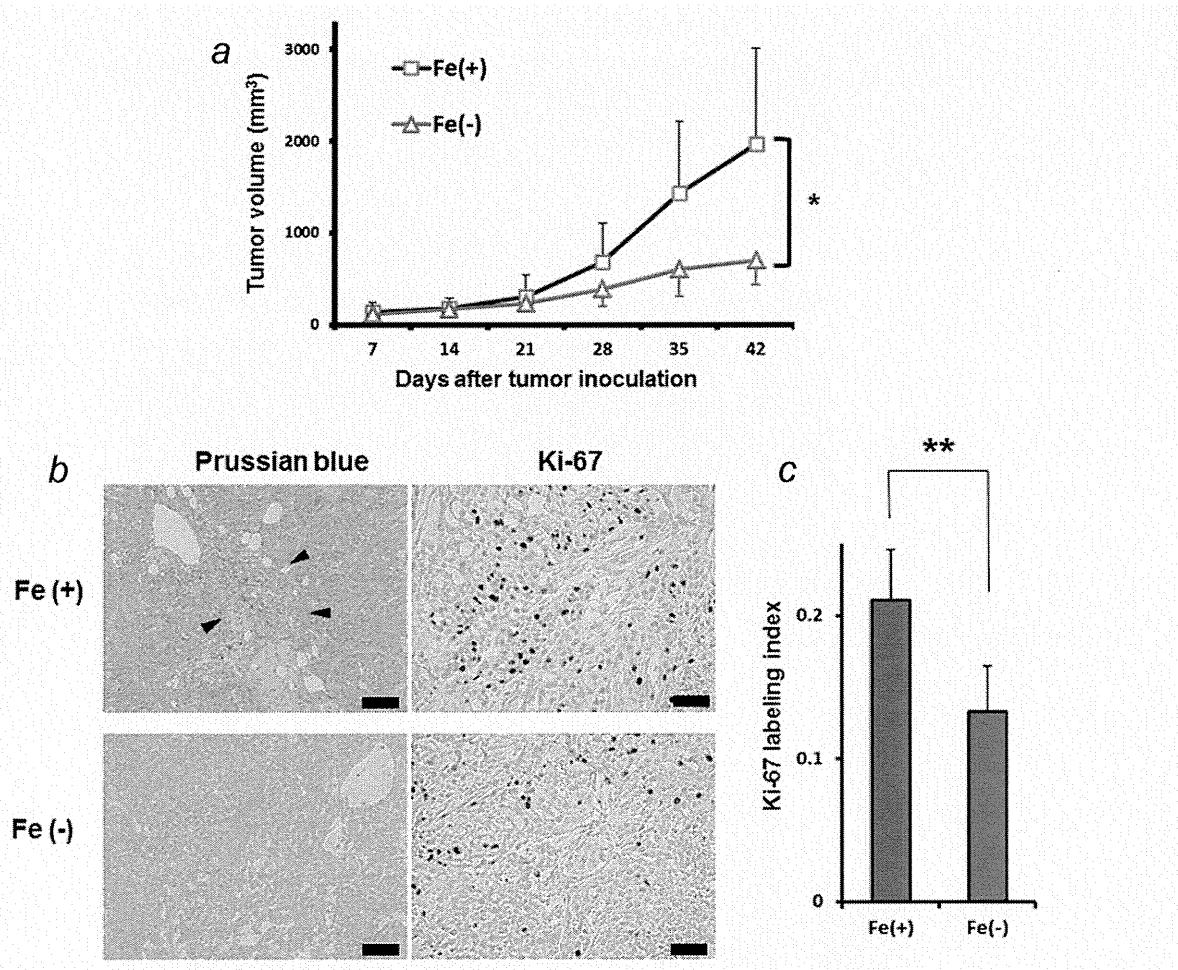

Figure 1. Iron depletion suppressed tumor growth *in vivo*. (*a*) An iron-deficient diet was started 3 weeks before inoculating A549 cells on the backs of mice. The iron-deficient diet was continuously fed until the end of the study. Tumor volume was measured as a cube (length × width × height) and was tracked up to 6 weeks. Tumor growth was suppressed in the iron-deficient diet group. Tumor growth was expressed as mean tumor volume ± SD. Statistical significance (*) was determined as $p < 0.01$. (*b*) Prussian blue and Ki-67 staining showed the positive spot area and proliferating cells were reduced in the iron-depleted condition. Scale bars: 50 mm. (*c*) Ki-67 labeling index was decreased in iron-depleted condition. Data are means ± SD. Statistical significance (**) was determined as $p < 0.05$.

Results

Iron-deficient diet produced iron-depleted mice

We first confirmed that the iron-deficient diet resulted in an iron-depleted mouse model. The 6-week-old male BALB/c nu/nu mice were randomized into two groups of eight mice each. Blood sampling was performed after 3 weeks. The iron-deficient diet reduced serum iron levels (Table 2). To confirm iron deficiency histologically, Prussian blue staining was done using surgically resected murine spleens. Although the positive blue spots were diffusely recognized in the normal diet group, no positive blue spot was recognized in the iron-deficient diet group (Supporting Information Fig. 1). A reduction of iron in the reticuloendothelial system proves that the iron-deficient diet reduced serum iron levels.

Iron depletion suppressed tumor growth

We investigated the tumor growth under an iron-depleted condition. A549 subcutaneous xenografts were produced on the backs of mice after 3 weeks of an iron-deficient diet and tumor size was measured once a week. Tumor growth was suppressed in the iron-deficient diet group (tumor volume: normal diet *vs.* iron-deficient diet = 1,375.9 ± 688.4 *vs.* 497.0 ± 192.2 mm^3; $p = 0.0037$) (Fig. 1*a*). There were no mice that died and no significant side effects were observed during the experiment. Moreover, diet intake and body weight were not significantly different between normal diet group and iron-deficient diet group (Supporting Information Fig. S2).

Iron depletion reduced iron levels in tumor tissue and suppressed cancer cell proliferation

To identify the differences in tumor progression in an iron-depleted condition, we performed histological and immunohistological examinations (Fig. 1*b*). As shown above, an iron-deficient diet reduced the serum and tumor tissue iron levels. Interestingly, there was only difference of positive spot area in stroma tissue of the tumor. Proliferating cells (G1, S, G2 and M cycling stages of cell division) were stained

