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Suppression of Tumor Growth and Downregulation of Platelet-derived Endothelial Cell Growth Factor/Thymidine Phosphorylase in Tumor Cells by Angiogenesis Inhibitor TNP-470

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We investigated the effects of the angiogenesis inhibitor TNP-470 on human lung squamous cell carcinoma cell lines H226B and H226Br both *in vivo* and *in vitro*. H226B was established from human lung squamous cell carcinoma and H226Br was established from a brain metastatic lesion of H226B in nude mice. Nude mice inoculated with these cells were treated with 30 mg/kg of TNP-470 subcutaneously every other day. At this dose, TNP-470 only significantly suppressed the growth of H226Br tumor, but not H226B tumor. Attempts to use a high dose of TNP-470 (100 mg/kg) resulted in a severe loss of body weight. Immunohistochemical studies showed marked tumor vascularization in H226Br tumor, but the formation of new blood vessels was suppressed by 30 mg/kg of TNP-470. Investigation of the mechanism of anti-angiogenic effects of TNP-470 *in vivo* showed that the expression and the activity of platelet-derived endothelial cell growth factor/thymidine phosphorylase (PD-ECGF/dThdPase) in H226Br tumor was significantly suppressed by 30 mg/kg of TNP-470. Furthermore, TNP-470 inhibited cell growth of cultured H226Br dose-dependently at concentrations of ≥1 μg/ml. Immunoblot analysis revealed H226Br cells gave a stronger PD-ECGF signal than H226B cells, and the expression of PD-ECGF/dThdPase in H226Br was also suppressed by treatment with TNP-470 at ≥0.1 μg/ml. No change in basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) was noted in these cell lines. Our results suggested that TNP-470 acts, at least in part, by downregulation of PD-ECGF/dThdPase in this cell line.

Key words: Angiogenesis — TNP-470 — PD-ECGF — Thymidine phosphorylase

Angiogenesis is an important process in solid cancer growth, progression, and metastasis.[1, 2] Recently, inhibition of angiogenesis has been used as a strategy for cancer therapy. The angiogenesis inhibitor TNP-470 is an analog of fumagillin derived from *Aspergillus fumigatus*. It strongly inhibits angiogenesis by the suppression of vascular endothelial cell proliferation. It has been reported that TNP-470 inhibits the cell cycle and suppresses the proliferation of vascular endothelial cells.[3] TNP-470 also has a direct inhibitory effect on cancer cells,[4–6] although the mechanism of this is not well understood. TNP-470 inhibits cancer proliferation and metastasis both *in vitro* and *in vivo*.[7–9]

Previous studies have shown that thymidine phosphorylase (dThdPase) is identical to platelet-derived endothelial cell growth factor (PD-ECGF), which has an angiogenic function.[10–12] PD-ECGF/dThdPase is expressed in various tumors, including gastric, lung, breast, and bladder cancers,[13–16] as well as in normal tissues.[17] Its expression plays an important role in cancer growth and metastasis, and influences the prognosis.[1, 18]

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In the present study, we investigated the effects of TNP-470 on human lung cancer cell lines by examining its effects on tumor growth, cell proliferation, expression of PD-ECGF/dThdPase, activity of PD-ECGF/dThdPase, and angiogenesis.

MATERIALS AND METHODS

Animals, cell lines and cell culture Female ICR nude mice (4 weeks old) were obtained from Charles River Japan, Inc. (Yokohama). The animals were used at 5 weeks of age. We also used two human lung cancer cell lines, H226B established from human lung squamous cell carcinoma, and H226Br established from brain metastatic lesion of H226B. H226B cells express wild-type p53 while H226Br cells express mutant-type p53.[19] These cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, and cultured at 37°C in a 5% CO_2 incubator.

Preparation of TNP-470 The angiogenic inhibitor TNP-470, derived from fumagillin, which is a naturally secreted antibiotic of *A. fumigatus* Fresenius,[4] was a kind gift from Takeda Chemical Industries, Ltd. (Osaka). TNP-470 was suspended in a vehicle of 3% or 10% ethanol and 5% gum

Arabic in saline *in vivo*.[20] It was dissolved in dimethylsulfoxide and diluted with culture medium *in vitro*.[21]

