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Inhibition of in vivo angiogenesis by *N*-β-alanyl-5-*S*-glutathionyl-3,4-dihydroxyphenylalanine

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Abstract

N-β-alanyl-5-*S*-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD), an antibacterial substance isolated from the flesh fly, inhibits human tumor growth in the nude mice model; however, the mechanism of its action is unclear. The in vivo antitumor effect includes the inhibition of tumor cell proliferation and suppression of angiogenesis. Angiogenesis is essential for tumor growth in vivo. In this study, we examined whether 5-S-GAD inhibits tumor cell-induced angiogenesis by performing the mouse dorsal air sac assay. We found that intraperitoneal administration of 5-S-GAD inhibited the angiogenesis induced by S180 mouse sarcoma cells. Furthermore, 5-S-GAD also inhibited vascular endothelial growth factor-induced angiogenesis in the Matrigel plug assay and embryonic angiogenesis in the chick embryo chorioallantoic membrane assay. However, 5-S-GAD did not show any effect on the proliferation, migration, and tube formation of vascular endothelial cells. These results provide the first evidence that a bioactive substance derived from the flesh fly has antiangiogenic activity in vivo, although the mechanisms involved could not be explained.

Keywords: Angiogenesis; Antiangiogenic agent; 5-S-GAD; Tumor therapy

1. Introduction

N-β-alanyl-5-*S*-glutathionyl-3,4-dihydroxyphenylalanine (5-S-GAD) was originally isolated from *Sarcophaga peregrina* (flesh fly) as a novel antibacterial substance (Leem et al., 1996). In addition to antibacterial activity, 5-S-GAD was found to show cytotoxicity toward certain human tumor cell lines in vitro (Hijikata et al., 1999; Akiyama et al., 2000). Among the 38 tumor cell lines examined, 1 melanoma (LOX-IMV1) and 2 breast carcinoma (MDA-MB-435S and MDA-MB-231) cell lines were sensitive to 5-S-GAD; the IC_{50} values of these cell lines were 0.5–20 μM (Akiyama et al., 2000). Moreover, the growth of tumor nodules arising from LOX-IMV1 cells and MDA-MB-435S cells was significantly inhibited when 5-S-GAD was administered intraperitoneally (i.p.) to tumor-bearing nude mice (Akiyama et al., 2000). These results indicate that 5-S-GAD is potentially useful for treating human tumors.

5-S-GAD is a conjugate of glutathione and β-alanyl-L-dihydroxyphenylalanine (Leem et al., 1996). The catechol moiety of 5-S-GAD is assumed to be readily converted to orthoquinone; and this conversion is accompanied by the production of hydrogen peroxide (H_2O_2). In fact, the antibacterial activity (Leem et al., 1996) and cytotoxicity of 5-S-GAD (Hijikata et al., 1999; Akiyama and Natori, 2003) in vitro were abolished in the presence of catalase—an enzyme that converts

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H_2O_2 into H_2O and O_2. These results indicate that the antibacterial activity and cytotoxicity of 5-S-GAD are due to the H_2O_2 produced from it. However, the mechanism of its antitumor effect in vivo remains unclear.

Angiogenesis–the formation of new blood vessels by sprouting from preexisting ones–plays an essential role in many important pathological and physiological settings. In healthy adults, the normal angiogenic activity is low, except for the reproductive cycle in women, but increases during injury and in diseases.

Diseases characterized or caused by excessive angiogenesis include cancer, rheumatoid arthritis, psoriasis, diabetic retinopathy, obesity, atherosclerosis, and inflammatory and infectious diseases; this list continues to grow (Carmeliet, 2003). Therefore, inhibition of angiogenesis is a promising strategy for the treatment of these disorders. In fact, many antiangiogenic agents have been identified, and several others are in clinical trials (Cherrington et al., 2000). Angiogenesis is essential for the growth of solid tumors greater than 2–3 mm in diameter and for tumor metastasis in vivo (Folkman, 1995).