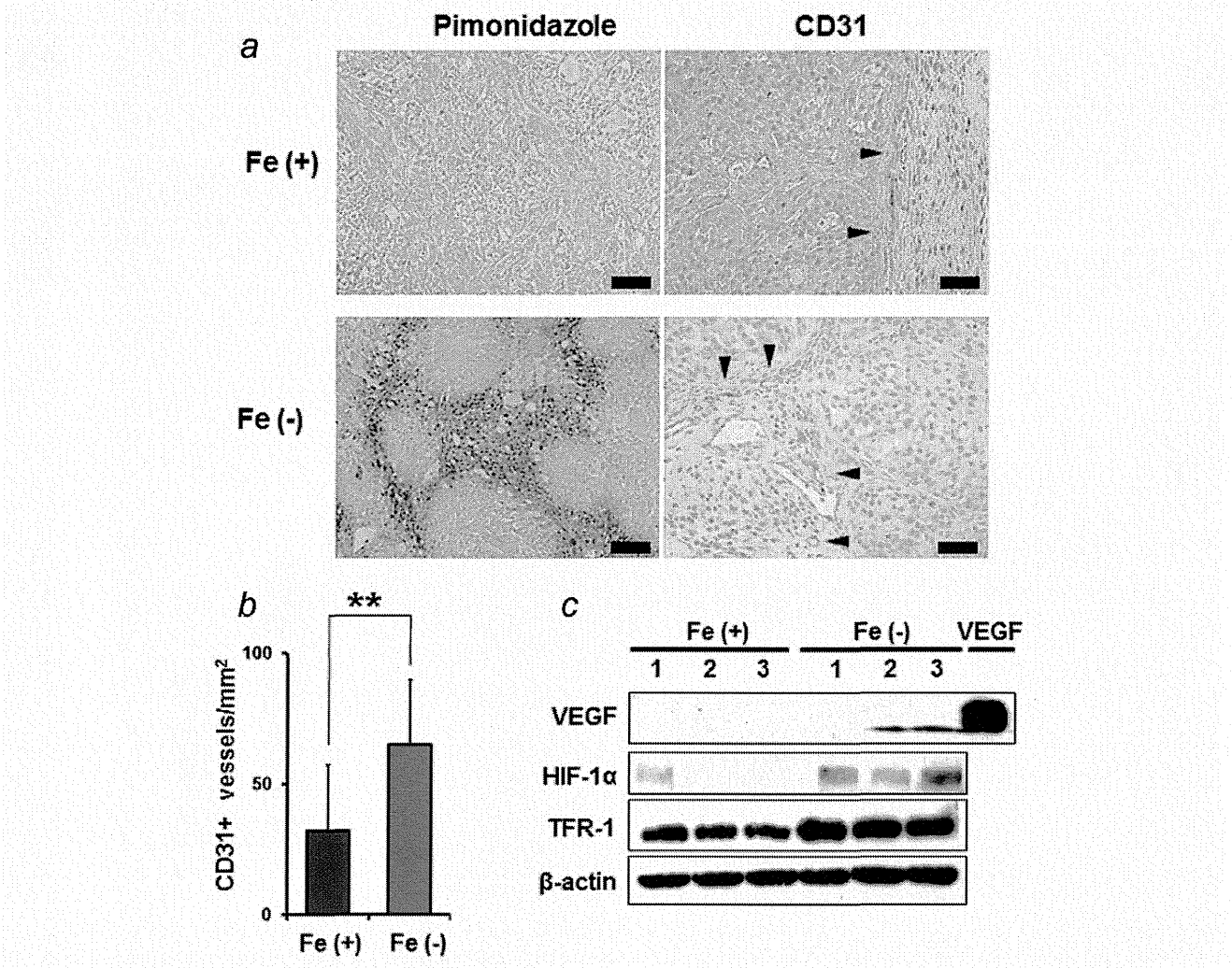

Figure 2. Iron depletion reciprocally induced angiogenesis *via* upregulation of HIF-1α. (*a*) Tumor tissues obtained as described in the legend to Figure 1 were analyzed for angiogenesis. Pimonidazole and CD-31 staining showed that positive spot area and positive stained vessels were increased in the iron-depleted condition. Scale bars: 50 mm. (*b*) MVD is increased in the iron-depleted condition. Data are means ± SD. Statistical significance (**) was determined as $p < 0.05$. (*c*) Western blot analysis of tumor-homogenized samples showed that VEGF and TfR-1 were upregulated in the iron-depleted condition. Similarly, HIF-1α was also upregulated at the nucleus protein level. Each tumor sample was obtained from three individual mice.

positive; G0 cycling stage cells were excluded. The Ki-67 labeling index revealed that an iron-deficient diet suppressed cancer cell proliferation compared to the normal diet group (Ki-67 labeling index: normal diet *vs.* iron-deficient diet = 0.211 ± 0.035 *vs.* 0.133 ± 0.032; $p = 0.0459$). Prussian blue staining was almost negative in the tissue in iron-deficient group.

Iron depletion followed by hypoxia and angiogenesis

We hypothesized that iron depletion induced a reduction of serum hemoglobin and tissue hypoxia. Iron depletion also upregulates angiogenesis in the tumor. To test these hypotheses, we investigated pimonidazole and CD-31 staining (Fig. 2*a*). Tumor hypoxia was increased in the group fed an iron-deficient diet. CD-31 staining was performed to investigate whether iron depletion induced angiogenesis. Microvessel density (MVD) was calculated to count CD-31-positive vessels (Fig. 2*b*). CD-31-positive vessels were increased in an iron-depleted condition. The MVD of the iron-deficient diet group was higher than that of the normal diet group (MVD: normal diet *vs.* iron-deficient diet = 32.02 ± 25.24 *vs.* 64.96 ± 24.71; $p = 0.045439$). This result suggested that iron depletion induced angiogenesis via hypoxia.

Angiogenesis was induced by iron depletion via HIF-1α upregulation

To identify the mechanism by which iron depletion induced angiogenesis *via* hypoxia, a Western blot analysis was performed using homogenized tissue samples. The expression of transferrin receptor 1 (TfR-1) was determined to confirm the effect of iron depletion in tumor tissue samples. As TfR-1