Tumor growth inhibition by TNP-470 *in vivo* The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Okayama University Medical School. H226B cells (1×10^7/body) in 0.2 ml of saline were inoculated on the right side of the back of mice, and H226Br cells (1×10^7/body) in 0.2 ml of saline were inoculated on the left side. TNP-470 was administered subcutaneously at a concentration of 100 mg/kg every other day. Control mice were injected with the vehicle only (10% ethanol and 5% gum Arabic in saline). Eight mice were used in each group. In another series of experiments, H226B or H226Br cells (1×10^7/body) in 0.2 ml of saline were inoculated on the right side of the back of the mice. TNP-470 was administered subcutaneously at 30 mg/kg every other day. Control mice were injected with the vehicle only (3% ethanol and 5% gum Arabic in saline). Tumor volume was calculated as follows:

$$\text{Tumor volume (mm}^3\text{)}=(1/2)\times a\times b^2$$

where a is the longest and b is the shortest diameter of the tumor.[8]

Immunohistochemical study We used DAKO EPOS anti-von Willebrand factor/HRP monoclonal antibody (DAKO Japan, Kyoto) to quantitate the extent of vascularization in tumor tissues obtained from mice treated with or without 30 mg/kg of TNP-470, by using the indirect immunoperoxidase method. Paraffin-embedded tissue samples were sectioned at 4 μm thickness and mounted on glass slides. The deparaffinized sections were incubated with 3% H_2O_2 in distilled water for 5 min. After having been washed with Tris-buffered saline (TBS, 0.05 *M* Tris/HCl, 0.15 *M* NaCl, pH 7.6), the sections were incubated with a proteolytic enzyme (trypsin) for 30 min at 37°C. After another washing with TBS, the sections were incubated with first antibodies for 60 min at room temperature. After further washing with TBS, the sections were incubated with the chromogenic substrate solution for 5 min. Finally, the sections were counterstained with Mayer's hematoxylin, mounted and coverslipped.[22]

Cell growth inhibition by TNP-470 H226B and H226Br cells were seeded in 6-well plates at 5×10^4 cells/well. TNP-470 diluted with the culture medium to a concentration of 0, 0.1, 1, or 10 μg/ml was added to each well on day 0. Viable cells were counted on day 2, 4 or 6 by the dye-uptake method.

Immunoblotting Cultured H226B and H226Br cells were harvested and directly resolved in sodium dodecyl sulfate (SDS) sample buffer (125 m*M* Tris, pH 6.8, 4% SDS, 20% glycerol, and 0.2% bromophenol blue). Equivalent amounts of total proteins obtained from direct lysis were loaded on 12% SDS polyacrylamide gels and transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). The membrane was blocked for 1 h at 37°C by rocking in TBS/milk buffer containing 50 m*M* Tris pH 7.0, 150 m*M* NaCl, and 5% skimmed milk. The membrane was incubated for 8 h at room temperature with the primary antibody in TBS/milk buffer. Anti-human basic fibroblast growth factor (bFGF) antibody, anti-human vascular endothelial cell growth factor (VEGF) antibody and anti-human PD-ECGF antibody (R&D Systems, Minneapolis, MN) were used as primary antibodies. Proteins were visualized using alkaline phosphatase-conjugated secondary antibody.[23] Lysates were also obtained from *in vivo* tissue samples by homogenization and the expressions of angiogenic factors were studied.

dThdPase enzyme assay The activity of dThdPase was measured by the method described by Nishida *et al*.[24] Tissue samples were first homogenized in 10 m*M* Tris-HCl buffer (pH 7.4) containing 15 m*M* NaCl, 1.3 m*M* $MgCl_2$ and 50 m*M* potassium phosphate, and then centrifuged at 105 000*g* for 90 min. The supernatant was dialyzed overnight against 20 m*M* potassium phosphate buffer (pH 7.4) and 1 m*M* 2-mercaptoethanol, and was then used as a source of crude dThdPase. The dThdPase reaction mixture (120 μl) contained 183 m*M* potassium phosphate (pH 7.4), 10 m*M* 5′-deoxy-5-fluorouridine, and the crude enzyme from tissue samples. The reaction was carried out at 37°C for 60 min, and the amount of 5-fluorouracil generated was measured after its separation from 5′-deoxy-5-fluorouridine in an ERC-ODS-1171 HPLC column, with 50 m*M* sodium phosphate buffer (pH 6.8) containing 5 m*M* 1-decanesulfonic acid:methanol=85:15 (v/v).

Statistical analysis All data were expressed as mean±SEM. Differences between groups were examined for statistical significance using Student's *t* test. A *P* value less than 0.05 was taken to indicate a statistically significant difference.