In this study, we examined whether 5-S-GAD could inhibit tumor cell-induced angiogenesis. On performing the mouse dorsal air sac assay, we found that 5-S-GAD inhibited the angiogenesis induced by S180 mouse tumor cells. We further investigated whether 5-S-GAD could inhibit other angiogenic systems by performing the Matrigel plug assay and chick embryo chorioallantoic membrane assay. 5-S-GAD also showed an antiangiogenic effect in these systems.

Blood vessels consist of an inner layer of endothelial cells. Small blood vessels are composed of only endothelial cells, whereas larger blood vessels are surrounded by mural cells (pericytes in medium-sized vessels and smooth muscle cells in large vessels). New capillaries are generated through a multistep process that includes the migration and proliferation of endothelial cells and the formation of a new vascular tube. Thus, endothelial cells play a central role in angiogenesis.

Therefore, we investigated the effect of 5-S-GAD on the migration, proliferation, and tube formation of endothelial cells in vitro. 5-S-GAD showed little effect on these processes. Thus, our results show that 5-S-GAD has an inhibitory effect on angiogenesis in vivo, but the mechanisms involved could not be explained.

2. Materials and methods

2.1. Materials

5-S-GAD was synthesized as described previously (Leem et al., 1996). Millipore rings (inner diameter, 10 mm; thickness, 2 mm) and Millipore filters (pore size, 0.45 μm) were obtained from Nihon Millipore (Yonezawa, Japan). Ethylene-vinyl acetate copolymer 40 pellets were kindly donated by Mistui-DuPont Polychemicals (Tokyo, Japan). Heparin sodium salt (low molecular weight) and Drabkin's reagent were purchased from Sigma (St. Louis, MO, USA). Matrigel was purchased from Becton Dickinson Labware (Bedford, MA, USA). Recombinant murine vascular endothelial growth factor (VEGF) was obtained from R&D Systems (Minneapolis, MN, USA).

2.2. Cells and animals

Malignant mouse tumor cells (S180), which were kindly donated by Dr. Chizuko Tashiro, Institute of Cancer (Tokyo, Japan), were maintained in female ICR mice aged 7–9 weeks. Human umbilical vein endothelial cells (HUVECs) were obtained from Cell Systems (Kirkland, WA, USA). The female ICR and C57BL/6 mice were purchased from Charles River Japan (Atsugi, Japan) and Japan SLC, Inc. (Hamamatsu, Japan), respectively.

2.3. Mouse dorsal air sac assay

The mouse dorsal air sac assay was performed as described previously (Nakamura et al., 1999). A chamber, which was prepared by covering both sides of a Millipore ring with Millipore filters of 0.45-μm pore size, was filled with a suspension of S180 tumor cells (2×10^7 cells) in 0.15 ml of phosphate buffered saline (PBS). This chamber was implanted into the subcutaneous dorsal air sac created in 15-week-old female ICR mice by injecting an appropriate volume of air. These treated mice were divided into 7 groups, each comprising 6 mice; each group was administered the vehicle (i.e., 0.5% carboxymethylcellulose sodium salt) alone, 100 mg/kg cytogenin per os (p.o.) (Oikawa et al., 1997), 0.78, 3.1, 12.5, 50, or 200 mg/kg 5-S-GAD i.p. The vehicle alone was administered i.p. to the negative control group implanted with the PBS-containing chamber. 5-S-GAD was first dissolved in 1N NaOH to achieve a concentration of 320 mg/ml (pH = 7) and diluted with a 0.9% NaCl solution immediately prior to injection. The agents were administered at 0.1 ml/10 g body weight once a day for 4 days starting from the day of implantation of the chamber. On day 5, the implanted chambers were removed from the subcutaneous fascia of the treated animals, and a black ring with the same inner diameter as that of the Millipore ring was placed at the same site. The angiogenic response was assessed under a dissecting microscope by determining the number of newly formed blood vessels greater than 3 mm in length within the area encircled by the black ring. The blood vessels that were newly formed due to the malignant tumor cells were morphologically distinguishable from the preexisting background vessels by their zigzag character. This experiment was conducted according to the Guidelines for Animal Experiments of Institute of Microbial Chemistry Ethics Committee on Animal Experimentation.