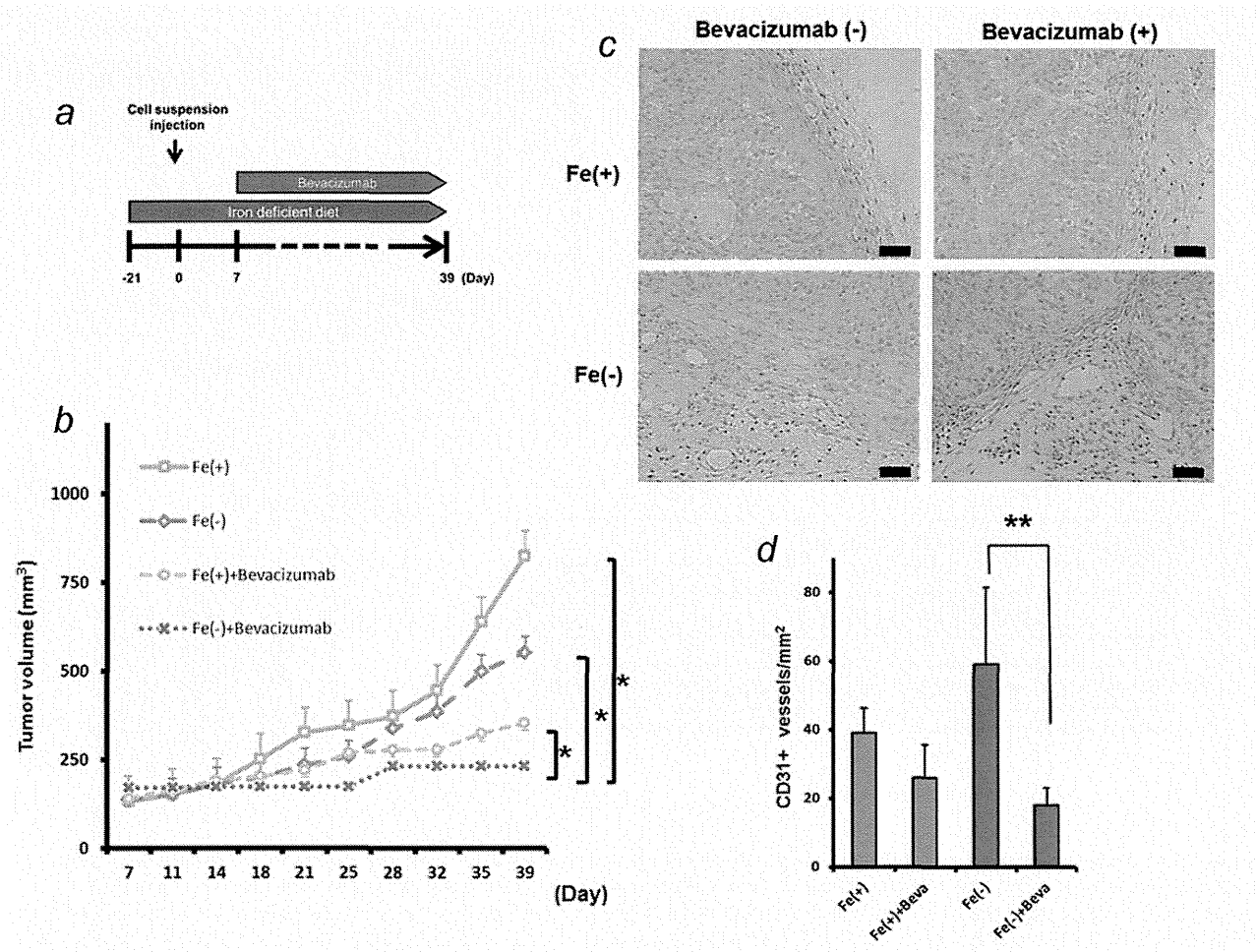

Figure 3. Bevacizumab synergistically suppressed tumor growth in an iron-depleted condition. (*a*) An iron-deficient diet was started 3 weeks before inoculating A549 cells on the backs of mice (day 21). The iron-deficient diet was continuously fed until the end of the study. (*b*) Bevacizumab (5 mg/kg) or saline as a control was administered ip twice/week. Tumor volume was measured as a cube (length × width × height) and was tracked up to 39 days. Five mice were used for each group. Tumor growth was expressed as mean tumor volume ± SD. Statistical significance (*) was determined as $p < 0.01$. Tumor growth was significantly inhibited in the combination group as compared to the control, bevacizumab or Fe (–) groups. (*c*) CD-31 staining revealed that positively stained vessels were increased in the iron-depleted condition. Scale bars: 50 mm. (*d*) Bevacizumab decreased MVD in both normal diet group and iron-deficient diet group.

expression changes based on the serum iron level, a decrease in serum iron levels increases TfR-1 expression.[19] Whole-cell lysate from homogenized tissue samples showed TfR-1 was upregulated in the iron-deficient diet group (Fig. 2*c*). Subsequently, the expression status of HIF-1α in nuclei was determined. HIF-1α is known to play a critical role in angiogenesis *via* hypoxia.[2,20] Western blot analysis of an extraction from nuclei showed the expression of HIF-1α in the iron-deficient diet group was higher than that of the normal group (Fig. 2*c*). This result suggested that iron depletion induced hypoxia *via* HIF-1α, which caused angiogenesis.

Bevacizumab synergistically suppressed tumor growth by inhibiting upregulated angiogenesis

As shown above, we found that iron depletion was followed by hypoxia and angiogenesis. Thus, an antiangiogenic therapeutic agent (bevacizumab) was predicted to have a synergetic effect on suppressing tumor growth in an iron-depleted condition. Bevacizumab was administrated ip 5 mg/kg twice a week to mice with subcutaneous tumors fed either an iron-deficient or a normal diet. This dose and schedule were cited in the previous reports.[21,22] Bevacizumab had a synergistic effect on inhibiting tumor growth on Day 39 (tumor volume: normal diet [857.6 ± 129.0 cm^3], iron-deficient diet [401.8 ± 126.6 cm^3], normal diet + bevacizumab [221.6 ± 63.8 cm^3], iron-deficient diet + bevacizumab [61.0 ± 27.5 cm^3]) (Figs. 3*a* and 3*b*). To calculate MVD, CD-31 staining was performed (Fig. 3*c*). We could confirmed that bevacizumab inhibited angiogenesis in spite of induction by iron depletion condition (MVD: normal diet [39 ± 7.3], iron-deficient diet [59 ± 22.4], normal diet + bevacizumab [26 ± 9.7], iron-