RESULTS

TNP-470 inhibits tumor growth *in vivo* At 56 days after inoculation, the average volume of H226Br tumors was 1937 mm^3 while that of H226B tumors was smaller (463 mm^3). Tumor growth of H226Br was significantly more rapid than that of H226B ($P<0.05$). At a concentration of 30 mg/kg, TNP-470 suppressed the growth of both tumors, although the effect was greater on H226Br (Fig. 1).

TNP-470 reduces body weight Loss of body weight, the major side effect of TNP-470, was observed when the angiogenic inhibitor was used at a dose of 100 mg/kg. At 56 days after inoculation, the mean body weights of naive, control and treated mice were 28, 25.2 and 19 g, respectively. This side effect was not observed when the dose of TNP-470 was 30 mg/kg.

TNP-470 suppresses tumor vascularization Tissue sam-

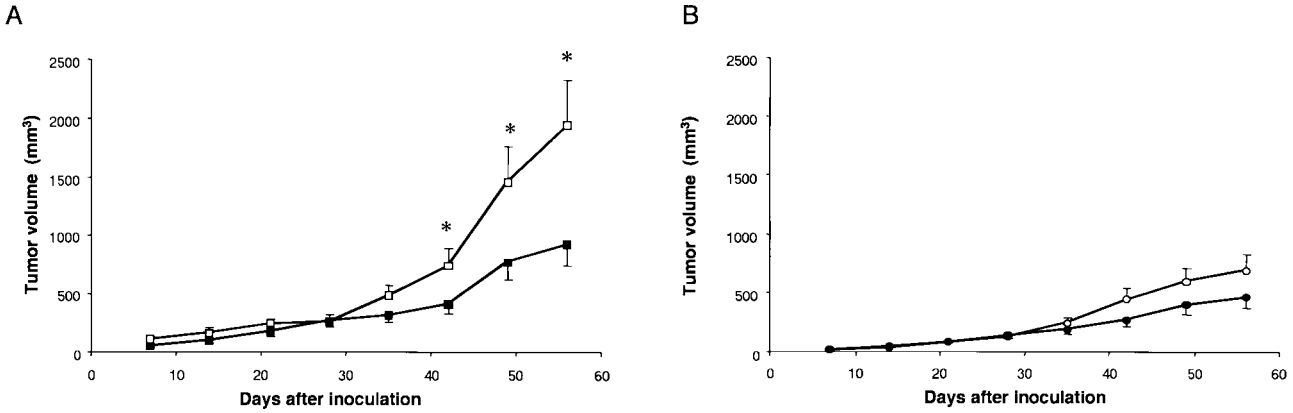

Fig. 1. Inhibitory effects of TNP-470 on the growth of H226Br (A) and H226B (B) tumors. H226B or H226Br cells were inoculated on the right side of the back of mice. TNP-470 was administered subcutaneously at 30 mg/kg every other day. Control mice received injections of the vehicle only. Data are mean±SD. A: * $P<0.05$, □ H226Br Control, ■ H226Br TNP-470 30 mg/kg. B: NS, ○ H226B Control, ● H226B TNP-470 30 mg/kg.

