

SK-216, an Inhibitor of Plasminogen Activator Inhibitor-1, Limits Tumor Progression and Angiogenesis

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Abstract

Plasminogen activator inhibitor-1 (PAI-1), which can be produced by host and tumor cells in the tumor microenvironment, is intimately involved in tumor progression. In the present study, to pursue the possibility that PAI-1 could be a therapeutic target in the management of malignancy, SK-216, a specific PAI-1 inhibitor, was orally administered to wild-type mice that were subcutaneously implanted or intravenously injected with either PAI-1-secreting Lewis lung carcinoma (LLC) or PAI-1-nonsecreting B16 melanoma cells. The systemic administration of SK-216 was found to reduce the size of subcutaneous tumors and the extent of metastases, regardless of PAI-1 secretion levels from the tumor cells. SK-216 also reduced the extent of angiogenesis in the tumors and inhibited VEGF-induced migration and tube formation by human umbilical vein endothelial cells *in vitro*. Then, to determine whether host or tumor PAI-1 was more crucial in tumor progression and angiogenesis, PAI-1-deficient or wild-type mice were subcutaneously implanted or intravenously injected with LLC or PAI-1 knockdown LLC cells. Tumor progression was shown to be controlled by the presence of host PAI-1 and not affected by the PAI-1 levels in the tumors. Similarly, host PAI-1 played a more crucial role in tumor angiogenesis than did tumor PAI-1. These observations suggest that regardless of the PAI-1 levels in the tumor, the systemic administration of SK-216 exerts an antitumor effect through its interaction with host PAI-1. This antitumor effect might be mediated by the antiangiogenic properties of SK-216. *Mol Cancer Ther*; 12(11); 2378–88. ©2013 AACR.

Introduction

The plasminogen activation system, represented by urokinase-type plasminogen activator (uPA), the cellular receptor for uPA (uPAR), and its specific inhibitor, the plasminogen activator inhibitor-1 (PAI-1), plays a crucial role in tumor growth, invasion, metastasis, and angiogenesis. The interaction between uPA and uPAR is believed to be a particularly efficient proteolytic system for endothelial and tumor cells to breakdown the extracellular matrix (ECM) during migration (1). In addition, through binding to uPA, uPAR transduces signals that promote cell migration and proliferation (2). Judging from these observations, PAI-1, a primary inhibitor of uPA, has long been considered a cancer inhibitor (3). However, recent evidence now shows an association between high expression of PAI-1 and poor prognosis in various types of tumors (4–

10). In addition, a large number of animal and/or *in vitro* studies have revealed the involvement of PAI-1 in tumor growth and metastasis through several possible mechanisms. Experiments using PAI-1-deficient (PAI-1^{-/-}) mice have shown the significance of host PAI-1 in regulating tumor angiogenesis (11–14). This process is thought to be mediated by the actions of PAI-1 on endothelial cells, thereby regulating plasmin-mediated proteolysis (15, 16), modulating migration (17, 18), and/or preventing apoptosis (19). PAI-1 is also known to be associated with cell motility. Binding of PAI-1 to the ECM protein vitronectin blocks the interaction between the integrins and the uPAR–uPA complex with vitronectin, thereby inhibiting adhesion and accelerating migration of cells (17, 18). Furthermore, recent studies have revealed that PAI-1 has a direct effect on pro-proliferative (20) and antiapoptotic signaling (21) in tumor cells. These observations clearly suggest an important role of PAI-1 in tumor progression.

In the tumor microenvironment, PAI-1 can be produced by host and tumor cells. There may be interactions between host and tumor PAI-1 and they likely differ in their relevance to tumor progression. However, whether host or tumor PAI-1 is more crucial to tumor progression is unknown. To date, deficiency of host PAI-1 has been clearly shown to reduce tumor progression through inhibiting tumor angiogenesis (11–14). In addition, recent studies have reported the inhibitory effects of reduced tumor PAI-1 levels on tumor progression (22, 23).

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To pursue the possibility that PAI-1 could be a therapeutic target in the management of malignancy, we first examined the effect of systemic administration of SK-216, a specific inhibitor for PAI-1, on tumor progression and angiogenesis. In this experiment, PAI-1-secreting Lewis lung carcinoma (LLC) cells and PAI-1-nonsecreting B16 melanoma cells were used to establish a subcutaneous tumor model and a tail vein metastasis model. Then, we determined whether host or tumor PAI-1 was more important in tumor progression and angiogenesis. Toward that end, we stably transfected LLC cells with short hairpin RNA (shRNA) to generate siRNA targeting PAI-1 (PAI-1-siRNA) or nonspecific scrambled siRNA (NS-siRNA), thereby yielding PAI-1 knockdown LLC (siPAI-1 LLC) cells or control LLC (siControl LLC) cells. After siPAI-1 LLC cells or siControl LLC cells were transplanted into PAI-1^{-/-} mice or wild-type (WT) mice, the degrees of tumor progression and angiogenesis were analyzed.

Materials and Methods

Cells and cell culture

LLC, B16 melanoma, and human embryonic kidney 293 cells were purchased from and authenticated by American Type Culture Collection. These cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin. Human umbilical vein endothelial cells (HUVEC) authenticated by Lifeline Cell Technology were purchased from Kurabo and cultured following the manufacturer's protocol. All cells were incubated at 37°C in a 5% CO₂ incubator and used within 6 months after resuscitation.

Reagents and animals

Matrigel was purchased from BD Biosciences. VEGF was obtained from Kurabo. SK-216 (Supplementary Fig. S1) was chemically synthesized and supplied by Shizuoka Coffein Co., Ltd.. Inhibitory activity of SK-216 on PAI-1 was investigated using previously published methods (24) and the IC₅₀ was determined to be 44 μmol/L as reported in international patent WO04/010996. Breeding pairs of the homozygous PAI-1^{-/-} mouse strain on a C57BL/6 background were purchased from The Jackson Laboratory. Age- and sex-matched WT C57BL/6 mice were purchased from the Charles River Laboratories. Animals were maintained according to guidelines for the ethical use of animals in research at Hiroshima University (Hiroshima, Japan).

Preparation of LLC cells stably expressing PAI-1-siRNA or NS-siRNA

To stably express siRNA in LLC cells, we used an shRNA expression vector containing a neomycin-resistant gene: pSINsi-mU6 (TaKaRa). Synthetic oligonucleotides to express shRNA were annealed and ligated into the linearized pSINsi-mU6 vector. The sequences of the oligonucleotides for shRNA to generate PAI-1-siRNA and

NS-siRNA were as follows: 5'-GATCCGCCAACAA-GAGCCAATCACATAGTGCTCCTGGTTGTGTGATTG-GCTCTTGTGGCTTTTTTAT-3' and 5'-GATCCGCT-TAATCGCGTATAAAGGCTAGTGCTCCTGGTTGGCC-TTATACGCGATTAAGACTTTTTTAT-3', respectively. These pSINsi-mU6 cassette vectors were transfected into 293 cells by the use of Retrovirus Constructive System Eco (TaKaRa), and the recombinant retroviral vectors containing the expression cassettes of PAI-1-siRNA and NS-siRNA were collected. These retroviral vectors were infected into LLC cells and followed by selection with G418 (Promega), LLC cells stably expressing PAI-1-siRNA or NS-siRNA (siPAI-1 or siControl LLC cells, respectively) were prepared.