2.4. In vivo Matrigel plug assay

Matrigel plug assay was performed as described previously (Min et al., 2004). C57BL/6 female mice were injected subcutaneously (s.c.) with 0.4 ml of growth factor-reduced Matrigel containing 20 U/ml heparin and 100 ng/ml recombinant murine VEGF with or without the indicated amount of 5-S-GAD. After 6 days, the skin of the mouse was pulled back and

the Matrigel plug was excised from it. The hemoglobin content of the Matrigel plugs was measured using Drabkin's reagent kit for the quantification of blood vessel formation. The hemoglobin concentration was determined from a standard curve obtained using human hemoglobin (Sigma). Groups of 4 or 5 mice were formed, and the experiment was repeated at least twice at each dose of 5-S-GAD. Some of the excised Matrigel plugs were fixed with 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. This experiment was approved by the Wako Animal Experiment Committee of the Safety Division of RIKEN and was carried out in accordance with the Guidelines and the Animal Experiment Handbook of Wako Institute of RIKEN.

2.5. Chick embryo chorioallantoic membrane assay

The chick embryo chorioallantoic membrane assay was performed as described previously (Nakamura et al., 1999). In brief, the chorioallantoic membranes of 5-day-old chick embryos were treated with ethylene-vinyl acetate copolymer 40 pellets containing or not containing various doses of 5-S-GAD and incubated at 37 °C for 2 days in a humidified egg incubator. Subsequently, an appropriate volume of a 20% fat emulsion was injected into the chorioallantois to clearly visualize the vascular network. The antiangiogenic response was assessed as positive when the avascular zone exceeded 3 mm in diameter; only the frequency was monitored. This experiment was approved by the Committee on the Ethics of Animal Experiments of the Tokyo Metropolitan Institute of Medical Science and was carried out in accordance with the Guidelines for Animal Experiments of the Tokyo Metropolitan Institute of Medical Science.

2.6. Effects of 5-S-GAD on HUVEC functions in vitro

The proliferation (Nakamura et al., 1999), migration (Oikawa et al., 2001), and tube formation (Oikawa et al., 1997) of HUVECs were analyzed as described previously. The secreted and cell-associated plasminogen activator activities were determined as described previously (Nakamura et al., 1999). The pro-matrix metalloproteinase (MMP)-2 activity was quantified by using gelatin zymography as described elsewhere (Munaut et al., 2003).

2.7. MMP enzyme assay

Recombinant human MMP-1, MMP-2, MMP-9, and the catalytic domain of human MT1-MMP (MMP-14) were produced by using mouse C127 cells, insect Tn cells, human HEK923 cells, and *E. coli* strain BL21, respectively. The enzyme assay was performed using a fluorogenic peptide substrate, namely, 2-*N*-methylaminobenzoic acid (Nma)-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Lys-N^{ε}-(2,4-dinitrophenyl) (Dnp)-NH_2 (Peptide Institute, Inc., Osaka, Japan). The MMP reaction was carried out in an assay buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, and 0.05% (w/v) Brij-35) in a total volume of 100 μl. The substrate was used at a concentration that was equivalent to the Km value of each enzyme. The reaction mixture containing the substrate was added to the wells of a 96-well white Microfluor plate (Packard Instrumental Company, Meriden, CT, USA) and preincubated at 37 °C for 30 min in the presence or absence of inhibitors. The reaction was initiated by the addition of the MMP enzyme, and the fluorescence at 460 nm was continuously monitored for 30 min with excitation at 355 nm by using a Fluoroskan Ascent Type 374 plate fluorometer (Labsystems, Helsinki, Finland). A time point located on the linear part of the reaction curve was chosen to determine the activity.

2.8. Hypoxia-inducible factor 1(HIF-1)-dependent reporter gene assay

This assay was performed as reported previously (Yamazaki et al., 2003).