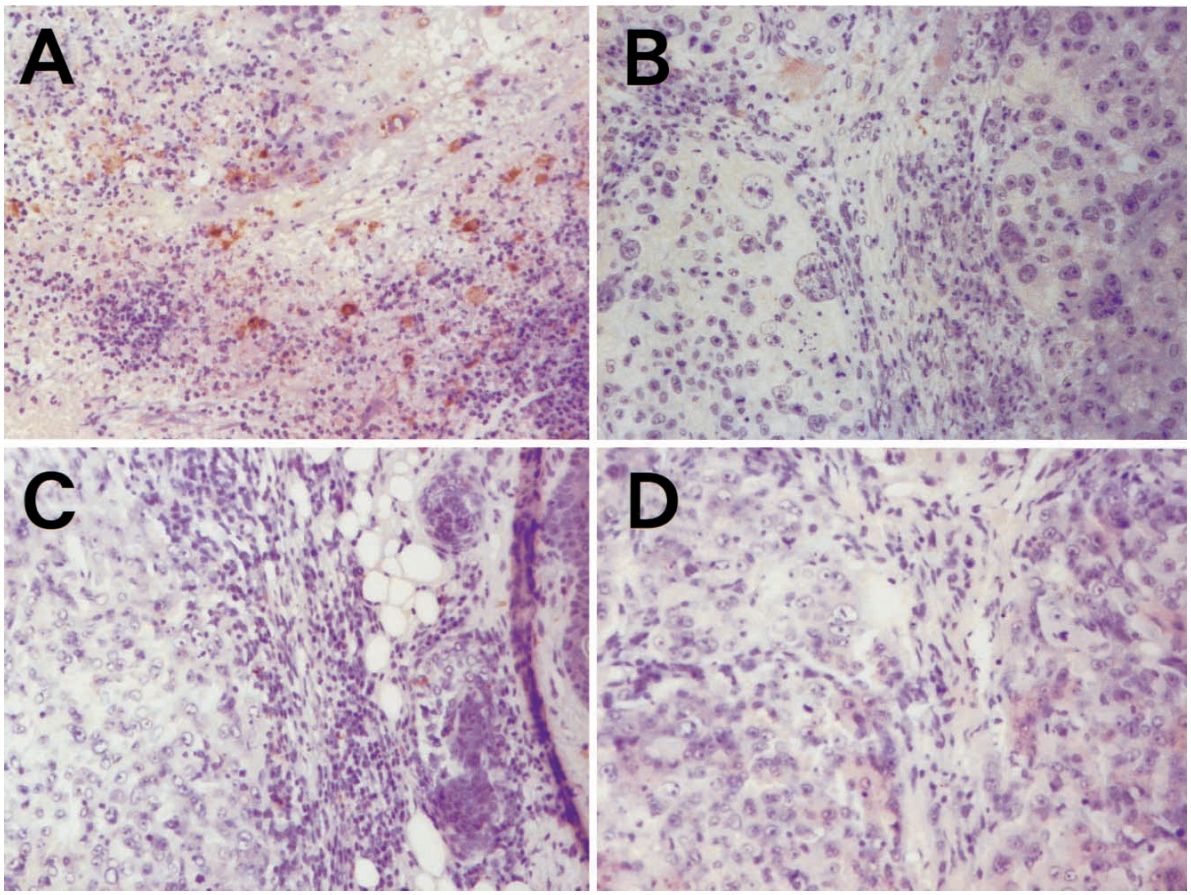

Fig. 2. Immunohistochemical study of tumor vascularization in H226B and H226Br. Tissue samples were harvested on day 56 for immunohistochemical examination. To quantitate the extent of vascularization in tumors from mice treated with or without TNP-470 at 30 mg/kg, we used DAKO EPOS anti-von Willebrand factor monoclonal antibody. A and C represent H226Br and H226B tumors from untreated mice, respectively. B and D represent H226Br and H226B tumors from mice treated with 30 mg/kg of TNP-470. Original magnification, ×66.

ples were harvested on day 56 and immunostained with anti-von Willebrand factor/HRP monoclonal antibody. Marked vascularization was noted in control H226Br tumors (Fig. 2A). Vascularization was suppressed in mice treated with 30 mg/kg of TNP-470 (Fig. 2B). H226B tumors showed less extensive vascularization (Fig. 2C) and the effect of the same dose of TNP-470 was less marked (Fig. 2D).

TNP-470 inhibits cell growth *in vitro* TNP-470 significantly suppressed the growth of H226Br cells *in vitro*