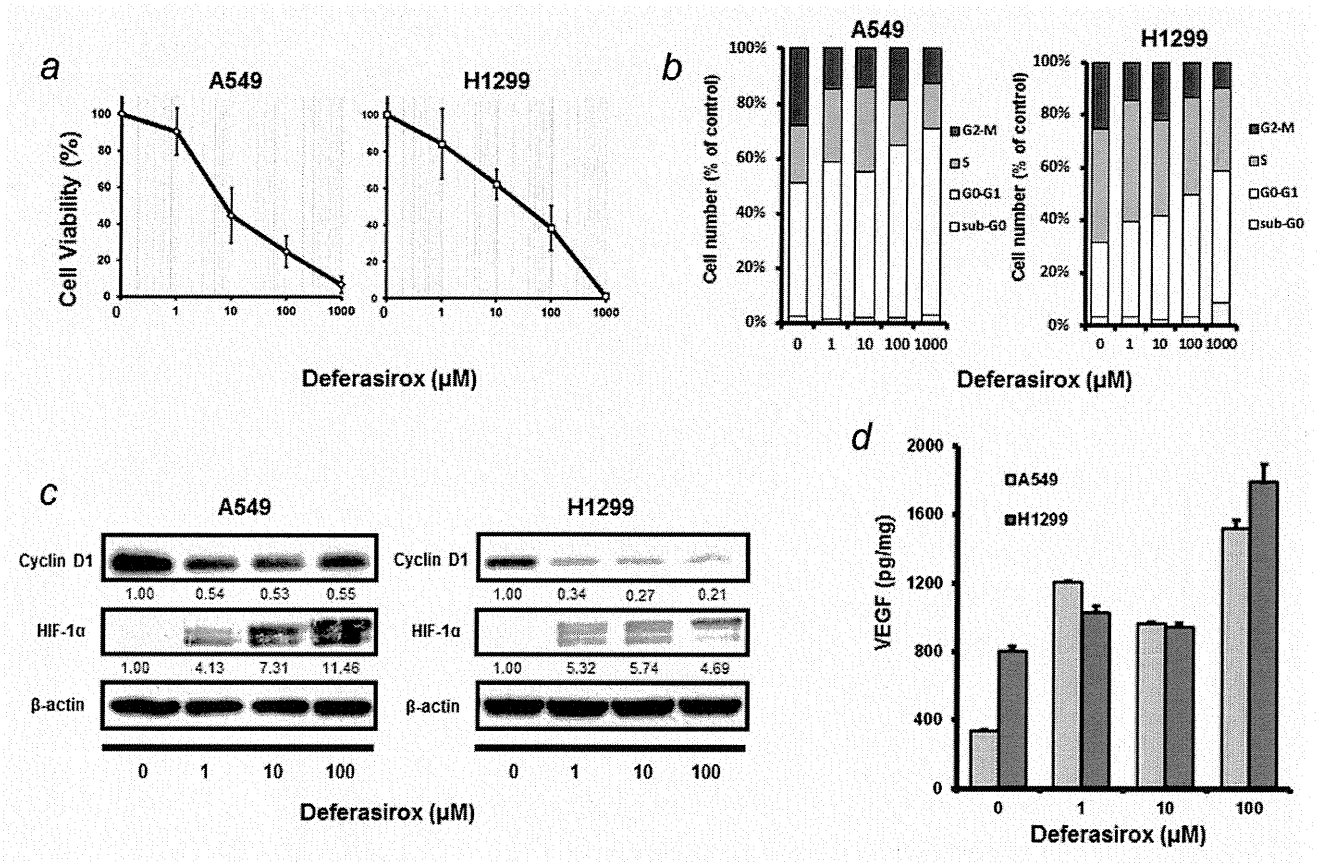

Figure 4. Iron depletion inhibited cell proliferation *via* cell-cycle arrest and induced VEGF secretion *in vitro*. (*a*) Cultured A549 cells and H1299 cells were treated with the indicated concentrations of deferasirox for 24 h and the cell viability was measured by the WST-1 method. (*b*) The cells were treated with different concentrations of deferasirox for 24 h and the cell-cycle distribution was analyzed by flow cytometry. Each histogram consists of the following four cell cycle populations; sub-G0 (black), G0-G1 (white), S (light gray) and G2-M (dark gray). (*c*) Whole-cell lysates and nuclear protein of these cells treated with the indicated concentrations of deferasirox were used for Western blot to determine its inhibitory effects on cell cycle and upregulation effects on HIF-1α. The expression level of each protein was calculated relative to its expression in mock-treated cells, whose expression level was designated as 1. (*d*) Supernatant treated with the indicated concentrations of deferasirox was harvested and VEGF secretion examined by ELISA.

deficient diet + bevacizumab [18 ± 5.1]) (Fig. 3*d*). None of the mice died owing to drug-induced toxicity and no other significant adverse events were observed.

Iron depletion inhibited cell proliferation via cell-cycle arrest in vitro

To reproduce an iron-depleted condition *in vitro*, an iron chelator was used (deferasirox, Exjade™). Deferasirox suppressed cancer cell proliferation in A549 and H1299 cells in a dose-dependent manner (Fig. 4*a*). To identify the mechanism of inhibition, a flow cytometry analysis for cell-cycle distribution was performed (Fig. 4*b*). Deferasirox increased the population of the G0-G1 phase in the A549 and H1299 cell lines. To confirm the results of the flow cytometry analysis, Western blot analysis was performed using a total protein extraction of the A549 and H1299 cells. The Western blot showed that deferasirox decreased cyclin D1 in a dose-dependent manner (Fig. 4*c*). Taken together, these results suggest that iron depletion inhibited cancer cell proliferation *via* cell-cycle arrest *in vitro*.

Iron depletion induced VEGF secretion in A549 and H1299 supernatant via upregulation of HIF-1α

To investigate whether iron depletion induces angiogenesis *via* HIF-1α, a Western blot analysis and ELISA assay were performed. Deferasirox treatment induced nuclear HIF-1α expression (Fig. 4*c*) and significantly increased VEGF secretion from A549 and H1299 cells, both in a dose-dependent manner (Fig. 4*d*). These results suggested that iron deficiency induced angiogenesis *via* HIF-1α and VEGF signaling. However, *in vitro* angiogenesis assay (tube formation assay) did not show the increase by deferasirox administration (Supporting Information Fig. S3). The reason was that deferasirox did not affect HUVEC in proliferation and VEGF secretion in the absence of tumor cells *in vitro*. Thus, the combination

effect of iron depletion with antiangiogenenic therapy is prominent only *in vivo* with tumor cells.

Discussion

Cancer cells have the ability to survive under severe conditions. One of the mechanisms for survival under hypoxic conditions is the activation of VEGF signaling *via* HIF-1α.[23] This mechanism also allows cancer cells to, sometimes, be able to resist chemotherapy.[24,25] Therefore, the effectiveness of chemotherapy can be compromised or attenuated. These experiences indicate that chemotherapy could be more effective under conditions where cancer cells would have difficulty resisting. Antiangiogenic drugs, especially bevacizumab, are known to have an excellent antitumor effect in the clinic[3–5] and are commonly used concurrently with antitumor drugs. When multiple antitumor and antiangiogenic drugs are prescribed, the financial burden on the patient is increased.[26,27] Therefore, a new strategy that would enhance the effect of antiangiogenic therapy would be beneficial. As iron depletion is known to reduce serum hemoglobin and oxygen supply to tissue in humans,[10,11] we hypothesized that iron depletion with antiangiogenic therapy could have a novel therapeutic effect for the treatment of cancer. Moreover, iron-controlled treatment has some advantages such as having its own antitumor effect, being easily controllable in daily living, and being inexpensive.

First, we examined the effect of iron depletion on the mice and tumor growth. The iron-depleted mice revealed low serum levels of hemoglobin, iron, red blood cells and ferritin compared to the mice fed a normal diet. Tumor growth was suppressed in the iron-depleted mice. Tumor tissue was extracted and examined by histology and immunohistology. Iron deposits in the stroma of the tumors were reduced in the iron-depleted mice. This phenomenon suggested that the iron condition of the tumor influenced not only the cancer cell but also stroma cells. Pimonidazole (Hypoxyprobe-1 kit) and CD-31 staining showed that iron depletion induced hypoxia and angiogenesis. To confirm hypoxia in the tumor and analyze the differences in cell signaling, we further examined the expression of HIF-1α. HIF-1α was predictably upregulated in iron-depleted tumors at the protein level. Taken together, these results suggested that iron depletion suppressed tumor growth and reciprocally induced angiogenesis *via* hypoxia. To the best of our knowledge, there is no previous report of this reciprocal phenomenon.

We next investigated the mechanism of iron deficiency and reciprocally induced angiogenesis *in vitro*. We used an iron chelator (deferasirox) to stimulate iron depletion owing to its usefulness *in vitro* and the expectation to apply it clinically. Deferasirox is the first orally bioavailable iron chelator with an indication for the treatment of iron overload in transfusion-dependent anemias. Deferasirox is the most useful iron chelator in the clinical setting and has been reported to have an antiproliferative effect in leukemia and hepatoma cells.[28,29] In our study, deferasirox inhibited the cancer cell proliferation in lung cancer cell lines A549 and H1299. We also examined apoptosis using the tunnel assay and found no significant difference in the deferasirox treatment group (data not shown). An ELISA assay and Western blot analysis showed that deferasirox treatment significantly increased VEGF secretion *via* upregulation of HIF-1α. Similar to the *in vivo* results, our *in vitro* study showed that iron depletion inhibited cancer cell proliferation and reciprocally induced angiogenesis. These results may suggest that the cancer cells overexpressed VEGF to escape the iron-depleted condition.

We then hypothesized that iron depletion increased the antitumor effect of an antiangiogenic drug. Bevacizumab, an antibody against VEGF, is the most common antiangiogenic drug used clinically and is approved for the treatment of many kinds of cancer. Bevacizumab is usually used as a combination with other antitumor drug. The reason is that bevacizumab is targeting only VEGF instead of the cancer cell. This is the first study of bevacizumab combination therapy with controlled internal iron condition. Bevacizumab had a dramatic synergistic antitumor effect with iron depletion in our *in vivo* study, indicating an inexpensive method to enhance the effectiveness of chemotherapy. Additionally, these findings may lead to the changes in the daily diet recommendations for patients being treated with an antiangiogenic drug. Iron depletion condition induced antiproliferative effect and angiogenesis. As a result, bevacizumab inhibited angiogenesis and provided strong antitumor effect. Of course, the mechanism is not completely explained and further *in vivo* studies are necessary. For example, *in vivo* live imaging of the tumor growth as well as angiogenesis may be extremely useful.[30–34]

Bevacizumab is an established antiangiogenic drug with clinical benefits for many kinds of cancer.[35,36] However, there is no reliable biomarker or method by which curative effect can be predicted.[37,38] Our study showed that bevacizumab combined with iron depletion was very effective; therefore, we hypothesized that serum iron level could be a novel bevacizumab biomarker. We previously conducted a retrospective study to investigate the correlation between serum hemoglobin level and bevacizumab response rate in 34 patients with colorectal cancer in our facility assessed by RECIST criteria between September 2007 and July 2010.[39] Patient characteristics are summarized in Supporting Information Table 1. The response rate of the low-Hb patient group was higher than that of high-Hb group (41.2 *vs.* 17.6%). This result combined with the results of our present study suggests that a prospective study of bevacizumab with iron control therapy in patients with cancer is warranted.