Quantitative real-time PCR

Total RNA was isolated with RNeasy Mini Kits (Qiagen). The isolated total RNA was reverse transcribed into cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems) following the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was conducted on an ABI Prism 7700 (Applied Biosystems) for mouse PAI-1 using β-actin as a control housekeeping gene.

Quantification of PAI-1 protein

Total PAI-1 secreted into culture medium for 24 hours was measured using an ELISA kit (Innovative Research) following the manufacturer's instructions. The minimum detection limit of this ELISA kit was 0.02 ng/mL.

Immunohistochemical staining of PAI-1

Immunohistochemical analysis of PAI-1 was conducted as described in the Supplementary Materials and Methods.

Subcutaneous tumor model

The indicated cells (1×10^6) were subcutaneously inoculated in the left flank of mice. For SK-216 experiments, the mice were given drinking water containing or lacking SK-216 (100 or 500 ppm). Until 14 days after the inoculation, the length and width of the tumors were measured using a caliper twice a week and tumor volume was calculated using the formula: width² × length × 0.5 (25).

Tail vein metastasis model

The indicated cells (3×10^5) were injected into mice through the tail vein. For SK-216 experiments, the mice were given drinking water containing or lacking SK-216 (100 or 500 ppm). Mice were euthanized 21 days after the cell injection and the number of grossly identified tumor nodules on the surfaces of the lungs was manually counted.

Evaluation of microvessel density in subcutaneous tumors

Tumor sections were incubated with a rabbit polyclonal antibody against mouse CD31 (Abcam) followed by 30-minute reaction with a biotinylated goat anti-rabbit immunoglobulin G (IgG) antibody (Vector Laboratories).

The immunoreaction was amplified with a Vectastain ABC kit (Vector Laboratories) and visualized by incubation with a 3,3'-diaminobenzidine solution acting as a chromogen. The sections were then counterstained with hematoxylin and dehydrated. Images were captured using a microscope at a magnification of $\times 200$ (model BZ-9000; Keyence) and the area of CD31-positive vessel-like structures was measured in five random microscopic fields per section using Dynamic cell count software BZ-HIC (Keyence).

Proliferation assay

HUVECs were suspended in medium ($1 \times 10^4/100 \mu\text{L}$) containing 10 ng/mL VEGF plus SK-216 at various concentrations. The cells were seeded into a 96-well tray and incubated. To determine cells' proliferation rates after 16 and 36 hours, the absorbance of the medium in each well was assessed using cell counting kit-8 (DOJINDO) following the manufacturer's instructions.

Cell migration assay

HUVEC migration was assessed using an Oris Universal Cell Migration Assembly kit (Platypus Technologies) following the manufacturer's instructions. Briefly, HUVECs (2×10^4) suspended in 100 μL of medium were seeded into each test well of the Oris plate with the well inserts (stoppers) and then incubated to allow cell attachment. After 4 hours, the stoppers in each well were removed. HUVECs were incubated with 10 ng/mL VEGF and SK-216 at various concentrations for 36 hours, and then were stained with calcein acetoxymethyl ester (Calcein AM) stock solution (2 mmol/L; DOJINDO). Images were captured at a magnification of $\times 40$ using a fluorescence microscope (model BZ-9000; Keyence) and the areas occupied by HUVECs (occupied area) and not occupied by HUVECs (background area) were measured using Dynamic cell count software BZ-HIC (Keyence). The percentage of occupied area was determined by the following formula: $100 \times (\text{background area at baseline} - \text{background area at 36 hours})/\text{background area at baseline}$.

Capillary-like tube formation assay

Seventy microliters of Matrigel was applied to each well of a 96-well plate and incubated for 30 minutes. HUVECs (1×10^4) suspended in 100 μL of medium were plated onto the Matrigel and incubated with 10 ng/mL of VEGF and SK-216 at various concentrations for 16 hours and then were stained with Calcein AM stock solution (2 mmol/L). Images were captured at a magnification of $\times 20$ using a fluorescence microscope (model BZ-9000) and the total area of the tube-like space was quantified using Dynamic cell count software BZ-HIC (Keyence).

Statistical analysis

Statistical analyses were undertaken using SPSS 17 (SPSS Japan). All the results are expressed as mean \pm SEM, and the Student *t* test or Mann-Whitney *U* test were used to evaluate statistical differences between the

groups. A *P* value more than 0.05 was considered to be statistically significant.

Results

Oral administration of SK-216, a PAI-1-specific inhibitor, reduced tumor progression in both the subcutaneous tumor model and the tail vein metastasis model

To determine whether PAI-1 could be a therapeutic target in the treatment of malignancy, a subcutaneous tumor model and a tail vein metastasis model were generated in C57BL/6 mice using C57BL/6-derived cell lines, LLC, and B16 melanoma cells. SK-216 was orally administered to the mice. Interestingly, B16 cells were found to secrete almost no PAI-1 in contrast with LLC cells (Fig. 1A). In consistence with the PAI-1 secretion levels *in vitro*, immunohistochemical staining of PAI-1 for subcutaneous tumors in PAI-1^{-/-} mice confirmed that PAI-1 was detectable in the tumor of LLC cells but not in that of B16 cells (Supplementary Fig. S2). The volumes of subcutaneous tumors were evaluated 14 days after the inoculation of LLC or B16 cells and the number of tumor nodules on lung surfaces was counted 21 days after injection. As shown in Fig. 1B and D, the volumes of subcutaneous tumors 14 days after the inoculation of LLC and B16 cells were significantly smaller in the SK-216-treated group than in the control group. In addition, the number of lung tumor nodules 21 days after the injection of LLC or B16 cells was significantly lower in the SK-216-treated group than in the control group (Fig. 1C and E). Interestingly, the effects of SK-216 on subcutaneous tumor growth in the subcutaneous tumor model showed a trend toward dose-dependency (Fig. 1B).