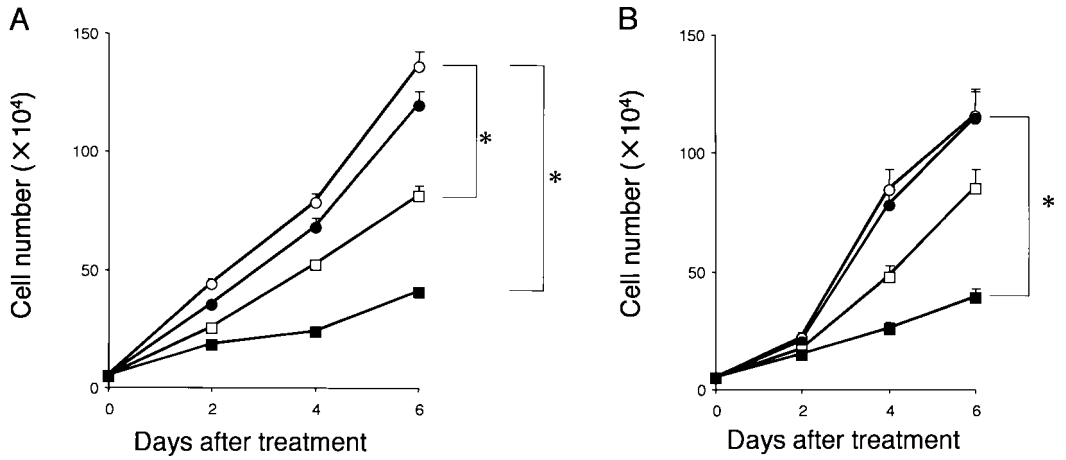

Fig. 3. Effect of TNP-470 on the cell growth of H226Br (A) and H226B (B) cell line *in vitro*. H226Br and H226B cells were seeded onto 6-well plates at 5×10^4 cells/well. TNP-470 diluted with the culture medium to a concentration of 0, 0.1, 1, or 10 μg/ml was added to each well on day 0. Viable cells were counted on day 2, 4, or 6 by the dye-uptake method. Data are mean±SD. $P<0.05$, ○ Control, ● TNP-470 0.1 μg/ml, □ TNP-470 1 μg/ml, ■ TNP-470 10 μg/ml.

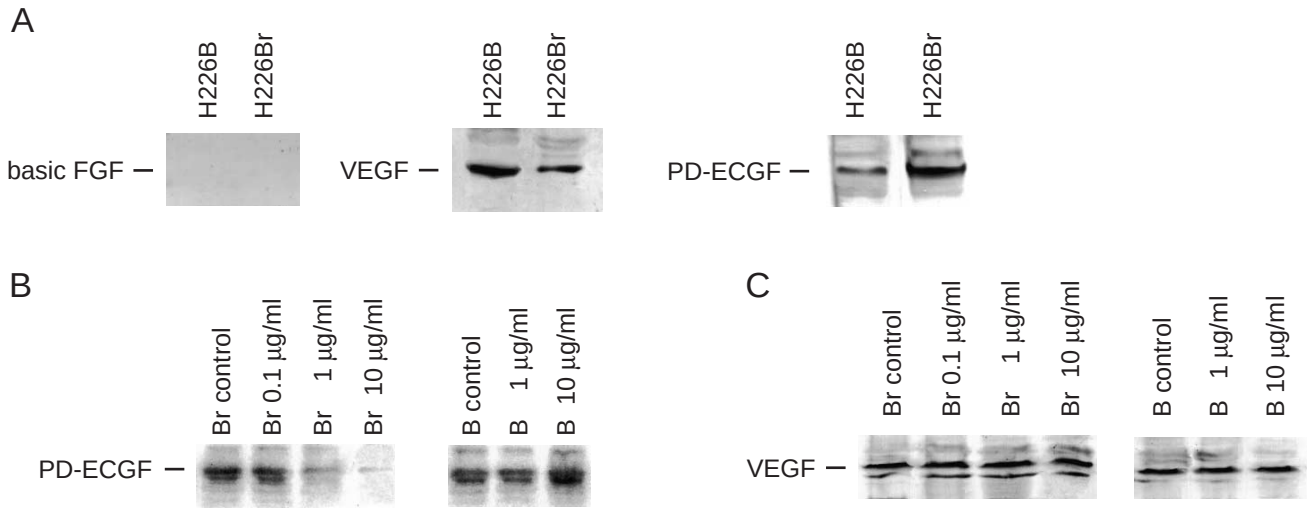

Fig. 4. Expression of angiogenic factors (A) and effect of TNP-470 on PD-ECGF expression (B) and on VEGF expression (C). Expressions of bFGF, VEGF and PD-ECGF in H226B and H226Br were evaluated by immunoblotting using anti-human bFGF antibody, anti-human VEGF antibody and anti-human PD-ECGF antibody (A). To evaluate the effect of TNP-470 on PD-ECGF expression, H226Br and H226B cells were treated with TNP-470 at the indicated concentrations. H226Br cells treated with TNP-470 at a concentration of 0, 0.1, 1, or 10 μg/ml for 6 days and H226B cells treated by TNP-470 at a concentration of 0, 1, or 10 μg/ml for 6 days were harvested and directly resolved in SDS sample buffer. Total proteins obtained from equivalent numbers of viable cells were separated on 12% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated with anti-human PD-ECGF antibody (B) or anti-human VEGF antibody (C). The same amounts of the same samples were loaded and different antibodies were used as the probe in experiments (B) and (C).