Conclusions

In conclusion, iron depletion inhibited the cancer cell proliferation and reciprocally induced angiogenesis *in vitro* and *in*

vivo. Bevacizumab had a dramatic synergistic antitumor effect with iron depletion. Treatment to create an iron-depleted condition could induce a novel therapeutic effect with antiangiogenic drugs in the treatment of cancer.

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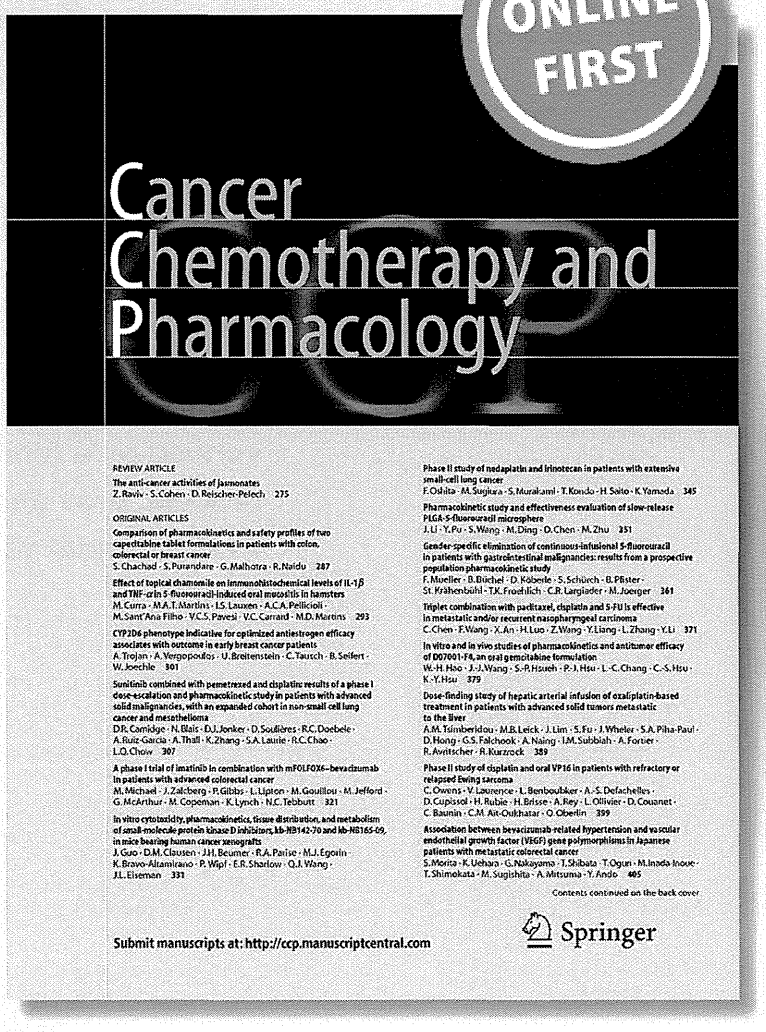

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ORIGINAL ARTICLE

Multicenter phase II study of S-1 and docetaxel combination chemotherapy for advanced or recurrent gastric cancer patients with peritoneal dissemination

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Abstract
Purpose Peritoneal dissemination is the most frequent and life-threatening mode of metastasis and recurrence in patients with gastric cancer. A multicenter phase II study was designed to evaluate the efficacy and tolerability of S-1 and docetaxel combination chemotherapy regimen for the treatment of advanced or recurrent gastric cancer patients with peritoneal dissemination.
Methods Nineteen patients with histologically confirmed unresectable or recurrent gastric cancer with peritoneal dissemination were enrolled. Oral S-1 at 80 mg/m^2/day was administered twice daily for 2 weeks, followed by 1 drug-free week. Docetaxel infusion at 40 mg/m^2 was performed on day 1, simultaneous with S-1 administration. The primary endpoints were overall survival (OS) and time to progression (TTP). The secondary endpoints were the response rates and safety status.
Results Patients received a median of 4 cycles of the S-1 and docetaxel regimen (range 1–43). The disease control rate was 73.7 % (14/19). Median overall survival was 459 days (15.3 months), while median time to progression was 212 days (7.1 months). Neutropenia was the most common type of toxicity (n = 7, 36.8 %).
Conclusions Combination chemotherapy with S-1 and docetaxel is a tolerable and effective treatment for advanced or recurrent gastric cancer patients with peritoneal dissemination.

Keywords Gastric cancer · Peritoneal dissemination · S-1 · Docetaxel · Phase II study

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Introduction

Gastric cancer is the second most common cause of cancer-related deaths, with 700,000 worldwide mortalities reported annually [1]. In Japan, the proportion of patients with gastric cancer has recently decreased; however, it remains one of the most refractory cancers [2]. The prognosis for patients with unresectable or recurrent gastric cancer is extremely poor. The reported median survival time of patients with untreated gastric cancer is 3–5 months [3, 4]. In particular, peritoneal dissemination is the most frequent and life-threatening form of metastasis and recurrence in patients with gastric cancer, and it is often refractory to chemotherapy [5–7]. Randomized clinical trials using combination chemotherapies such as docetaxel, cisplatin and fluorouracil (DCF) or epirubicin, cisplatin and fluorouracil (ECF) were carried out on non-curable gastric cancer patients; however, the median survival time (MST) was only 8.9 and 9.2 months, respectively [8, 9]. These

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outcomes were considered unsatisfactory and, therefore, the search for more effective regimens is warranted.

The anticancer drug S-1 is an orally active combination of tegafur (a pro-drug converted to fluorouracil), gimeracil (a dihydropyrimidine dehydrogenase inhibitor that degrades fluorouracil) and oteracil (a fluorouracil phosphorylation inhibitor in the gastrointestinal tract that reduces fluorouracil toxicity) in a molar ratio of 1:0.4:1 [10, 11]. S-1 has been evaluated in two randomized controlled trials conducted in Japan: the JCOG9912 trial and the S-1 plus cisplatin versus S-1 randomized controlled trial for the treatment for gastric cancer (SPIRITS) [12, 13]. The former demonstrated that S-1 is superior to continuous infusion of 5-fluorouracil with respect to overall survival (OS). The latter demonstrated that S-1 plus cisplatin (CDDP) is superior to S-1 alone with respect to OS and that this combination could be an effective standard chemotherapy regimen for advanced or recurrent gastric cancer.

The novel drug docetaxel has also shown promise for the treatment of advanced gastric cancer, both as monotherapy [14] and in combination with other agents [15–17]. Two previous phase II studies evaluated the effects of S-1 and docetaxel combination chemotherapy and reported overall response rates of 56.3 and 57.8 %, and median OS of 14.3 and 15.3 months, respectively [18, 19]. These studies demonstrated that the combination of S-1 and docetaxel is a promising therapy for advanced gastric cancer; however, the specific effect of this regimen on gastric cancer patients with peritoneal dissemination remains unclear as both measurable and non-measurable lesions were included in previous studies. Paclitaxel as well as docetaxel is expected to be effective for peritoneal dissemination because of their high affinity for the peritoneum [20].