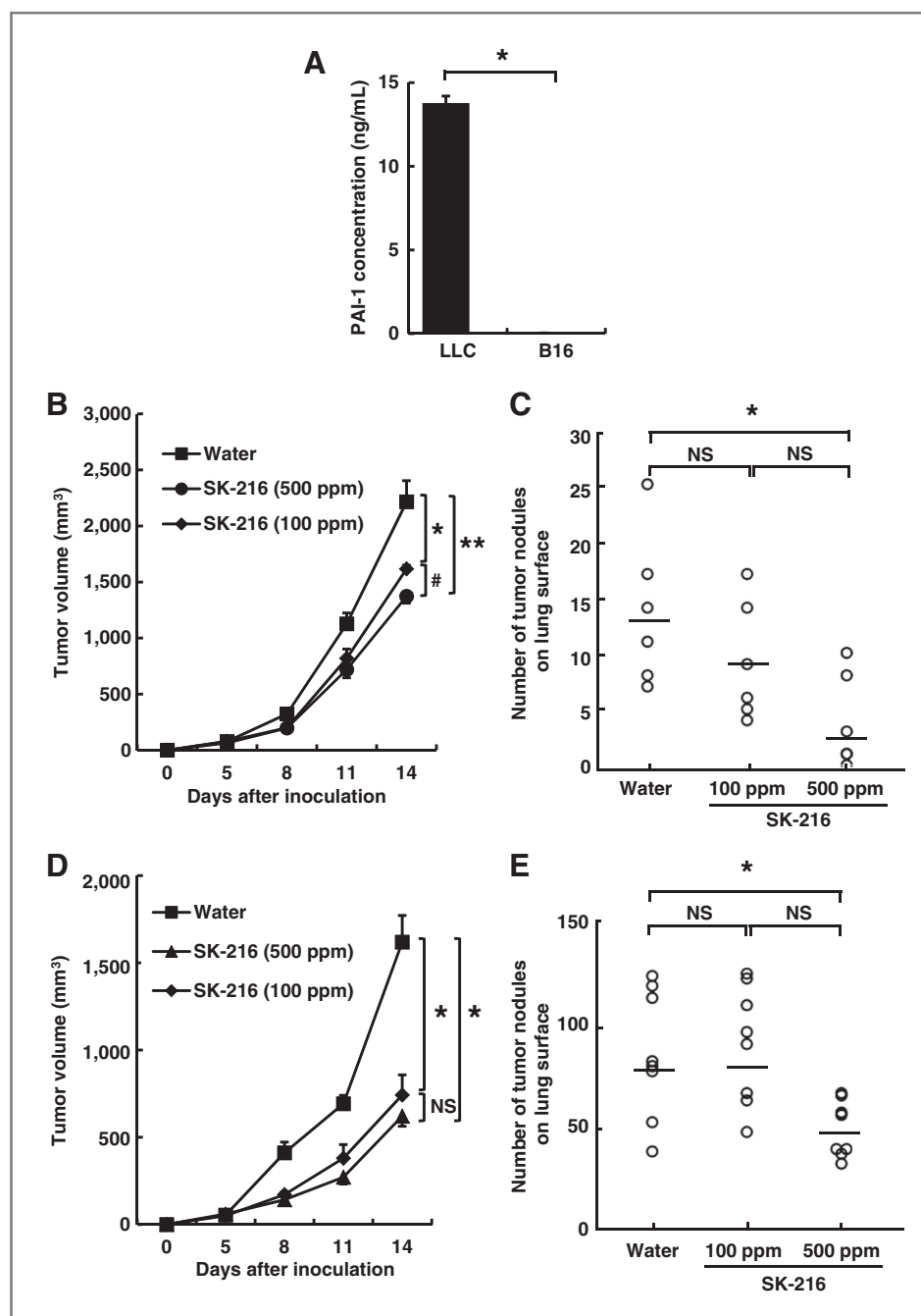
SK-216 reduced the degree of angiogenesis in subcutaneous tumor

To determine whether SK-216 affected the degree of angiogenesis in subcutaneous tumor, we undertook immunohistochemical staining of the excised subcutaneous tumors of LLC and B16 cells with anti-CD31 monoclonal antibody (mAb). In both subcutaneous tumors of LLC and B16 cells in WT mice, the areas of CD31-positive vessels were significantly lower in the SK-216-treated group than in the control group (Fig. 2A and C).

Host but not tumor PAI-1 was crucial for tumor progression in the subcutaneous tumor model and the tail vein metastasis model

The systemic administration of SK-216 effectively reduced the size of subcutaneous tumors and the extent of lung metastases regardless of the presence or absence of PAI-1 secretion by the tumor cells. To further investigate the significance of tumor PAI-1 in tumor progression, we established two LLC-derived cell lines that differed in expression levels of PAI-1, namely siControl LLC and siPAI-1 LLC cells. As shown in Fig. 3A, qRT-PCR revealed that the expression level of PAI-1 mRNA was significantly decreased in siPAI-1 LLC cells compared with siControl

Figure 1. Effects of systemic administration of SK-216 on tumor progression in the subcutaneous tumor model and the tail vein metastasis model. **A**, comparison of PAI-1 secretion levels between LLC and B16 cells. LLC or B16 cells (1×10^4) were seeded in 96-well plates and cultured for 24 hours. Concentrations of PAI-1 in culture media were measured by ELISA. Data represent the mean values (\pm SEM) of triplicate samples and were analyzed with the Student *t* test. *, $P < 0.01$ versus LLC cells. **B** and **D**, evaluation of tumor sizes in the subcutaneous tumor model using PAI-1-secreting LLC cells and PAI-1-nonsecreting B16 cells. Volumes of subcutaneous tumors were measured twice a week for 2 weeks after the inoculation of LLC (B) or B16 (D) cells into WT mice. Mice were given drinking water or SK-216 (100 or 500 ppm). The data represent the mean values (\pm SEM) of 6 mice per group and were analyzed with the Student *t* test. *, $P < 0.01$; **, $P < 0.05$ versus control group. #, $P < 0.05$ versus the group treated with 100 ppm of SK-216. NS, not significant. **C** and **E**, evaluation of lung metastases in the tail vein metastasis model using PAI-1-secreting LLC cells or PAI-1-nonsecreting B16 cells. The number of tumor nodules on the lung surface of WT mice was counted 21 days after injection of LLC (C) or B16 (E) cells through the tail vein. Mice were given drinking water or SK-216 (100 or 500 ppm). Each bar represents the mean value of 6 or 8 mice per group. The data were analyzed with the Student *t* test. *, $P < 0.05$ versus control group. NS, not significant.



LLC cells. Similarly, PAI-1 protein levels in the culture media were approximately one third decreased in siPAI-1 LLC cells compared with siControl LLC cells (Fig. 3B). *In vitro*, no differences in proliferation or migration between siControl LLC and siPAI-1 LLC cells were shown (data not shown).

