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ORIGINAL ARTICLE

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Combined effects of docetaxel and fluoropyrimidines on tumor growth and expression of interleukin-6 and thymidine phosphorylase in breast cancer xenografts

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Abstract *Purpose*: Although several combination treatments with docetaxel and other antitumor agents have been tested in experimental and clinical studies, their synergistic effects are still ill-defined. The degree of synergism between docetaxel and two oral fluoropyrimidines, tegafur and 5′-deoxy-5-fluorouridine (5′-dFUrd), was investigated in the KPL-4 human breast cancer xenograft model. *Methods*: Because this KPL-4 cell line secretes interleukin-6 (IL-6) and induces cachexia, the effects of the combined treatment on serum IL-6 levels and cachectic markers were investigated. In addition, the expression levels of thymidine phosphorylase (dThdPase), a key enzyme for converting 5′-dFUrd to 5-fluorouracil, were determined. Female nude mice bearing KPL-4 tumors were treated orally with 5′-dFUrd (60 mg/kg, five times a week) or tegafur (100 mg/kg, five times a week) and by intraperitoneal injection of docetaxel (5 or 10 mg/kg, once a week). *Results*: Although docetaxel (5 mg/kg) alone did not decrease either tumor growth or serum IL-6 levels, docetaxel (5 mg/kg) plus 5′-dFUrd or tegafur enhanced tumor growth inhibition and decreased serum IL-6 levels more than 5′-dFUrd or tegafur alone. Docetaxel (5 mg/kg) alone slightly increased the percentage of dThdPase-positive tumor cells, but the combined treatment with docetaxel plus 5′-dFUrd or tegafur significantly decreased the percentage of dThdPase-positive cells in the KPL-4 tumors. *Conclusion*: These findings indicate that docetaxel may stimulate dThdPase expression in tumor tissues and may enhance the antitumor activity of oral fluoropyrimidines. In addition, combined treatment with docetaxel and oral fluoropyrimidines may decrease serum IL-6 levels and may ameliorate IL-6-induced cancer cachexia.

Keywords Breast cancer · Thymidine · Phosphorylase · IL-6 · Fluoropyrimidines · Docetaxel

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Introduction

Taxanes, such as docetaxel and paclitaxel, are promising antitumor agents for the treatment of patients with advanced breast cancer. Combined treatments with docetaxel and other antitumor agents, such as doxorubicin, cyclophosphamide and 5-fluorouracil (5-FU), have been explored in experimental and clinical studies [1, 2, 3, 4, 5, 6]. However, whether they act synergistically is still a matter of controversy. Recently, an experimental study has shown that combined treatment with docetaxel/paclitaxel and 5′-deoxy-5-fluorouridine (5′-dFUrd) synergistically inhibits the growth of human colon and breast cancer xenografts due to the induction of dThdPase, which is a key enzyme for activation of 5′-dFUrd [7].

Oral fluoropyrimidines, such as 5′-dFUrd and tegafur, have been used for the treatment of patients with various malignancies in Japan. A recent study has shown that the oral administration of a combination of tegafur and uracil, known as UFT, results in a 5-FU blood level equivalent to that following continuous intravenous 5-FU infusion [8]. In addition, another oral fluoropyrimidine derivative, capecitabine, has been approved for use in the treatment of patients with paclitaxel-refractory breast cancer in the United States [9]. These findings prompted us to explore the effects of combined treatments with docetaxel and oral fluoropyrimidines on human breast cancer xenografts.

We have recently established a unique human breast cancer cell line, KPL-4, which was derived from a

patient with recurrent breast cancer and inflammatory skin metastasis [10]. Interestingly, this cell line secretes a cachectic factor, interleukin-6 (IL-6) and induces cachexia in female nude mice. Our previous study suggested that IL-6 may play an important role in cancer-induced cachexia [11]. In addition, our pilot study indicated that this cell line expresses thymidine phosphorylase (dThdPase), which is known to be a key enzyme in the conversion of 5′-dFUrd to an active metabolite, 5-FU, and to be an angiogenic factor, platelet derived-endothelial cell growth factor (PD-ECGF), in the transplanted tumors. Therefore, we decided to use this KPL-4 xenograft model to investigate the effects of combined treatments with docetaxel and oral fluoropyrimidines on tumor growth, serum IL-6 levels, cachectic markers and the levels of expression of dThdPase by tumor cells.