2.9. Flt-1 tyrosine kinase assay

The activity of VEGF tyrosine kinase receptor Flt-1 was measured using membrane fractions derived from Flt-1-over-expressing Tn5 cells as described previously (Sawano et al., 1997). In brief, the membrane fractions (2 μg) with or without 5-S-GAD were incubated at 25 °C for 10 min in a kinase assay buffer, and the reaction mixture was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was further treated with 1 N NaOH for 2 h at 55 °C, and the bands of phosphorylated Flt-1 were visualized by autoradiography. SU5416 (Itokawa et al., 2002), which is an Flt-1 tyrosine kinase inhibitor, was used as a positive control.

2.10. Statistics

The mouse dorsal air sac assay data were analyzed by using the Wilcoxon rank-sum test for comparison between two groups and the nonparametric Dunnett multiple comparison procedure for comparison among three or more groups. The results of the Matrigel plug assay were analyzed by Student's *t* test for comparison between two groups. The chorioallantoic membrane assay data were analyzed by Fisher's exact probability test.

3. Results

3.1. Effect of 5-S-GAD on tumor cell-induced angiogenesis

First, we examined the effect of i.p. administration of 5-S-GAD on tumor cell-induced neovascularization in the mouse dorsal air sac assay. The observations in representative experiments are shown in Fig. 1. In the positive control group, which was implanted with S180 cell-containing chambers and administered the vehicle alone, there was a drastic induction of formation of new blood vessels characterized by zigzag lines (Fig. 1B). In the negative control group, which was implanted with PBS-containing chambers and administered the vehicle alone, there was slight or no formation of neovessels (Fig. 1A).

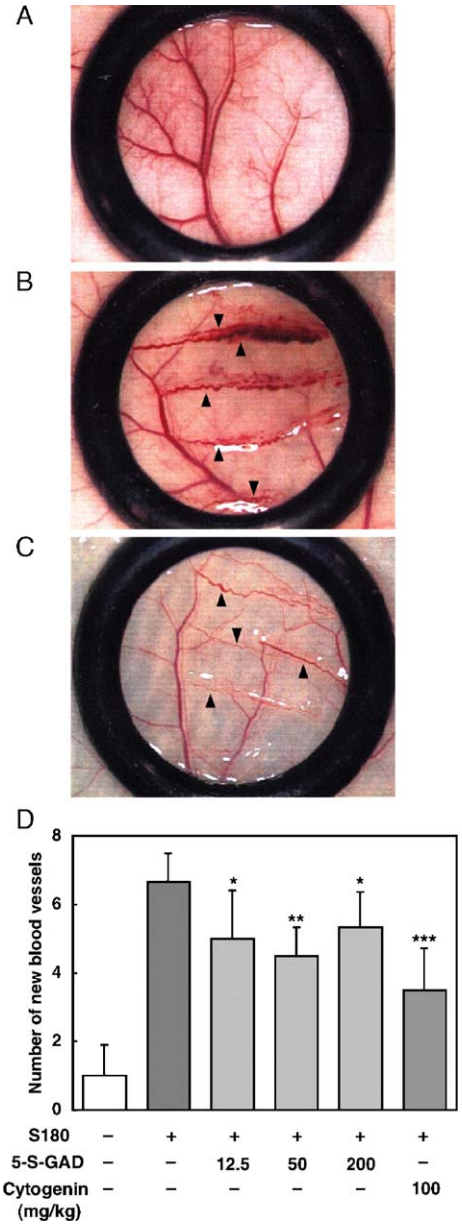

Fig. 1. Inhibitory effect of 5-S-GAD on tumor cell-induced angiogenesis in the mouse dorsal air sac assay. Angiogenic response was observed under a microscope (A–C) and assessed based on the number of newly formed vessels (D). (A) Mice implanted with a chamber containing PBS were treated with the vehicle alone. Mice implanted with a chamber containing S180 tumor cells were treated with the vehicle (B) and with 5-S-GAD at 12.5 mg/kg i.p. (C). The arrowheads indicate the newly formed blood vessels characterized by zigzag lines. The inner diameter of the black ring is 10 mm. (D) The number of newly formed vessels was counted. The means with standard deviations are shown ($n=6$). *$P<0.05$, **$P<0.01$, and ***$P<0.001$ vs. control.