In this study, we evaluated an S-1 and docetaxel combination regimen for the treatment of advanced or recurrent gastric cancer patients with peritoneal dissemination, which has not been defined as a measurable lesion in conventional phase II studies.

Methods

Patient eligibility

Patients who met the following criteria were included: histologically proven unresectable or recurrent gastric adenocarcinomas; presence of positive peritoneal cytology or macroscopic peritoneal dissemination diagnosed by imaging or staging laparoscopy; no prior therapy within the previous 28 days; adequate hepatic, cardiac, renal and bone marrow function (hemoglobin $\geq$ 8 g/dl; white blood cell count, 3,500–12,000/mm^3; neutrophil count $\geq$ 1,500/mm^3; platelet count $\geq$ 100,000/mm^3; total bilirubin $\leq$ 1.5 mg/dl; and aspartate aminotransferase and alanine aminotransferase $\leq$2.5 $\times$ the upper limit of the institution); Eastern Cooperative Oncology Group (ECOG) performance status of 0–1 estimated life expectancy >3 months; between 20 and 75 years old; ability to take medications orally; no abnormal electrocardiography findings; and written informed consent. Patients were not excluded, even if they had other metastatic sites (such as distant lymph nodes, liver, lung or bone). This study was approved by the Institutional Review Board of the participating institutions.

Treatment strategy

The trial was a multicenter, open-labeled, non-randomized and non-controlled phase II study of S-1 and docetaxel. S-1 was orally administered twice daily at 80 mg/m^2 for 2 weeks, followed by a drug-free interval of 1 week (one cycle). Docetaxel was intravenously administered at 40 mg/m^2 on day 1, simultaneous with S-1 administration. Dexamethasone was infused before docetaxel to prevent an allergic response. Granulocyte colony-stimulating factor (G-CSF) was used therapeutically in the event of grade 4 febrile neutropenia. The primary end points of this study were the overall survival (OS) and time to progression (TTP). The secondary end points were the response rates and safety. Treatment continued until evidence of tumor progression, unacceptable toxicity, patient refusal or the physician's decision to stop treatment. Toxicity was graded according to CTCAE v3.0. S-1 was reduced to 50 mg/m^2 in cases of grade 4 leukopenia or neutropenia, $\geq$grade 3 thrombocytopenia or $\geq$grade 3 non-hematologic toxicity except anorexia, nausea and vomiting occurred. Docetaxel was maintained at 40 mg/m^2. Treatment was resumed after recovery from toxic events.

Evaluation of the disease

Tumor response of measurable lesions was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 [21]. Tumor markers, including carcinoembryonic antigen (CEA) and carbohydrate antigen 19–9 (CA19-9), were monitored monthly. Non-measurable lesions were assessed by upper gastrointestinal endoscopy, ultrasonography, computed tomography, upper gastrointestinal radiography and cytology. In addition to objective tumor response based on RECIST guidelines, the amount of malignant ascites was assessed by CT according to the Japanese Classification of Gastric Carcinoma—2nd English Edition [22]. All those evaluations were taken into account to calculate tumor response rate of measurable and non-measurable lesions.

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Statistical analysis

The required number of patients was calculated according to the Southwest Oncology Group One Arm Survival program [23]. The JCOG 9603 study reported that the median survival time was 155 days (95 % confidence interval (CI), 131–225 days) in the sequential treatment with Methotrexate and 5-FU in patients with peritoneal dissemination which is cytologically confirmed malignant ascites [24]. Tahara et al. [25] reported that the median survival time was 259 days in a retrospective study of 95 patients with peritoneal dissemination of gastric cancer treated with sequential MTX/5-FU. Based on these evidences, assuming a null hypothesis of 150 days and an alternative hypothesis of 259 days with one-sided type-I error of 0.05 and power of 0.8, with an accrual time of 2 years and follow-up of 1 year after closure of recruitment, it was necessary to enroll 23 assessable patients. TTP and OS were calculated using the Kaplan–Meier method. TTP was calculated as the interval between chemotherapy initiation and disease progression. OS was measured as the interval between chemotherapy initiation and death. Disease control rate (DCR) was defined by complete and partial responses and stable disease.

Results

Patient characteristics

A total of 19 patients with histologically proven unresectable or recurrent gastric cancer with peritoneal dissemination were enrolled onto this clinical trial from October 2005 to September 2010 (Table 1). The patients comprised 13 males and 6 females, with a median age of 54.3 years (range 21–73 years). The ECOG performance status for all patients was 0 or 1. Most patients had undifferentiated adenocarcinoma (17/19, 89.5 %). Fourteen patients (73.7 %) had no prior therapy. Of the 5 patients (26.3 %) who received prior therapies, 2 patients underwent gastrectomy (10.5 %) and 3 patients had gastrectomy followed by S-1 therapy (5.3 %), S-1 plus CDDP followed by gastrectomy (5.3 %) and S-1 plus CDDP followed by S-1 monotherapy (5.3 %). The lesions were measurable in 2 patients (10.5 %) and non-measurable in 17 patients (89.5 %).

Table 1 Patient characteristics ($n = 19$)

Characteristic	Number of patients (%)
Gender	
Male	13 (68.4)
Female	6 (31.6)
Age	
Median	54.3
Range	24–73
Performance status by ECOG	
0	16 (84.2)
1	3 (15.8)
2	0 (0)
3	0 (0)
Disease status	
Advanced	15 (78.9)
Recurrent	4 (21.1)
Histological type	
Differentiated	2 (10.5)
Undifferentiated	17 (89.5)
Prior therapy	
None	14 (73.7)
Gastrectomy	2 (10.5)
Gastrectomy, S-1	1 (5.3)
S-1 + CDDP, Gastrectomy	1 (5.3)
S-1 + CDDP, S-1	1 (5.3)
Measurable lesion	
Yes	2 (10.5)
No	17 (89.5)

ECOG eastern cooperative oncology group; *CDDP* cisplatin

Clinical outcome

The median number of courses per patient was 4 (average = 6.9), and the range was 1–43 courses (Table 2). All patients were evaluated for clinical response by upper gastrointestinal endoscopy, ultrasonography, computed tomography, upper gastrointestinal radiography, cytology and clinical findings. Objective responses consisted of a partial response in 2 patients (10.5 %), stable disease in 12 patients (63.2 %) and progressive disease in 5 patients (26.3 %); the disease control rate (DCR) was 73.7 % (14/19) (Table 3). Overall survival analysis by Kaplan–Meier was 57.9 % at 1 year and 25.2 % at 2 years (Fig. 1). The median survival time (MST) was 459 days (15.3 months). The median TTP was 212 days (7.1 months), with a 1-year progression-free rate of 26.3 % (Fig. 2).