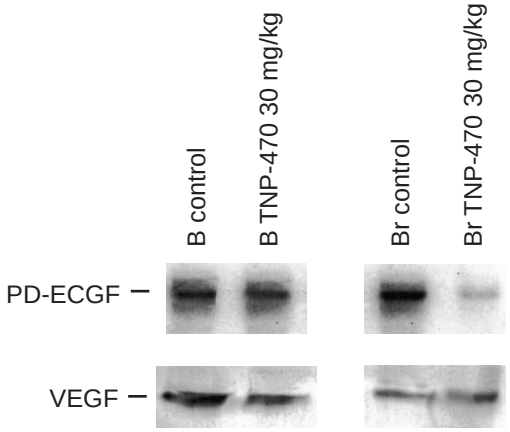

Fig. 5. Immunoblot analysis of angiogenic factors in H226B and H226Br tumors. Tissue samples from nude mice treated with or without 30 mg/kg of TNP-470 were harvested on day 56, and lysates were obtained by homogenization. Equivalent amounts of total protein were loaded on 12% SDS polyacrylamide gel and transferred to a membrane. The same amounts of the same samples were loaded and the membrane was probed with anti-human PD-ECGF antibody or anti-human VEGF antibody to evaluate the expression of angiogenic factors.

when used at a concentration of 1 μg/ml ($P<0.05$) or 10 μg/ml ($P<0.05$) (Fig. 3A). H226B cell growth was also suppressed by treatment with TNP-470 (Fig. 3B).

TNP-470 suppresses PD-ECGF expression on H226Br We also examined the expression of angiogenic factors on H226B and H226Br cells *in vitro*. Immunoblot analysis using anti-human PD-ECGF antibody showed a stronger signal of PD-ECGF in H226Br cells than in H226B cells (Fig. 4A). On the other hand, the expression of bFGF was not evident in H226B or H226Br cells, while the expression of VEGF was similar in the two cell lines. The addition of TNP-470 reduced the expression of PD-ECGF/dThdPase in H226Br dose-dependently, but had no effect on the expression in H226B cells (Fig. 4B). TNP-470 did not influence the expression of VEGF in H226B or H226Br cells (Fig. 4C).

TNP-470 suppresses PD-ECGF expression in H226Br tumors We performed immunoblot analysis to examine the *in vivo* expression of PD-ECGF and VEGF in H226B and H226Br. Immunoblotting using anti-human PD-ECGF antibody showed PD-ECGF expression in both H226Br and H226B tumors. Treatment with TNP-470 reduced the expression of PD-ECGF/dThdPase in H226Br, but not in H226B. On the other hand, the expression of VEGF was similar in both H226B and H226Br tumors and TNP-470 did not influence the expression of VEGF in either case (Fig. 5).

TNP-470 suppresses the activity of dThdPase Finally,

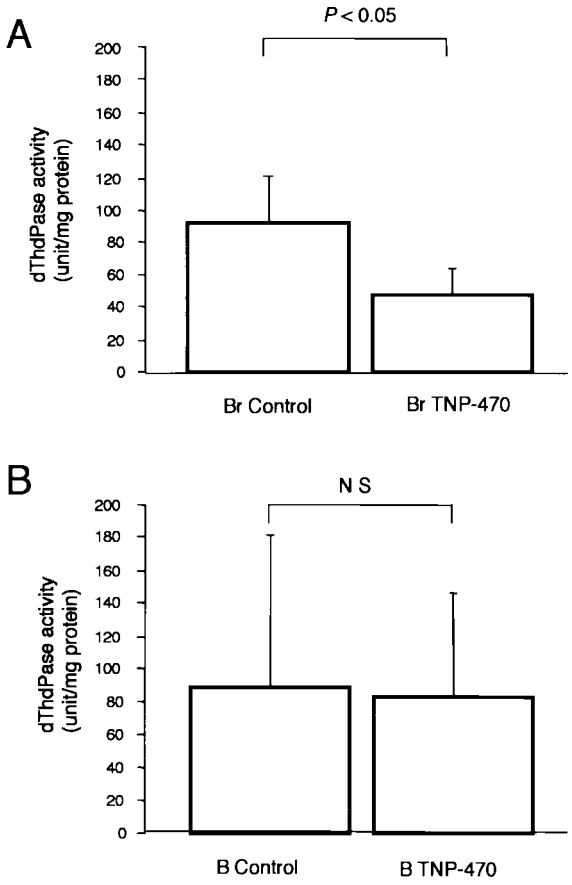

Fig. 6. Inhibitory effect of TNP-470 on dThdPase activity. Tissue samples were harvested on day 56 from untreated mice or from those treated with 30 mg/kg of TNP-470 every other day, and the activity of dThdPase was measured in terms of the generation of 5-fluorouracil from 5′-deoxy-5-fluorouridine as described in the text. Data are mean±SD. A, H226Br cell line; B, H226B cell line; NS, not significant.