Materials and methods

Reagents

5′-dFUrd was kindly provided by Nippon Roche (Tokyo, Japan), tegafur by Taiho Pharmaceutical (Tokyo, Japan), and docetaxel by Rhone-Poulenc Rorer (Antony, France).

Cell line and cell culture

The KPL-4 cell line was derived from the malignant pleural effusion of a Japanese patient with recurrent breast cancer and inflammatory skin metastasis, and its characterization has been reported elsewhere [10]. In brief, this cell line expresses all the Erb B family receptors and IL-6. Neither estrogen nor progesterone receptors are expressed. Injection of these cells into the mammary fat pad of female nude mice produces rapid-growing tumors and induces severe cachexia. Immunoreactive human IL-6 is detected in both the culture medium and serum of the recipient mice. KPL-4 cells are routinely cultured in Dulbecco's modified Eagles' medium supplemented with 5% fetal bovine serum (ICN Biochemicals, Costa Mesa, Calif.).

Animal experiments

Semiconfluent KPL-4 cells were trypsinized and harvested, and viable cells were counted in a hemocytometer using trypan blue exclusion. Approximately 2×10^6 viable KPL-4 cells/site were injected into the right and left mammary fat pads of 6-week-old female nude mice (CLEA Japan, Tokyo, Japan). 5′-dFUrd and tegafur were dissolved in 40 m*M* citrate buffer (pH 6.0) containing 5% arabic gum and given orally. Docetaxel was dissolved in saline containing 2.5% ethanol and 2.5% polysorbate 80 and given intraperitoneally (i.p.). In our pilot study, various doses of each antitumor agent were tested in this KPL-4 xenograft model. In brief, we found that 30 mg/kg docetaxel i.p. once every week was toxic and killed nude mice within 4 weeks but 15 mg/kg docetaxel i.p. once every week effectively inhibited the growth of KPL-4 tumors. Oral administration of 120 mg/kg 5′-dFUrd or 150 mg/kg tegafur (five times a week) was somewhat toxic but effectively inhibited the growth of KPL-4 tumors. Therefore, we decided to use the following doses of the respective agents in the present study.

In the docetaxel-treated group, 5 or 10 mg/kg docetaxel was administered i.p. once every week for 2 to 4 weeks (days 14, 21 and 28) after cell injection. In the 5′-dFUrd- and tegafur-treated group, 100 mg/kg tegafur or 60 mg/kg 5′-dFUrd were administered orally five times a week (days 14–34). In the combined treatment groups, docetaxel and 5′-dFUrd and tegafur were, respectively, administered in the same manner. The same volumes of the vehicles were administered to the control group in the same manner. Five mice (ten tumors) were treated in each group. Body weight was measured once a week. All mice were killed by cervical dislocation 5 weeks after cell injection. Serum samples were collected and stored at –80°C until use. After measurement of tumor weight, one half of each tumor sample was fixed with 5% buffered formalin and embedded in paraffin for morphologic analysis.

The animal protocols for these experiments were approved by the Animal Care and Use Committee of Kawasaki Medical School.

Measurement of dThdPase

The other half of the resected KPL-4 tumors were stored at –80°C until use. The tumor samples were homogenized in 10 m*M* Tris-HCl buffer (pH 7.4) containing 15 m*M* NaCl, 1.5 m*M* $MgCl_2$ and 50 m*M* potassium phosphate and then centrifuged at 10,000 *g* for 15 min. The dThdPase levels of the supernatant were measured using an enzyme-linked immunosorbent assay as described by Nishida et al. [12]. In brief, an anti-dThdPase monoclonal antibody (104B) was coated onto a 96-well microtiter plate (Nunc-immunoplate Maxisorp; Nunc, Roskilde, Denmark). Test samples and standard solutions of recombinant dThdPase serially diluted with a blocking buffer (3% w/v skimmed milk in PBS) were dispensed onto the plate. The plate was incubated at 37°C for 2 h and was then washed with diluted washing buffer (KPL, Gaithersburg, Md.) and incubated with another anti-dThdPase monoclonal antibody (232-2) at 37°C for 2 h. The plate was then incubated with 2000-fold diluted anti-mouse IgG conjugate with horseradish peroxidase (KPL) for 60 min at room temperature, washed and incubated with a substrate solution containing 3,3′,5,5′-tetramethylbenzidine and H_2O_2 (TMB microwell peroxidase substrate system, KPL) for 5–10 min at room temperature. The amount of dThdPase was estimated by measuring absorbency at 450 nm with a plate reader (model 3550; Bio-Rad, Hercules, Calif.) and was calibrated by measurement of the standard solutions. The levels of dThdPase are expressed as units per milligram protein, where 1 U is equivalent to the amount of dThdPase that can generate 1 mg 5-FU from 5′-dFUrd in 1 h. The values were then normalized to the total protein concentration in each sample. The monoclonal antibodies were not crossreacted with mouse dThdPase or mouse uridine phosphorylase.