Next, to determine the relationship between host and tumor PAI-1 in tumor growth, siControl LLC or siPAI-1 LLC cells were subcutaneously inoculated or injected through the tail vein into WT or PAI-1^{-/-} mice. As shown in Fig. 3C, the volumes of subcutaneous tumors 14 days

after the inoculation of siControl LLC or siPAI-1 LLC cells were significantly smaller in PAI-1^{-/-} mice than in WT mice. In WT mice, there were no significant differences in the volumes of subcutaneous tumors when inoculated with siControl LLC or siPAI-1 LLC cells. The same outcomes were observed in PAI-1^{-/-} mice. Downregulated expression of PAI-1 was confirmed in subcutaneous tumors initiated by siPAI-1 LLC cells compared with that of siControl LLC cells as determined by immunohistochemistry (Supplementary Fig. S3). Similar to the results in the subcutaneous tumor model, in the tail vein metastasis

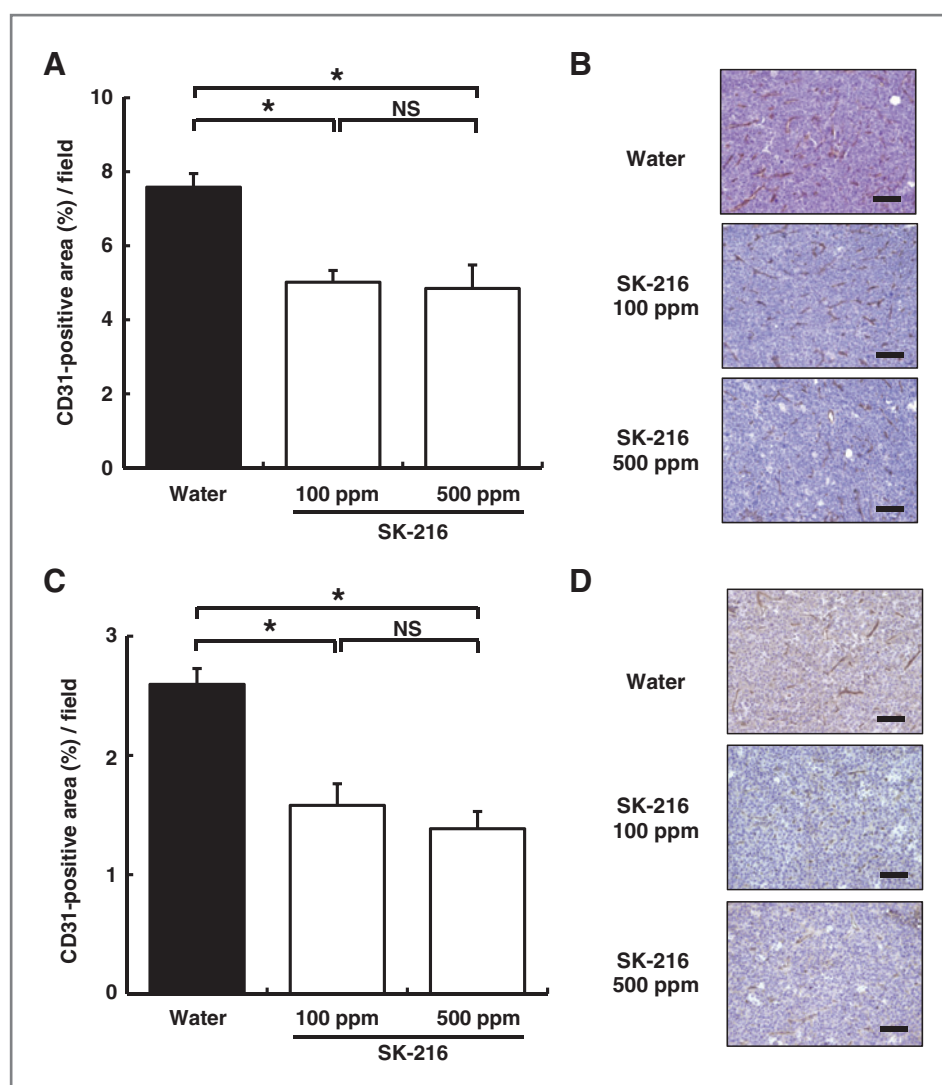


Figure 2. Evaluation of angiogenesis in subcutaneous tumors of WT mice subcutaneously inoculated with LLC (A) or B16 (C) cells. Mice were given either water or SK-216 (100 or 500 ppm). The area of CD31-positive vessels was calculated as described in Materials and Methods. Data represent the mean values (\pm SEM) of 6 mice in each group and were analyzed with the Student *t* test. *, $P < 0.01$ versus control group. NS, not significant. B and D, representative immunohistochemical staining of CD31 in subcutaneous tumors. Scale bar, 100 μ m.

model, the number of tumor nodules on the lung surface 21 days after the injection of cells was significantly lower in PAI-1^{-/-} mice than in WT mice (Fig. 3D). In both WT and PAI-1^{-/-} mice, there were no significant differences in the numbers of lung nodules between siControl LLC and siPAI-1 LLC cells. These results strongly suggest that host PAI-1 but not tumor PAI-1 is the determinant for the degree of tumor progression in both the subcutaneous tumor model and the tail vein metastasis model. To substantiate the significance of host PAI-1 in tumor progression, similar models were generated in WT and PAI-1^{-/-} mice using PAI-1–nonsecreting B16 cells. As shown in Fig. 3E and F, the volumes of subcutaneous tumors and the number of lung surface nodules were significantly smaller in PAI-1^{-/-} mice than in WT mice.

Deficiency of host PAI-1 reduced the degree of tumor angiogenesis

Previous studies clearly showed the important role of host PAI-1 in tumor angiogenesis (11–14). To confirm

the significance of host PAI-1 in tumor angiogenesis, the extent of angiogenesis in subcutaneous tumors of PAI-1–secreting LLC cells and nonsecreting B16 cells were compared between WT and PAI-1^{-/-} mice. Immunohistochemical staining of the excised subcutaneous tumor sections with anti-CD31 mAb showed apparently reduced areas of CD31-positive vessels in both subcutaneous tumors of LLC cells and B16 cells in PAI-1^{-/-} mice compared with those in WT mice (Fig. 4A and C).

Host but not tumor PAI-1 was determinant for the effects of SK-216 on tumor growth and angiogenesis

To evaluate by which of host or tumor PAI-1 the antitumor effect of SK-216 was more affected, WT and PAI-1^{-/-} mice subcutaneously inoculated with siControl LLC or siPAI-1 LLC cells were treated or untreated with SK-216. As shown in Fig. 5A, the volumes of subcutaneous tumors in WT mice were significantly smaller in the SK-216–treated groups than in the untreated groups. However, these differences were not observed in PAI-1^{-/-}