Adverse events

Toxicities and adverse events (AE) that occurred in all patients are presented in Table 4. Among the grade 3 and 4 hematologic toxicities, neutropenia was the most common (7/19, 36.8 %), followed by febrile neutropenia (2/19, 10.5 %). All adverse events were manageable with G-CSF. Non-hematologic toxicity was limited, with three incidences of grade 3 anorexia (5.3 %), nausea (5.3 %) and allergic reaction (5.3 %). All non-hematologic toxicities

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Table 2 Treatment courses

Number of cycles	
Median	4
Range	1–43
Mean	6.9

Table 3 Response assessment ($n = 19$)

Number of patients (%)	
Complete response	0 (0)
Partial response	2 (10.5)
Stable disease	12 (63.2)
Progressive disease	5 (26.3)

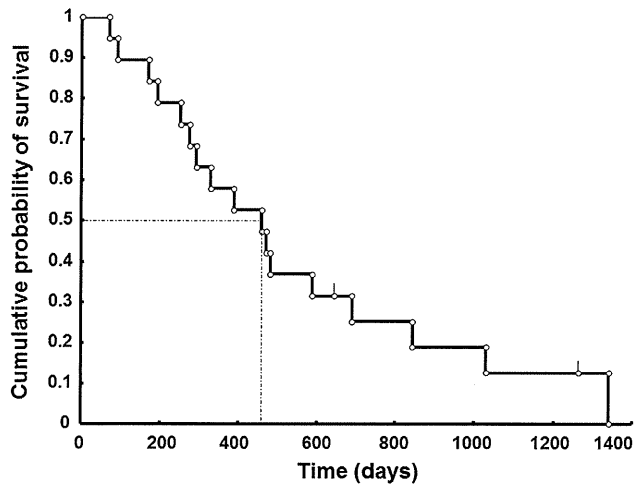

Fig. 1 Kaplan-Meier plot for overall survival time of all 19 patients. The MST was 459 days (15.3 months), with a 1-year survival of 57.9 % and a 2-year survival of 25.2 %

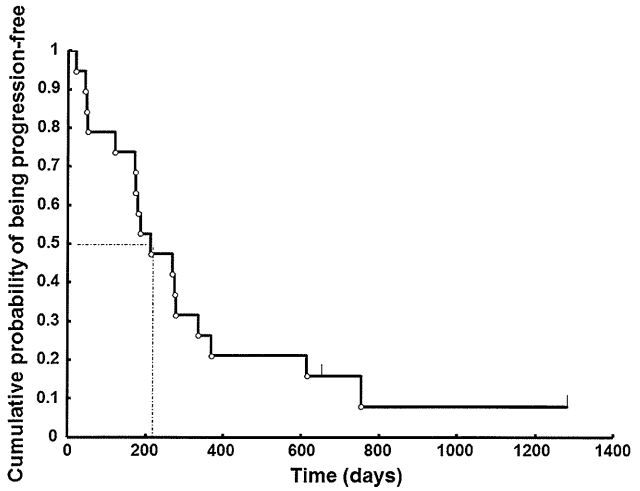

Fig. 2 The TTP of all 19 patients. The median time to progression was 212 days (7.1 months), with a 1-year progression-free rate of 26.3 %

were manageable with optimal treatment. All patients recovered with conservative care, and there were no treatment-related deaths.

Discussion

Peritoneal dissemination is the most common and significant cause of mortality in patients with advanced or recurrent gastric cancer. To improve the overall prognosis of gastric cancer patients, the treatment outcome of patients with peritoneal dissemination must be improved. Various treatment regimens have been developed for advanced or recurrent gastric cancer; however, the efficacy is still less than ideal [5–7] and, therefore, a safe, effective and tolerable regimen is required. In the present study, we conducted a prospective multicenter phase II study to assess the efficacy and safety of oral S-1 plus systemic docetaxel in gastric cancer patients with peritoneal dissemination.

The oral anticancer drug S-1 inhibits the phosphorylation of fluorouracil in the gastrointestinal tract and reduces gastrointestinal toxicities [10, 11]. It has been reported that S-1 monotherapy is associated with a response rate of 45 % and a 2-year survival rate of 17 % in advanced gastric cancer [26, 27]. These findings led us to consider that S-1 in combination with other chemotherapeutic drugs might

Table 4 Toxicity profile

Toxicity	Grade (number of patients)				3 or 4 (%)
	1	2	3	4	
Hematologic					
Leukopenia	2	8	1	5.3	
Neutropenia	1	3	5	2	36.8
Anemia	2	3			
Thrombocytopenia	2				
Febrile neutropenia	2	10.5			
Nonhematologic					
Alopecia	3	8			
Anorexia	7	1	1	5.3	
General fatigue	2				
Nausea	3	2	1	5.3	
Vomiting	1	1			
Diarrhea	4				
Constipation	2				
Allergic reaction	1	1	5.3		
Sensory neuropath	1	2			
Dysguesia	3				
Hyperpigmentation	3				
Stomatitis	1	1			

Toxicity was graded according to Common Terminology Criteria for Adverse Events (CTCAE) v3.0

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be more effective than S-1 alone. Potential partners for S-1 include cisplatin, irrinotecan (CPT-11) and taxane such as paclitaxel and docetaxel. The SPIRITS trial showed a survival benefit of S-1 plus cisplatin over S-1, with response rates of 31–54 % and OS of 11–13 months, resulting in the establishment of a standard care for advanced gastric cancer in Japan [13]. In contrast, the GC0301/TOP-002 trial failed to prove the advantages of S-1 and irrinotecan therapy over S-1 monotherapy [28]. Recently, the combination of S-1 and paclitaxel was reported to be an effective regimen for the treatment of advanced gastric cancer with peritoneal dissemination [29]. Paclitaxel was administered intravenously at 50 mg/m^2 and intraperitoneally at 20 mg/m^2 on days 1 and 8, respectively. S-1 was administered at 80 mg/m^2/day for 14 consecutive days, followed by 7 days rest. Although the outcome–a 1-year OS rate of 78 % and overall response rate of 56 %–appears encouraging, patients nevertheless have to be implanted with ports by mini-laparotomy under local anesthesia.

Docetaxel has synergistic antitumor activity in vivo with S-1 and is not associated with any overlapping toxicities [30]. In addition, docetaxel exhibits high antitumor activity in poorly differentiated adenocarcinoma [31]. The S-1 with docetaxel regimen has been evaluated in two previous phase II studies. Yoshida et al. [18] have reported the activity and tolerability of docetaxel and S-1 combination therapy for advanced or recurrent gastric cancer. Patients were treated with intravenous docetaxel (40 mg/m^2) on day 1 and oral S-1 (80 mg/m^2/day) on days 1–14. The overall response rate was 56.3 %, while the tumor control rate was 93.8 %. The median OS was 14.3 months, and the median TTP was 7.3 months. Kunisaki et al. [19] reported a phase II study of biweekly docetaxel and S-1 combination chemotherapy as a first-line treatment for advanced gastric cancer. Patients with histologically proven unresectable or recurrent gastric cancer were eligible for inclusion. Patients received oral S-1 twice daily for 1 week, followed by a drug-free interval of 1 week. Docetaxel (40 mg/m^2) was administered intravenously on days 1 and 15. The overall response rate was 57.8 %, the median OS was 15.3 months, the median TTP was 6.9 months and the median response duration was 8 months.