Measurement of IL-6

IL-6 concentrations in serum were measured using a chemiluminescent enzyme immunoassay kit (Fujirebio, Tokyo, Japan) according to the manufacturer's recommendations [9]. Briefly, a mouse anti-human IL-6 monoclonal antibody (HH61-10) was used as the first antibody and a mouse anti-human IL-6 monoclonal antibody labeled with alkaline phosphatase (HH 61-2 Fab′) as the second antibody. After removing the unbound second antibody, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1, 2-dioxetane disodium salt was added. Chemiluminescence was measured with a Lumipulse luminometer (Fujirebio). As the standard, 20–1000 pg/ml human recombinant IL-6 was used. In this assay, no crossreactivity against recombinant mouse IL-6 was observed [13].

Measurement of glucose in mouse serum

The concentration of glucose in mouse serum was measured using an assay kit (Glucose CII test; Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's recommendations.

Immunohistochemical staining

Paraffin sections of tumor samples fixed with 5% buffered formalin and embedded in paraffin were dewaxed with xylene, hydrated with PBS, treated with hydrogen peroxide for elimination of endogenous

peroxidase and then processed by the immunoperoxidase procedure. Mouse anti-dThdPase monoclonal antibody (654-1) [12], which was kindly provided by Nippon Roche, was used as the first antibody. The reaction was visualized by streptavidin-biotin techniques following the manufacturer's recommendations (Nichirei, Tokyo, Japan). Control experiments were performed by substituting normal mouse IgG for the first antibody. No marked immunostaining was observed in KPL-4 tumor cells in the control experiments. To quantify dThdPase expression levels, three microphotographs of representative areas in each sample were taken at a magnification of ×100. A total of 1000 tumor cells were then examined in each sample and the percentages of dThdPase-positive cells were calculated for each group.

Statistical analysis

All values are expressed as means ± SD. The values among the control and treated groups were compared by ANOVA using StatView software (ATMS, Tokyo, Japan). Two-sided *P*-values < 0.05 were considered statistically significant.

Results

In vivo antitumor effect

The weights of tumors of all the treated groups, except those treated with docetaxel (5 mg/kg) alone and tegafur alone, were significantly less than those of the control group 5 weeks after cell injection (Fig. 1). Docetaxel (5 mg/kg) alone had no antitumor effect, but the combined treatment with docetaxel (5 mg/kg) and 5′-dFUrd or tegafur showed an antitumor effect more than the respective single-agent treatment. The mean tumor weights as a percentage of those in the control group were 72% for 5′-dFUrd alone, 80% for tegafur alone, 114% for docetaxel (5 mg/kg) alone, 53% for 5′-dFUrd plus docetaxel (5 mg/kg) and 45% for tegafur plus docetaxel (5 mg/kg). No additive antitumor effects were observed in the groups treated with docetaxel (10 mg/kg) plus 5′-dFUrd or tegafur.

Mouse body weight and serum glucose levels

KPL-4 tumors induced body weight loss in the control group, but a similar body weight loss was also observed in all the treated groups. No significant differences in the changes in body weight or in carcass weight (data not shown) were observed among the control and treated groups. In contrast, low levels of serum glucose were observed in the control group and the group treated with docetaxel (10 mg/kg) (the serum glucose level of 6–7-week-old female nude mice was approximately 180 mg/ml in a separate experiment). Serum glucose levels were significantly higher in the groups treated with docetaxel (5 or 10 mg/kg) plus 5′-dFUrd- and docetaxel (10 mg/kg) plus tegafur than in the control group (Fig. 2).