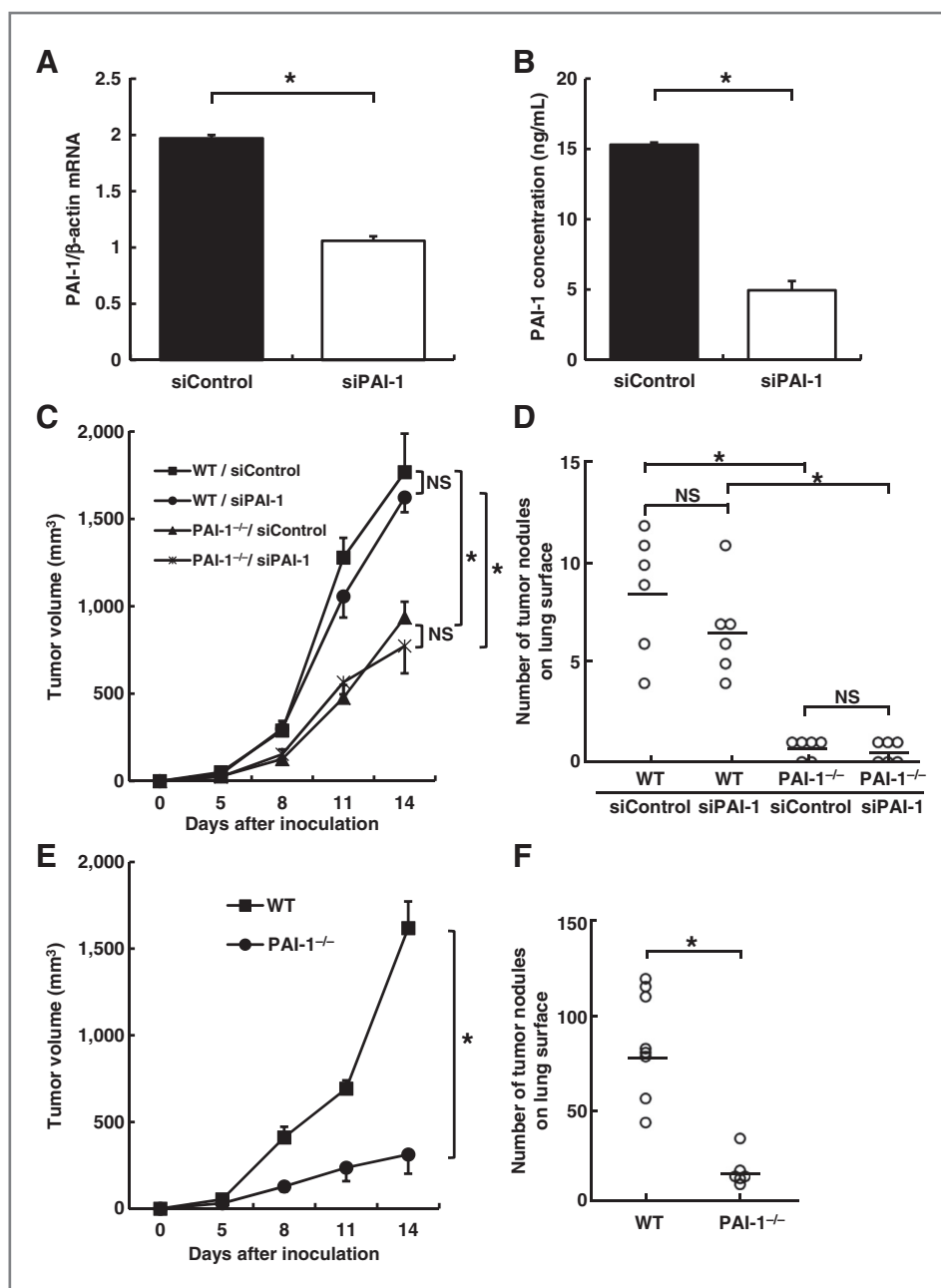


Figure 3. Evaluation of the knockdown efficiency of PAI-1 in LLC cells stably transfected with siRNA against PAI-1. A, siControl and siPAI-1 LLC cells were established as described in Materials and Methods. Expression levels of PAI-1 mRNA in siControl and siPAI-1 LLC cells were evaluated by qRT-PCR. B, concentrations of PAI-1 in culture media of siControl and siPAI-1 LLC cells were measured by ELISA. Data represent the mean values (\pm SEM) of triplicate samples and were analyzed by the Student *t* test. *, $P < 0.01$ versus siControl LLC cells. Effects of host and/or tumor PAI-1 expression levels on tumor progression in two tumor models. C, evaluation of tumor sizes in a subcutaneous tumor model using siControl and siPAI-1 LLC cells. Volumes of subcutaneous tumors were measured twice a week for 2 weeks after inoculation of siControl or siPAI-1 LLC cells into WT or PAI-1^{-/-} mice. Data represent the mean values (\pm SEM) of 6 mice per group and were analyzed with the Mann-Whitney *U* test. *, $P < 0.01$ versus WT mice. NS, not significant. D, evaluation of lung metastases in the tail vein metastasis model using siControl and siPAI-1 LLC cells. The number of tumor nodules on the lung surface of WT and PAI-1^{-/-} mice was counted 21 days after injection of siControl or siPAI-1 LLC cells through the tail vein. Each bar represents the mean value of 6 mice per group. The data were analyzed with the Mann-Whitney *U* test. *, $P < 0.01$ versus WT mice. NS, not significant. E, evaluation of tumor sizes in the subcutaneous tumor model using B16 cells. Volumes of subcutaneous tumors were measured twice a week for 2 weeks after the inoculation of B16 cells into WT or PAI-1^{-/-} mice. Data represent the mean values (\pm SEM) of 6 mice per group and were analyzed with the Mann-Whitney *U* test. *, $P < 0.01$ versus WT mice. F, evaluation of lung metastases in the tail vein metastasis model using B16 cells. The number of tumor nodules on the lung surface of WT and PAI-1^{-/-} mice was counted 21 days after injection of B16 melanoma cells through the tail vein. Each bar represents the mean value of 6 or 8 mice per group. The data were analyzed with the Mann-Whitney *U* test. *, $P < 0.01$ versus WT mice.