we investigated the activity of dThdPase in H226B and H226Br tumors in mice treated with 30 mg/kg of TNP-470 for 56 days. The activity of dThdPase in H226Br tumor was 92 units/mg protein in the control group, but significantly decreased to 47 units/mg protein during treatment with TNP-470 ($P<0.05$) (Fig. 6A). On the other hand, the activity of dThdPase in H226B was not suppressed by TNP-470 treatment (Fig. 6B).

DISCUSSION

Angiogenesis plays an important role in the growth of solid tumors,[1, 25] and exerts various effects on tumor progression, invasion, and metastasis. Several angiogenic factors have been reported, and the inhibition of neoangio-

genesis in tumors has already been proposed as a therapeutic strategy against solid cancers.[1, 2]

The angiogenesis inhibitor TNP-470 is an analog of fumagillin derived from *A. fumigatus*. It mainly acts by strongly suppressing the proliferation of vascular endothelial cells. Previous studies have shown that the latter effect of TNP-470 on vascular endothelial cells is due to inhibition of the cell cycle. TNP-470 also inhibits cancer cell growth through a direct effect on tumor cells.[4] Although the mechanism of this effect has not yet been established, inhibition of myristoylation of nitric oxide synthase is one possibility.[26] It is reported that TNP-470 inhibits the growth of gastric,[9] breast, prostate,[27] and colorectal[21] cancers, both *in vitro* and *in vivo*. TNP-470 also inhibits liver metastatic cancers[7, 9, 21, 28] and the combined use of TNP-470 and an anticancer agent is effective in inhibiting cancer metastasis.[29]

In this study, we investigated the effect of TNP-470 on human lung cancer cell lines H226B and H226Br both *in vivo* and *in vitro*. Our results showed that inoculation of tumor cells resulted in a rapid growth of H226Br tumors compared to H226B. Nude mice inoculated with these cells were injected subcutaneously with 30 mg/kg of TNP-470. TNP-470 suppressed tumor growth of both H226B and H226Br when used at a concentration of 100 mg/kg. However, at this dose, TNP-470 caused a marked reduction in body weight, presumably due to toxicity. On the other hand, TNP-470 suppressed the growth of H226Br tumor when used at a concentration of 30 mg/kg without producing a marked effect on body weight.

We also investigated the anti-angiogenic effects of TNP-470 in these tumors by immunohistochemical staining using DAKO EPOS anti-von Willebrand factor antibody. A marked degree of neovascularization was noted in H226Br tumors, although H226B tumors showed a lower degree of angiogenesis. TNP-470 suppressed tumor vascularization at a dosage that was not associated with toxicity. To investigate the mechanism of the anti-angiogenic effect of TNP-470, we quantitated the expression of angiogenic factors on H226B and H226Br cells. In our *in vitro* experiments, we demonstrated a strong expression of PD-ECGF on H226Br cells compared to H226B cells. In contrast, the expression levels of VEGF on these cell lines were similar.

PD-ECGF is identical to dThdPase,[10] and PD-ECGF/dThdPase exhibits angiogenic activity.[11, 12] Angiogenesis is an important factor in cancer progression.[18] Previous studies have shown that PD-ECGF/dThdPase is expressed in gastric, lung, breast, and bladder cancers.[13–16, 30] PD-ECGF/dThdPase is also expressed in normal tissues, macrophages, stromal cells, glial cells, and some epithelia.[17] In cancer tissues, its expression plays an important role in tumor growth and metastasis, and influences prognosis.[1, 15, 18, 25]

In our *in vitro* experiments, TNP-470 inhibited cell growth of H226Br and H226B at concentrations of 1 and 10 μg/ml. H226Br cells expressed PD-ECGF/dThdPase and this expression was suppressed by TNP-470 at concentrations of 0.1, 1 and 10 μg/ml, yet the expression of PD-ECGF in H226B cells was not suppressed by TNP-470. These results indicate that TNP-470 suppresses the proliferation of H226Br cells and also suppresses the expression of PD-ECGF at lower concentrations.