The formation of such neovessels was prevented by 5-S-GAD at a dose of 12.5 mg/kg/day (Fig. 1C). This dose is low and nontoxic since a single i.p. administration of 5-S-GAD up to 8 g/kg does not result in toxic death. We also examined the effect of 5-S-GAD at doses of 0.78 or 3.1 mg/kg; however, we were unable to detect any significant antiangiogenic effects (data not shown). We noticed that the neovessels observed in the 5-S-GAD-treated group tended to be thinner than those in the control group (Fig. 1B and C); however, the cause is unknown.

These angiogenic responses were assessed under a dissecting microscope by counting the tumor cell-induced neovessels (Fig. 1D). 5-S-GAD significantly but moderately suppressed the new vessel formation at doses of 12.5, 50, and 200 mg/kg, and its maximum effect was approximately 30% inhibition at 50 mg/kg. As a positive control for the antiangiogenic agent, we also examined the effect of cytogenin administered p.o. (Oikawa et

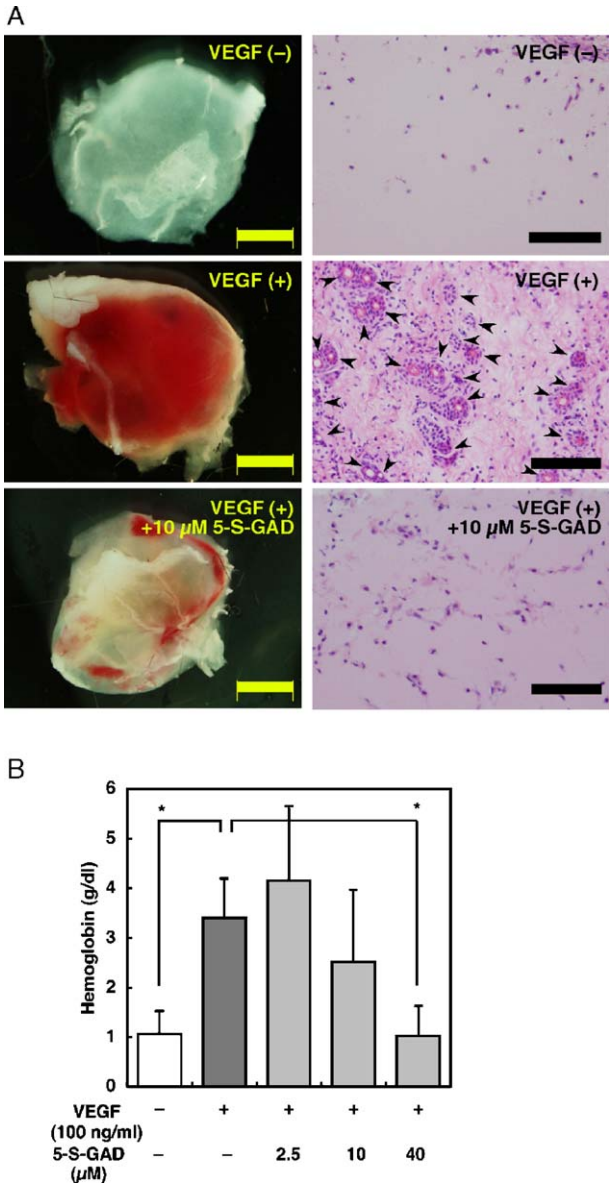

Fig. 2. Inhibitory effect of 5-S-GAD on VEGF-induced angiogenesis in the mouse Matrigel plug assay. Matrigel containing 100 ng/ml VEGF with or without 5-S-GAD was injected into C57BL/6 female mice, and the Matrigel plugs were excised after 6 days. (A) The photographs show the entire Matrigel plugs (left panels) and representative sections of the plugs stained by hematoxylin and eosin (right panels). The arrowheads indicate vessel-like structures. Yellow bars=2.0 mm. Black bars=100 μm. (B) The total hemoglobin content in the Matrigel plugs was quantified as an indicator of blood vessel formation. The data are expressed as means+S.E. The numbers of examined animals are as follows: VEGF (−) ($n=16$), 100 ng/ml VEGF ($n=26$), VEGF+2.5 μM 5-S-GAD ($n=8$), VEGF+10 μM 5-S-GAD ($n=8$), and VEGF+40 μM 5-S-GAD ($n=15$). Statistically significant differences were determined by Student's t test (*$P<0.05$).

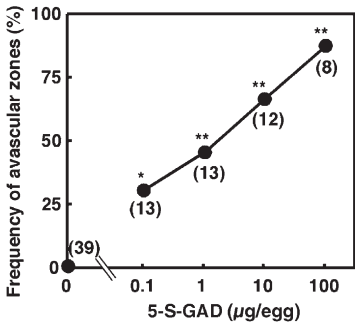

Fig. 3. Inhibitory effect of 5-S-GAD on embryonic angiogenesis. The chorioallantoic membranes of 5-day-old chick embryos were treated with ethylene-vinyl acetate copolymer 40 pellets containing various doses of 5-S-GAD. After 2 days, the antiangiogenic effect was assessed by measuring the avascular zones. The points show the frequency (%) of the avascular zones exhibiting a significant antiangiogenic response. The values in parentheses indicate the number of membranes examined. *$P<0.01$ and **$P<0.001$ vs. control (i.e., 0 μg 5-S-GAD) pellet-treated membranes, which did not show any avascular zone.