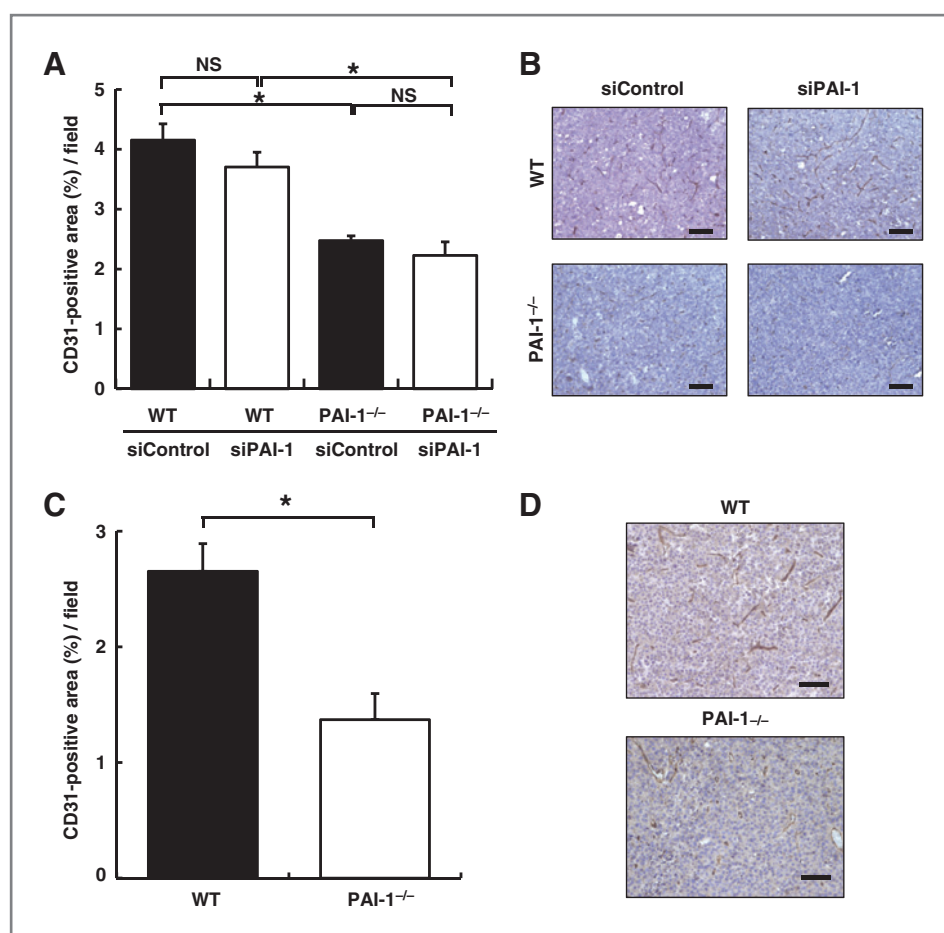


Figure 4. Evaluation of angiogenesis in subcutaneous tumors of WT and PAI-1^{-/-} mice subcutaneously inoculated with siControl or siPAI-1 LLC cells (A) or B16 cells (C). The area of CD31-positive vessels was calculated as described in Materials and Methods. Data represent the mean values (\pm SEM) of 6 mice per group and were analyzed with the Student *t* test. *, *P* < 0.01 versus WT mice. NS, not significant. B and D, representative immunohistochemical staining for CD31 in a subcutaneous tumor. Scale bar, 100 μ m.

mice (Fig. 5B). In both WT and PAI-1^{-/-} mice, there were no significant differences in the volumes of subcutaneous tumors between siControl LLC and siPAI-1 LLC cells (Fig. 5A and B). Similar to the results of subcutaneous tumor volumes, the differences in the areas of CD31-positive vessels, between the SK-216-treated and untreated groups were observed in WT mice (Fig. 5C) but not in PAI-1^{-/-} mice (Fig. 5D). In both WT and PAI-1^{-/-} mice, there was no significant difference in the areas of CD31-positive vessels between subcutaneous tumors consisting of siControl LLC and siPAI-1 LLC cells (Fig. 5C and D).

SK-216 did not affect proliferation of HUVECs but inhibited migration and tube formation of HUVECs *in vitro*

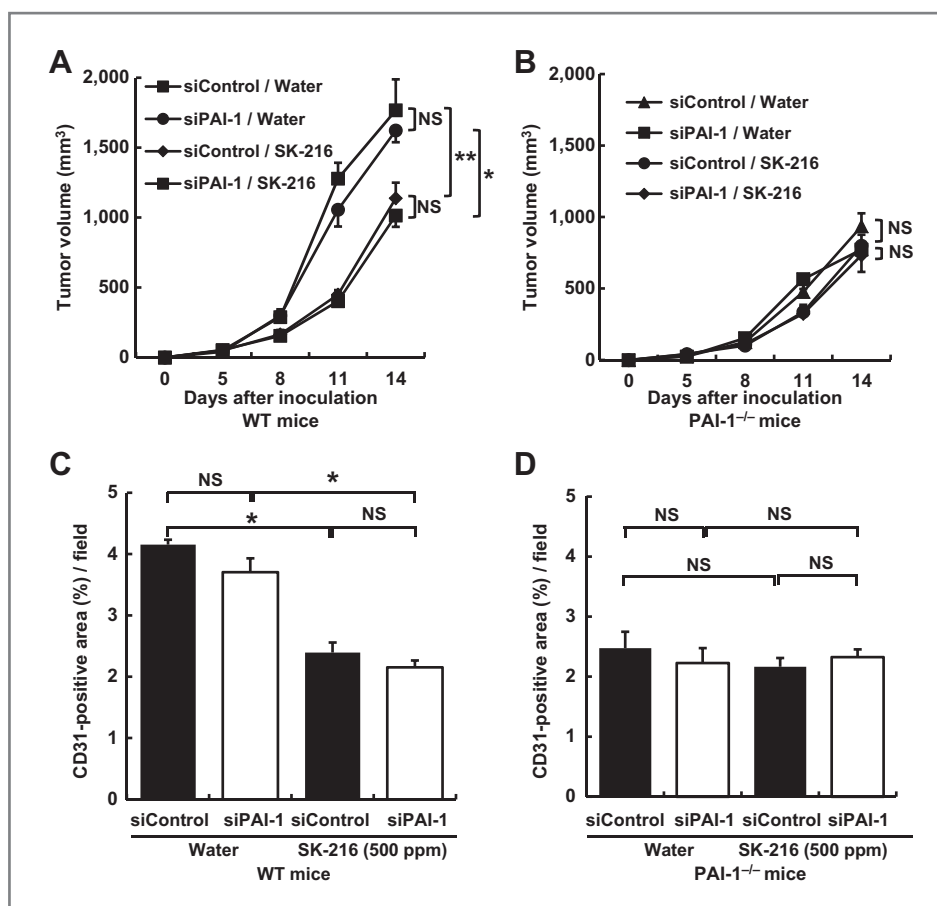
On the basis of the inhibitory effect of SK-216 on angiogenesis in tumors of LLC and B16 cells *in vivo*, we assessed the *in vitro* effects of SK-216 on proliferation, migration, and tube formation of endothelial cells. Because production of VEGF in both LLC and B16 cells was confirmed (data not shown), the primary angiogenic factor in the tumors of LLC and B16 cells was thought to be VEGF. Therefore, we used VEGF to stimulate proliferation, migration, and tube formation of HUVECs in the *in vitro* assays. The proliferation assay showed that the presence

of SK-216 at various concentrations in culture for 16 or 36 hours did not affect the cells' proliferation rates (Fig. 6A and B). As shown in Fig. 6C, however, the monolayer migration assay revealed that SK-216 inhibited the VEGF-induced migration of HUVECs in a dose-dependent manner. The statistically significant inhibition of HUVECs migration by SK-216 was observed at concentrations of 40 and 50 μ mol/L. Furthermore, SK-216 was shown to inhibit VEGF-induced tube formation of HUVECs in a dose-dependent manner (Fig. 6E). The statistically significant inhibition of HUVECs tube formation by SK-216 was observed at concentrations of 30, 40, and 50 μ mol/L.