Serum IL-6 levels

Serum human IL-6 levels in the group treated with docetaxel (5 mg/kg) were significantly higher than in the control group (81.9 ± 14.2 vs 42.1 ± 18.0 pg/ml, $P < 0.005$). In contrast, serum human IL-6 levels in the other treated groups, except those treated with docetaxel (10 mg/kg) alone and tegafur alone, were significantly lower than in the control group (Fig. 3). In particular, the groups treated with docetaxel (5 or 10 mg/kg) plus 5′-dFUrd showed a dramatic reduction in serum human IL-6 levels (6.4 ± 3.7 or 4.7 ± 2.8 pg/ml, respectively).

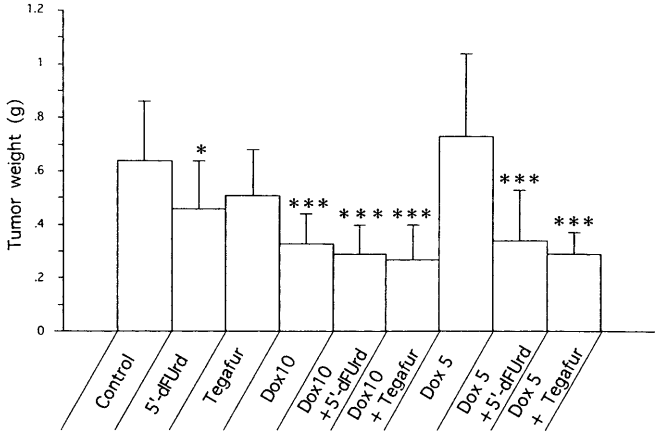

Fig. 1 Antitumor effects of various treatments on KPL-4 human breast cancer xenografts in female athymic nude mice. 5′-dFUrd (60 mg/kg, orally, five times a week) alone, tegafur (100 mg/kg, orally, five times a week) alone, docetaxel (5 or 10 mg/kg, i.p., once a week) alone or their combinations were administered to the mice 2–4 weeks after cell injection. Tumor weights of the KPL-4 tumors were measured 5 weeks after cell injection. Values are means + SD, $n = 10$ each. $^{*}P < 0.05$, $^{***}P < 0.005$ vs control

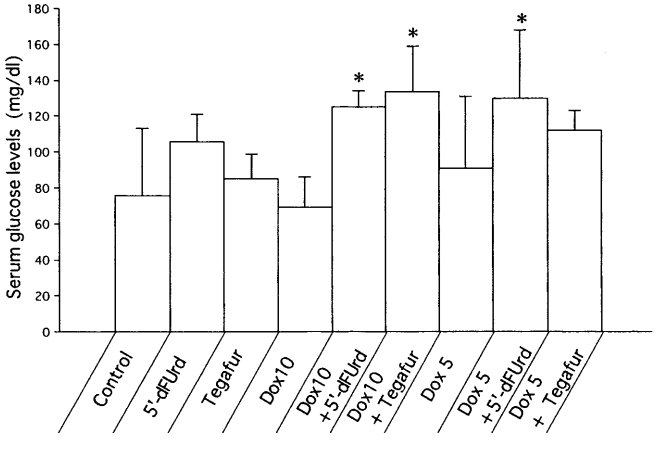

Fig. 2 Effects of various treatments on serum glucose levels of mice bearing KPL-4 human breast cancer xenografts. 5′-dFUrd alone, tegafur alone, docetaxel alone or their combinations were administered to the mice 2–4 weeks after cell injection. Serum glucose levels were measured 5 weeks after cell injection. Values are means + SD, $n = 10$ each. $^{*}P < 0.05$ vs control

dThdPase expression levels in tumor samples

dThdPase concentrations measured by ELISA were significantly lower in the groups treated with 5′-dFUrd- and docetaxel (5 or 10 mg/kg) plus 5′-dFUrd than in the control group (13.0 ± 3.4 U/mg protein for 5′-dFUrd alone, 14.2 ± 6.5 U/mg protein for 5 mg/kg docetaxel plus 5′-dFUrd, 12.2 ± 2.8 U/mg protein for 10 mg/kg docetaxel plus 5′-dFUrd and 34.5 ± 5.1 U/mg protein for the control; Fig. 4). In the immunohistochemical analysis, dThdPase expression was significantly greater in tumor cells from the group treated with docetaxel (5 mg/kg) alone than in tumor cells from the control group ($P < 0.05$). In contrast, significant decreases in the percentages of dThdPase-positive cells were observed in the groups treated with the 5′-dFUrd alone, tegafur alone and docetaxel (5 or 10 mg/kg) plus 5′-dFUrd or tegafur (Fig. 5). Overall, the results of this immunohistochemical analysis correlated well with those of the biochemical analysis described above.