To estimate the *in vivo* expression of angiogenic factors, we carried out immunoblot analysis of PD-ECGF and VEGF. The expression of PD-ECGF was weak in H226Br tumors treated with TNP-470 compared to tumors of untreated mice. The expression of PD-ECGF was also found in H226B tumors, but TNP-470 treatment did not influence the expression. To investigate further the mechanism of the anti-angiogenic effect of TNP-470 *in vivo*, we measured the activity of PD-ECGF/dThdPase in H226Br tumors. The activity of this enzyme in H226Br tumor was suppressed by TNP-470 at a concentration of 30 mg/kg. *In vivo* expression levels of PD-ECGF in H226B and H226Br were similar according to the results of activity and immunoblot analysis, although differences in PD-ECGF expression were noted between these cell lines in *in vitro* experiments. One possible explanation of this result is that *in vivo* samples taken from tumors contained stromal tissue. Infiltrating cells and stromal cells could be sources of PD-ECGF,[17] and this may account for the apparently similar levels of PD-ECGF expression in H226B and H226Br tumors on day 56. During tumor development the production of angiogenic factor by cancer cells might be most important for neoangiogenesis, and the downregulation of PD-ECGF in H226Br by TNP-470 resulted in the difference in tumor growth and angiogenesis. A significant difference in the size of H226Br tumors was observed between TNP-470-treated and untreated groups. This corresponded well with the result of the *in vitro* experiment showing that TNP-470 only suppresses the expression of PD-ECGF in H226Br, but not in H226B. The expression of VEGF was not altered by TNP-470 either *in vivo* or *in vitro*. These results suggest that downregulation of PD-ECGF is a possible underlying mechanism of the anti-angiogenic effect of TNP-470 *in vivo*.

Another question raised by the results of these experiments is why TNP-470 suppresses the expression of PD-ECGF in H226Br, but not in H226B. H226Br was established from a brain metastatic lesion in nude mice bearing H226B tumor. Because H226Br cells express mutant-type p53 while H226B cells express wild-type p53, we expected that a difference in VEGF expression between these cell lines caused the difference in tumor growth. Kieser *et al*. demonstrated that mutant p53 upregulated the expression of VEGF[31] and transfection of wild-type p53 gene downregulated VEGF expression.[32] Furthermore,

downregulation of VEGF by TNP-470 was observed *in vitro* in a pancreatic cancer cell line.[33] Although H226B and H226Br have different p53 status, the expression of VEGF was similar in the two cell lines, both *in vitro* and *in vivo*, and TNP-470 did not alter the expression of this angiogenic factor. Therefore, we examined the expression of other angiogenic factors *in vitro*. Surprisingly, H226Br cells expressed more PD-ECGF than H226B cells, and TNP-470 only downregulated the expression of PD-ECGF in H226Br cells. This result was consistent with a report that the frequency of dThdPase expression was significantly lower in p53-negative (wild-type) colorectal carcinomas than in positive (mutant-type) carcinomas.[34] The immunoblot experiment using the same samples and a different probe convincingly demonstrated that TNP-470 suppressed the expression of PD-ECGF only in H226Br but not in H226B both *in vitro* and *in vivo*.

TNP-470 clearly has other actions against tumor growth. Cell cycle arrest of endothelial cell is one of its reported effects. The importance of our study is the finding of a differential effect of TNP-470 on the growth of these two cell lines. The molecular mechanism of this difference is not known yet. A recent study revealed that methionine aminopeptidase 2 was one of the targets of TNP-470.[35] The inhibition of myristoylation of nitric oxide synthase was also suggested as a possible mechanism of the anti-angiogenic effect of TNP-470.[26] These targets could be related to the different actions of TNP-470 against the two cell lines. To explain this cell line-specific alteration of PD-ECGF expression by TNP-470 we have to examine the molecular mechanism of the expression of PD-ECGF at the transcriptional and translational levels in H226Br under the conditions of TNP-470 treatment.

In conclusion, we have demonstrated in this study that TNP-470 inhibited tumor cell proliferation and tumor growth of human lung cancer cells by inhibiting the formation of new blood vessels within growing tumors. This anti-angiogenic factor also suppressed the expression of PD-ECGF/dThdPase enzyme. Our results indicated that this suppression plays an important role in the anti-tumor and anti-angiogenic effects of TNP-470 *in vivo*.

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