Discussion

A growing body of evidence suggests that PAI-1 is closely involved in tumor progression and angiogenesis. In the present study, using a subcutaneous tumor model and a tail vein metastasis model, we have shown that systemic administration of SK-216, a specific PAI-1 inhibitor, was effective in suppressing both tumor progression and angiogenesis. This effect of SK-216 was found to be independent of the presence or absence of tumor PAI-1, suggesting the importance of host PAI-1 as a molecular target of SK-216. When the relevance for tumor progression

Figure 5. Influences of the presence or absence of host PAI-1 and the secreting levels of PAI-1 by tumor cells on effects of SK-216 on subcutaneous tumor growth and angiogenesis. A and B, evaluation of tumor sizes in the subcutaneous tumor model using siControl and siPAI-1 LLC cells. Volumes of subcutaneous tumors were measured twice a week for 2 weeks after inoculation of siControl or siPAI-1 LLC cells into WT (A) or PAI-1^{-/-} (B) mice. Mice were given water or SK-216 (500 ppm). The data represent the mean values (\pm SEM) of 6 mice per group and were analyzed with the Student *t* test or Mann-Whitney U test as appropriate. *, *P* < 0.01; **, *P* < 0.05 versus control group. NS, not significant. C and D, evaluation of angiogenesis in subcutaneous tumors of WT (C) and PAI-1^{-/-} (D) mice subcutaneously inoculated with siControl or siPAI-1 LLC cells. Mice were given water or SK-216 (500 ppm). The area of CD31-positive vessels was calculated as described in Materials and Methods. Data represent the mean values (\pm SEM) of 5 or 6 mice per group and were analyzed with the Student *t* test. *, *P* < 0.05 versus control group. NS, not significant.



and angiogenesis was compared between host and tumor PAI-1, we found that host but not tumor PAI-1 played a determinant role in these processes. These results also support the suggestion that SK-216 inhibited tumor progression and angiogenesis primarily through interacting with host PAI-1. In *in vitro* studies, SK-216 inhibited the VEGF-induced migration and tube formation of HUVECs.

There is only one previous study that used SK-216 for animal tumor models (26). In that study, SK-216 was shown to suppress the spontaneous formation of intestinal polyps in the adenomatous polyposis coli gene-deficient mouse. About another PAI-1 inhibitor, PAI-039, there is a report that it could reverse PAI-1's protection against apoptosis in human cancer cell lines (23). In the present study, we have shown the antitumor effect of SK-216 using a subcutaneous tumor model and a tail vein metastasis model. The most interesting finding was that the systemic administration of SK-216 could suppress tumor growth and lung metastasis irrespective of the presence or absence of PAI-1 secretion by the tumor cells. From the experiment that WT and PAI-1^{-/-} mice subcutaneously inoculated with siPAI-1 LLC cells or siControl LLC cells were treated or untreated with SK-216, we also found that host but not tumor PAI-1 was determinant for the effect of SK-216 on tumor growth. These results suggest that the antitumor effect of SK-216 is likely exerted

through interaction with host-derived PAI-1. In addition, in the present study, we have shown that the presence of host PAI-1 was a determinant in tumor growth and lung metastasis but the expression level of PAI-1 in tumor cells was not associated with either the degree of tumor growth or lung metastasis. Although we did not determine the precise mechanism by which host PAI-1 was involved in tumor progression, these results suggest that host PAI-1 was the primary molecular target for SK-216 in the animal tumor models used in the present study.

Although the crucial role of host PAI-1 in tumor progression has been reported (11–14), two recent studies showed the involvement of tumor PAI-1 in tumor growth. Nishioka and colleagues showed that reducing PAI-1 expression in either the tumor or the host could suppress tumor progression (22). In contrast, Fang and colleagues reported that both host PAI-1 and tumor PAI-1 had to be reduced to inhibit tumor progression (23). These two reports are inconsistent with our finding that the level of tumor PAI-1 did not affect the extent of tumor progression, and, unfortunately we do not have data to explain this difference. In animal tumor models using different tumor cells from those used in the present study, tumor PAI-1 might be associated with tumor progression. Because reduction of tumor PAI-1 expression or activity seemed to be advantageous for inhibiting tumor progression, we