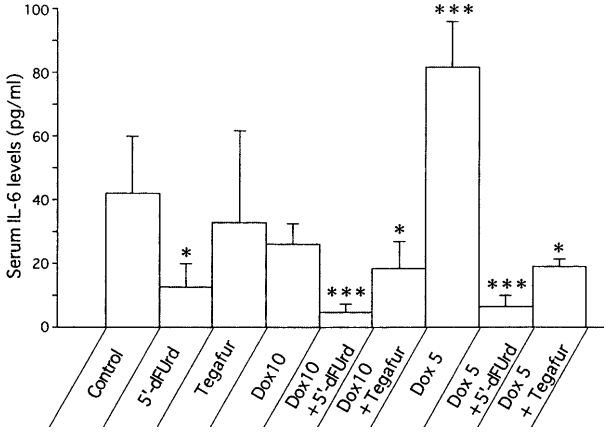

Fig. 3 Effects of various treatments on serum IL-6 concentrations in female athymic nude mice bearing KPL-4 human breast cancer xenografts. 5′-dFUrd alone, tegafur alone, docetaxel alone or their combinations were administered to the mice 2–4 weeks after cell injection. Values are means + SD, $n = 3$ each. $^{*}P < 0.05$, $^{***}P < 0.005$ vs control

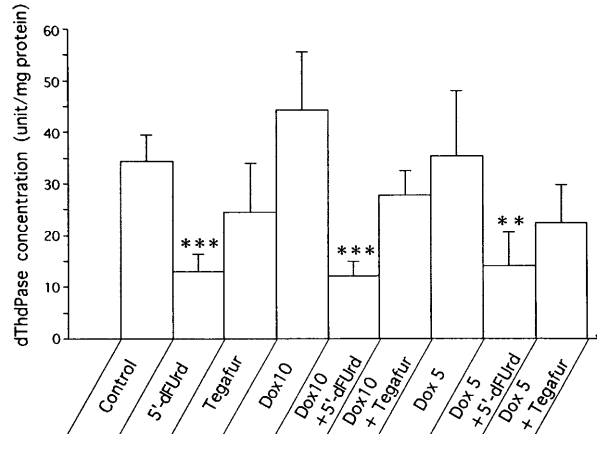

Fig. 4 Effects of various treatments on dThdPase concentrations in KPL-4 human breast cancer xenografts transplanted into female athymic nude mice. 5′-dFUrd alone, tegafur alone, docetaxel alone or their combinations were administered to the mice 2–4 weeks after cell injection. Values are means + SD, $n = 3$ each. $^{**}P < 0.01$, $^{***}P < 0.005$ vs control

Discussion

A number of combined treatments with docetaxel and other antitumor agents have been investigated with the aim of establishing the degree of synergism among these agents in their antitumor effects against human malignancies [1, 2, 3, 4, 5, 6]. However, suitable partners for docetaxel have not yet been defined. Recently, a new candidate for such a partnership, an oral fluoropyrimidine, capecitabine, has been tested in clinical trials [14]. This approach originated from the following experimental findings: (1) dThdPase is preferentially expressed in human malignancies [15]; (2) some antitumor agents, such as docetaxel, upregulate dThdPase activity in various human tumor xenografts [6]; (3) the stimulated dThdPase activity efficiently converts 5′-dFUrd, which is the main metabolite of capecitabine, to 5-FU in tumor tissues [14]; (4) the increased concentration of 5-FU in tumor tissues results in a strong tumor regression.

In the present study, docetaxel (5 mg/kg) alone slightly upregulated dThdPase expression in KPL-4 breast cancer cells (Fig. 5). In addition, docetaxel (5 mg/kg) enhanced the antitumor effect of 5′-dFUrd against KPL-4 transplanted tumors (Fig. 1). Furthermore, a small percentage of tumor cells showed positive dThdPase staining in the group treated with docetaxel plus 5′-dFUrd (Fig. 5). These findings suggest that dThdPase-expressing tumor cells might be selectively killed by 5′-dFUrd. However, docetaxel (5 mg/kg) also enhanced