al., 1997). The antiangiogenic effect of 5-S-GAD was weaker than that of cytogenin.

3.2. Effect of 5-S-GAD on VEGF-induced angiogenesis in vivo

Since 5-S-GAD shows cytotoxicity to some tumor cells, it might have suppressed angiogenesis by inhibiting the growth of S180 cells. To determine whether 5-S-GAD directly blocks angiogenesis, we examined whether it could inhibit the VEGF-induced angiogenesis in the Matrigel plug assay. VEGF is one of the critical factors that promote angiogenesis. Matrigels containing VEGF with or without 5-S-GAD were injected s.c. into mice, and the Matrigel plugs were excised after 6 days.

The Matrigel plugs containing VEGF alone were apparently red (Fig. 2A, left middle panel) due to neovascularization. We confirmed the formation of numerous blood vessels by histological analysis of the plugs (Fig. 2A, right middle panel). On the other hand, the Matrigel plugs treated with both VEGF and 5-S-GAD were only partially red, indicating decreased blood vessel formation (Fig. 2A, bottom panels). The Matrigel plugs without VEGF were colorless and showed the absence of vasculature (Fig. 2A, top panels).

Furthermore, we measured the hemoglobin content in the plugs as an indicator of the number of newly formed blood vessels. The hemoglobin content in the plugs treated with VEGF increased, while 5-S-GAD suppressed this effect in a dose-dependent manner (Fig. 2B). The hemoglobin content in the plugs treated with both VEGF and 40 μM 5-S-GAD decreased to the same level as that in the plugs without VEGF. These results show that 5-S-GAD directly inhibits VEGF-induced angiogenesis in vivo.

3.3. Effect of 5-S-GAD on embryonic angiogenesis

Next, we examined whether 5-S-GAD also inhibits embryonic angiogenesis by performing a chick embryo chorioallantoic membrane assay. This assay is used not only as an in vivo angiogenesis model but also for the screening of angiogenesis inhibitors (Oikawa, 1995). In this assay, the newly formed vascular network is observed in the chorioallantoic membrane. When the diameter of the avascular zone in the membrane exceeded 3 mm, we judged that the antiangiogenic response was positive.