On the basis of these results, we assessed the efficacy of the S-1 and docetaxel regimen for the treatment of advanced gastric cancer with peritoneal dissemination. We focused on the most treatment-refractory patient population having a wide dispersion of cancer cells in the abdominal cavity. Our phase II study demonstrated that S-1 and docetaxel combination therapy to be a highly effective for the treatment of histologically proven unresectable or recurrent gastric cancer patients with peritoneal dissemination, with an acceptable and manageable toxicity profile. The combination achieved promising results in terms of median TTP (7.1 months) and median OS (15.3 months). Although the present study was originally planned as a study involving 23 patients, patient enrollment had been delayed and finally terminated before the projected number of patients was achieved. This was caused by the eligibility criteria for this study. Most patients with peritoneal metastasis tend to have relatively poor performance status and impaired organ function. In addition, it was also difficult for them to be fully informed and consent to join the study. They were considered to be critical issues delaying patient enrollment. Even with the actual sample size of 19 patients, the median survival time of 459 days (95 % confidence interval: 274–645) was observed, which was longer than initially anticipated. Non-hematologic toxicities were generally mild, and grade 3 non-hematologic toxicities, including anorexia, nausea and allergic reaction, occurred in only 5.3 % patients. The predominant toxicity was myelo-suppression, and grade 3–4 neutropenia occurred in 36.8 % patients; however, both hematologic and non-hematologic toxicities were manageable by conservative treatments. Recently, Kim et al. reported the results of a randomized phase III study of S-1 and docetaxel for unresectable or recurrent gastric cancer (JACCRO GC03 study: START trial), in which the combination had no significant survival benefit over S-1 alone; however, in patients with non-measurable disease, the OS and TTP of the combination group were significantly superior to those treated with S-1 alone [32], which is consistent with our results. Our study represents the first multicenter, prospective phase II study of S-1 plus docetaxel targeting only patients with peritoneal dissemination.

In conclusion, the present study demonstrates that S-1 and docetaxel combination chemotherapy is tolerable and effective for the treatment of advanced or recurrent gastric cancer patients with peritoneal dissemination.

Conflict of interest All authors declare no potential conflict of interest.

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ORIGINAL ARTICLE

The effects of the Kampo medicine (Japanese herbal medicine) "Daikenchuto" on the surgical inflammatory response following laparoscopic colorectal resection

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Abstract
Purposes The inflammatory response after surgery is associated with various postoperative complications. The aim of the present prospective study was to evaluate the effects of Daikenchuto (DKT) (a Japanese herbal medicine) on the inflammatory response in patients following laparoscopic colorectal resection.
Methods Thirty patients who underwent laparoscopic colectomy for colorectal carcinoma were divided into two groups: a DKT intake group (D group, $n = 15$) and a control group (C group, $n = 15$). The D group took 7.5 g/day of DKT from the day after surgery until the 7th postoperative day. The body temperature, heart rate, WBC count, lymphocyte count, C-reactive protein (CRP) level, β-D-glucan level and Candida index were compared between the two groups.
Results The patients' mean age in the D group was significantly younger than that in the C group. D3 lymph node dissection was performed more often in the D group. The time until first flatus was significantly shorter in the D group (1.8 ± 0.5 days) than in the C group (2.7 ± 0.5 days). The CRP level was significantly lower in the D group (4.6 ± 0.6 mg/dl) than in the C group (8.3 ± 1.1 mg/dl) on the 3rd postoperative day.
Conclusions Postoperative DKT administration significantly suppressed the CRP level and shortened the time until first flatus. DKT administration also significantly suppressed postoperative inflammation following surgery for colorectal cancer.

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Introduction

The inflammatory response after surgery is associated with various postoperative complications. For example, an increase in inflammatory cytokines is responsible for a significant portion of the loss of the epithelial barrier function [1]. Impaired integrity of the intestine causes bacterial translocation (BT) which is a particularly severe complication [2]. BT is known to be a key factor leading to conditions causing high morbidity, such as sepsis and multiple organ failure [3]. A reduction of postoperative inflammation is associated with fewer postoperative complications. To suppress the postoperative inflammatory response, less invasive surgery, such as laparoscopic-assisted surgery, has become increasingly common, and various drugs have also been used to reduce surgical stress. Laparoscopic colectomy has been carried out at many institutions and is considered to be a less invasive therapy. Preventing the release of inflammatory cytokines at an early stage is very important for preventing sepsis and bacterial translocation.

Daikenchuto (DKT) has been used to treat adhesive bowel obstruction and a feeling of coldness in the abdomen [4]. Several studies have shown that DKT accelerates gastrointestinal transit [5–11]. Recently, other effects of DKT have been reported; it induces increases in intestinal blood flow [12], reduces the blood ammonia level after hepatectomy [13], and has anti-inflammatory effects [14]. We previously reported that DKT prevented BT by maintaining the intestinal barrier integrity through its anti-inflammatory and anti-apoptotic effects in a rat BT model [15]. We hypothesized that DKT may also prevent the inflammatory response in

postoperative patients. The aim of the present prospective randomized trial was to evaluate the effects of DKT on the inflammatory response in patients following colorectal resection. To judge the effects of DKT in a controlled fashion and diminish the effects of individual differences in surgical stress, we focused on laparoscopic resection.

Methods

Participants

Thirty patients who underwent laparoscopic colectomy for colorectal carcinoma in the Department of Digestive Surgery of Tokushima University from July 2007 to June 2008 were enrolled in this study. The study was performed as an alternate study. The patients were divided into two groups: a DKT-treated group (D group, $n = 15$) and a control group (C group, $n = 15$). The patients in C group did not take any drugs or a placebo. The inclusion criteria were as follows: histologically confirmed adenocarcinoma of the colorectum, no involvement of other organs, a performance status of 0 or 1, no prior abdominal surgery, and no prior chemotherapy or radiotherapy for any malignancy. The exclusion criteria ware as follows: unstable anemia, myocardial infarction within 6 months before registration, uncontrolled hypertension or diabetes mellitus, severe respiratory disease, and intestinal leakage or obstruction. The patients' mean age was 62 ± 12 years in D group and 70 ± 5 years in C group. The clinical stages of colorectal carcinoma according to the *Japanese Classification of Colorectal Carcinoma* were stage 0, I, II, IIIA, IIIB and IV in 0, 6, 5, 3, 0 and 1 patient in D group and 4, 3, 3, 1, 2 and 2 in C group, respectively. The factor that led to patients being classified to have stage IV disease was liver metastasis. None of the patients had any significant associated diseases, such as cirrhosis, renal failure, pulmonary diseases, or cardiac diseases. The patients' mean body mass index (BMI) was 21 ± 3.7 in D group and 24 ± 4.2 in C group. There were no significant differences between the two groups in terms of these patient characteristics except for age (Table 1).

Study design

D group took 7.5 g/day of DKT from the day after surgery to the 7th postoperative day, while C group did not take DKT. This amount of DKT was half of the recommended dose, because some patients had difficulty in taking 15 g after surgery in a previous study. Both groups drank water from the day after surgery and began eating from the 5th postoperative day. The patients' body temperature, heart rate, WBC count, lymphocyte count, C-reactive protein (CRP) level, β-D-glucan level and Candida index were checked preoperatively and on the 1st, 3rd, 5th, and 7th

Table 1 Patient characteristics

	D group ($n = 15$)	C group ($n = 15$)	P value
Gender (male/female)	8/7	8/7	0.99
Age (years)			
Mean ± SE	62 ± 12	70 ± 5	0.04
Range	41–80	61–86	
Tumor location			
Cecum	2	1	
Ascending colon	1	4	
Transverse colon	0	2	
Descending colon	1	0	
Sigmoid colon	4	2	
Rectosigmoid	1	1	
Rectum	6	5	0.43
Clinical staging, n (%)			
0	0	4	
I	6	3	
II	5	3	
IIIA	3	1	
IIIB	0	2	
IV	1 (liver meta)	2 (liver meta)	0.08
BMI			
Mean ± SE	21 ± 3.7	24 ± 4.2	0.32
Range	17–28	20–34	