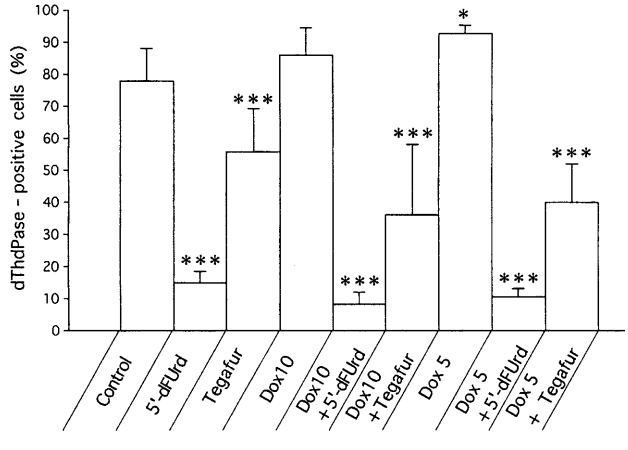

Fig. 5 Effects of various treatments on dThdPase expression measured by immunocytochemistry in KPL-4 human breast cancer xenografts transplanted into female athymic nude mice. 5′-dFUrd alone, tegafur alone, docetaxel alone or their combinations were administered to the mice 2–4 weeks after cell injection. Values are means + SD ($n = 5$ each) of the percentage of dThdPase-positive tumor cells in each group. $^{*}P < 0.05$, $^{***}P < 0.005$ vs control

the antitumor effect of another oral fluoropyrimidine, tegafur (Fig. 1). Although it has been reported that tegafur is partly converted by dThdPase to 5-FU in tumor tissues [16], the enhanced antitumor effect of docetaxel plus oral fluoropyrimidines may not be simply explained by the changes in dThdPase activity in tumor tissues. Further experiments are needed to elucidate the mechanisms of action responsible for these synergistic effects.

It has been suggested that elevated blood levels of tumor necrosis factor-α, IL-1, IL-6, IL-11, interferon-γ and leukemia inhibitory factor may induce cachexia in patients with malignancies [17, 18, 19, 20, 21, 22]. Our previous studies have indicated that one of these cachectic factors, IL-6, may play an important role in the development of cachexia in nude mice bearing KPL-4 breast cancer cells. In addition, the downregulation of IL-6 expression in tumor cells and a subsequent decrease in serum IL-6 levels may provide relief from cachexia [11]. In the present study, 5′-dFUrd alone or 5′-dFUrd plus docetaxel significantly decreased serum IL-6 levels (Fig. 3). It is known that cachexia induces changes in various parameters, such as body weight and serum levels of glucose [23, 24, 25]. Therefore, changes in body weight and serum glucose levels were determined in the present study. Although these treatments did not lead to a recovery of body weight loss induced by KPL-4 tumors in the mice, their serum glucose levels, which is a marker of cachexia, significantly recovered (Fig. 2). Several adverse effects including gastrointestinal disturbance are well known in patients receiving 5′-dFUrd or docetaxel. These adverse effects may hinder the anticachectic effect of these agents. Another possibility is that IL-6 is not the sole cachectic factor responsible for the cachexia induced by KPL-4 tumors and some other cachectic factors might be regulated by these agents in a different manner.

dThdPase is known to function not only as an essential enzyme for the activation of 5′-dFUrd but also as a potent angiogenic factor, PD-ECGF [26], in tumor tissues. It has been suggested that PD-ECGF is expressed in breast cancer tissues and its expression levels correlate with the grade of tumor angiogenesis [27]. However, some other angiogenic factors, such as vascular endothelial growth factors and fibroblast growth factors, are also known to be expressed in breast tumor tissues [28]. Our pilot study revealed high expression levels of vascular endothelial growth factors and a possible angiogenic factor, interleukin-8 [29] (unpublished data). In addition, docetaxel (10 mg/kg) did not decrease dThdPase (PD-ECGF) expression levels in KPL-4 tumors in spite of its antitumor effect in the present study. Therefore, it is unlikely that PD-ECGF expression levels directly influence the antitumor effect in this model.

In conclusion, combined treatment with docetaxel and oral 5′-dFUrd or tegafur enhanced the growth inhibitory effect in KPL-4 transplanted tumors and decreased serum IL-6 levels. Expression levels of dThdPase in tumor cells were slightly induced by docetaxel in vivo. Upregulation of dThdPase in tumor cells by docetaxel may, at least in part, enhance the antitumor effect of 5′-dFUrd. These findings suggest that combined treatment with docetaxel and oral fluoropyrimidines may be a new strategy to produce both antitumor and anticachectic effects in breast cancer patients.

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