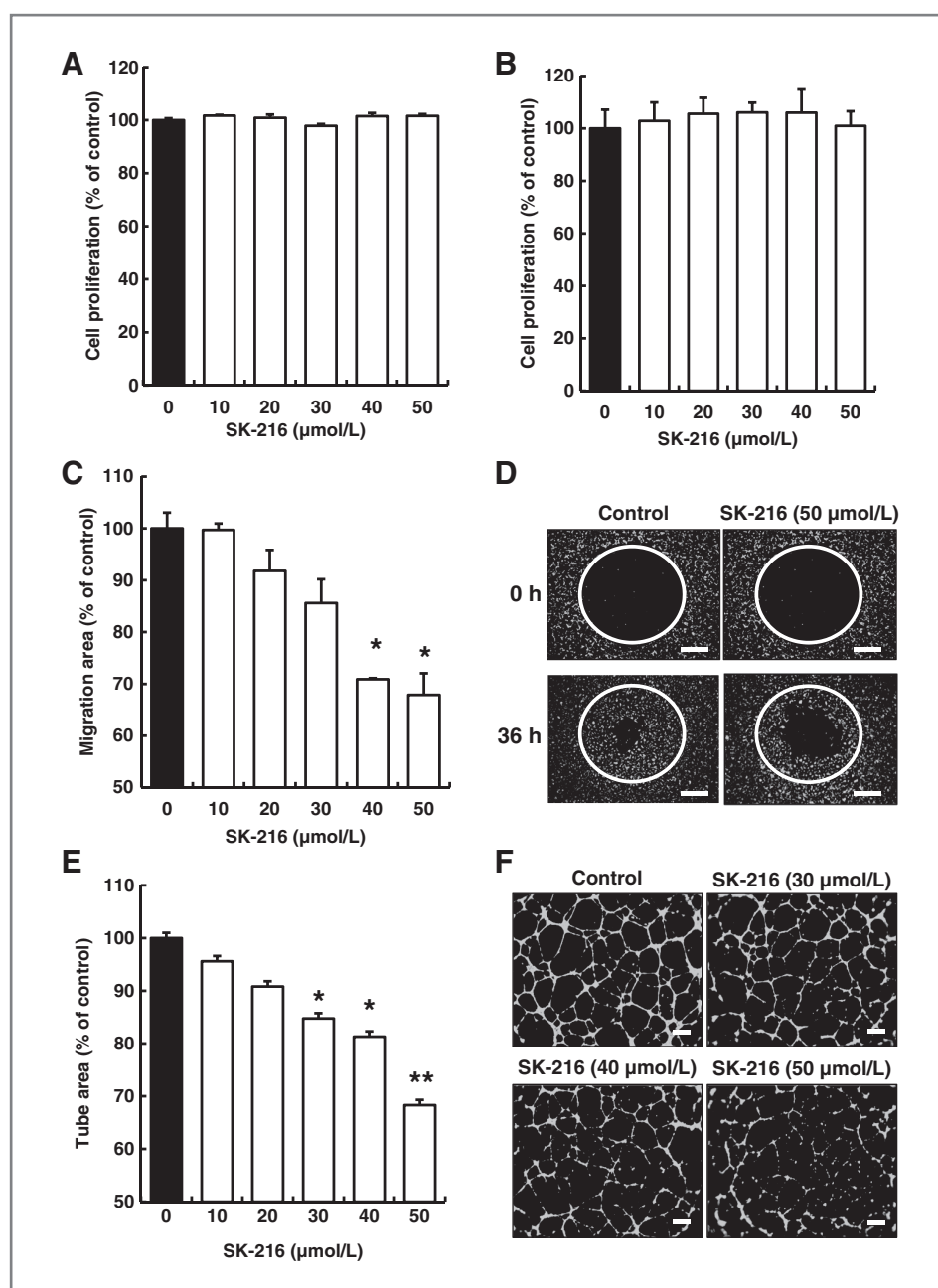


Figure 6. Effects of SK-216 on the degrees of proliferation, migration, and tube formation of HUVECs. A and B, proliferation of HUVECs induced by VEGF was quantified after 16 hours (A) and 36 hours (B) in the presence of SK-216 at various concentrations. C and E, migration (C) and tube formation (E) of HUVECs induced by VEGF in the presence of SK-216 at various concentrations were evaluated as described in Materials and Methods. D and F, representative images of HUVECs in cell migration assay (D) and capillary-like tube formation assay (F). HUVECs were stained with Calcein AM stock solution. Data represent the mean values (\pm SEM) of triplicate samples and were analyzed with the Student *t* test. *, $P < 0.05$ versus control; **, $P < 0.01$ versus control; scale bar, 250 μm .

believe that this difference should not be an obstacle to the use of SK-216 as a systemic antitumor agent.

We note that there was a study that was inconsistent with our results. Eitzman and colleagues reported that the expression level of host PAI-1 did not affect the extent of tumor growth in the foot pad or the formation of lung metastases by B16 cells (27). Unfortunately, we cannot readily explain this discrepancy. We speculate that differences between the sites where B16 cells were implanted and/or the number of cells used for experiments between Eitzman and colleagues' and our studies resulted in these inconsistent data.

Independent of tumor cells' expression of PAI-1, PAI-1 production is thought to be increased by soluble factors in the tumor microenvironment. It has been reported that VEGF produced by tumor cells and/or stromal host cells promoted PAI-1 secretion by endothelial cells (28). In addition, inflammatory cytokines such as interleukin (IL)-1, IL-6, and TNF- α from immune cells (29) and TGF- β from fibroblasts (30) are all known to induce PAI-1 expression in endothelial cells (31) and hepatocytes (32). Moreover, extravascular synthesis of PAI-1 by adipocytes (33), macrophages (34), and fibroblasts (35, 36) is promoted. Elevated levels of circulating PAI-1 in

tumor-bearing patients (37–39) seem to reflect overproduction of PAI-1 in the tumor environment. Considering the strong association between the abundance of PAI-1 in the tumor microenvironment and the aggressiveness of the tumor (6–9), systemic administration of SK-216 could be a reasonable therapeutic approach for the treatment of malignancy.

In the present study, the extent of angiogenesis in tumors generated in PAI-1^{-/-} mice was significantly lower than that in WT mice. This result confirms the previous observations that indicated the significance of host PAI-1 in regulating tumor angiogenesis (11–14). Indeed, a previous study showed that PAI-1 produced by tumor cells, even at high concentrations, could not compensate for the absence of host PAI-1 in tumor angiogenesis (13). These observations suggest that host PAI-1 could become a novel molecular target for the reduction in tumor angiogenesis. Interestingly, the systemic administration of SK-216 reduced angiogenesis in tumors of PAI-1-secreting LLC cells and PAI-1-nonsecreting B16 cells, similar to that observed in PAI-1^{-/-} mice. From the experiment that WT and PAI-1^{-/-} mice subcutaneously inoculated with siPAI-1 LLC cells or siControl LLC cells were treated or untreated with SK-216, we also found that host but not tumor PAI-1 was determinant for the effect of SK-216 on angiogenesis. These results suggest that systemic administration of SK-216 reduced tumor angiogenesis through inhibition of host PAI-1 activity. In addition, the direct inhibitory effect of SK-216 on VEGF-mediated migration and tube formation of HUVECs was also shown in the present study. Although the precise mechanism of host PAI-1 involvement in tumor angiogenesis was not determined, these observations suggest that inhibition of host PAI-1 activity would result in the reduction of tumor angiogenesis, raising the possibility that systemic administration of SK-216 could become a novel antiangiogenic therapeutic in the treatment of malignancy.

Because the induction of angiogenesis is an important mechanism by which tumors promote their own continued growth and metastasis (40), inhibition of tumor angiogenesis represents an attractive therapeutic approach in the treatment of malignancy. VEGF plays a major role in tumor angiogenesis, however, the contribution of other factors, such as PDGF, FGF, and angiopoietins, has been confirmed (41–44). Currently, only VEGF-targeted antiangiogenic agents are clinically available for the treatment of malignancy. They include bevacizumab (Avastin; Genentech/Roche) targeting VEGF and two kinase inhibitors, sorafenib (Nexavar; Bayer) and sunitinib (Sutent; Pfizer), targeting the VEGF receptor sig-

nal pathway. Thus, the development of antiangiogenic therapeutics with different targets seems necessary. The reduction of angiogenesis in the subcutaneous tumors of LLC and B16 cells by SK-216 raises the possibility that SK-216 could be used as an alternative antiangiogenic agent. The target of this antiangiogenic approach was found to be host-derived PAI-1. We believe that systemic administration of SK-216 proposes a new concept of antiangiogenic therapeutics that targets host-derived factors.

In conclusion, using systemic administration of SK-216, a specific inhibitor for PAI-1, we showed that it limited tumor progression and angiogenesis *in vivo*, independent of the presence or absence of PAI-1 secretion by the tumor cells. In addition, the results of the present study indicate that host (but not tumor) PAI-1 plays a determinant role in these processes. These results suggest the possibility that host PAI-1 was the main molecular target for SK-216. Furthermore, SK-216 was shown to have an inhibitory effect on migration and tube formation by HUVECs *in vitro*. Taken together, these observations strongly suggest that systemic administration of SK-216 reduced tumor progression mainly through its interaction with host PAI-1 and that this antitumor effect might be mediated by the antiangiogenic properties of SK-216.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: T. Masuda, T. Senoo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Masuda, T. Senoo, S. Akita
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Masuda, T. Senoo
Writing, review, and/or revision of the manuscript: T. Masuda, N. Hattori, T. Senoo, N. Ishikawa, K. Fujitaka, Y. Haruta, H. Murai, N. Kohno
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Senoo, S. Akita
Study supervision: N. Hattori, T. Senoo, K. Fujitaka, N. Kohno

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