Fig. 3 shows the dose–response relationship between 5-S-GAD and the appearance of avascular zones. 5-S-GAD suppressed embryonic angiogenesis in a dose-dependent manner, whereas the negative control group treated with only ethylene-vinyl acetate copolymer 40 pellets did not show antiangiogenic effects ($n=39$). The ID_{50} value for 5-S-GAD was 2.62 nmol (1.5 μg)/egg.

3.4. Effects of 5-S-GAD on endothelial cell functions in vitro

The generation of new capillaries is a multistep process that includes the migration and proliferation of endothelial cells and the formation of a new vascular tube. We investigated the effect of 5-S-GAD on the proliferation, migration, and tube formation of angiogenic HUVECs. 5-S-GAD had a slight or no effect on the proliferation and migration of the endothelial cells and tube formation at concentrations up to 100 μM (Table 1).

3.5. Effects of 5-S-GAD on expression and activities of plasminogen activator and MMP

Extracellular proteolysis is an essential process in angiogenesis because extracellular matrix degradation is a key feature in the migration of endothelial cells. MMP and the plasminogen activator/plasmin system play a major role in this process (Pepper, 2001). In particular, MMP-2 was clearly shown to be involved in angiogenesis (Itoh et al., 1998), and its expression is known to increase in endothelial cells in response to angiogenic stimuli and in a variety of tumors.

We examined the effect of 5-S-GAD on the activities of secretory and cell-associated plasminogen activator and the secretion of pro-MMP-2 from HUVECs. We also tested whether 5-S-GAD inhibits the enzymatic activities of MMP-1, MMP-2, MMP-9, and MT1-MMP in vitro. However, 5-S-GAD did not

Table 1
The effects of 5-S-GAD on the functions of human umbilical vein endothelial cells and the molecular activities associated with angiogenesis

Effect on functions of human umbilical vein endothelial cells	
Proliferation	No effect (0.01–100 μM)
Migration	27% inhibition at 1000 μM (10–1000 μM)
Tube formation	No effect (1–100 μM)
Activity of plasminogen activator (secretory or cell-associated)	No effect (0.01–100 μM)
Pro-MMP-2 secretion	No effect (0.01–100 μM)
Effect on molecular activities	
Enzyme activities of MMP-1, MMP-2, MMP-9, MT1-MMP	No effect (0.001–10 μM)
HIF-1-dependent transcription	No effect (0.8–20 μM)

The concentrations of 5-S-GAD are indicated in parentheses.

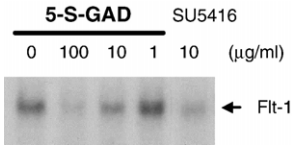

Fig. 4. Effect of 5-S-GAD on Flt-1 receptor autophosphorylation analyzed by an in vitro tyrosine kinase assay. Membrane fractions obtained from Tn5 cells overexpressing Flt-1 were incubated in a kinase assay buffer with or without test compounds (5-S-GAD and SU5416) at 25 °C for 10 min and analyzed by electrophoresis. The bands of phosphorylated Flt-1 were detected by autoradiography after alkaline treatment.

have any effect on the expression and the activities of these enzymes (Table 1).

3.6. Effect of 5-S-GAD on HIF-1-dependent reporter activity induced by hypoxia

Among the various triggers of angiogenesis, tissue hypoxia is a particularly important stimulus for the induction of new vessels. The transcription of the *VEGF* gene, which is one of the most important angiogenic factors, is enhanced under hypoxic conditions and is controlled by the HIF-1 (Forsythe et al., 1996). We examined the effect of 5-S-GAD on HIF-1 dependent transcription activity under hypoxic conditions by performing an established reporter assay (Yamazaki et al., 2003). However, 5-S-GAD did not inhibit the activity at concentrations up to 20 μM (Table 1).

3.7. Effect of 5-S-GAD on the autophosphorylation of the VEGF receptor Flt-1

Since 5-S-GAD has been shown to be a potent inhibitor of protein tyrosine kinases, including v-Src and BCR-ABL (Hijikata et al., 1997; Leem et al., 1998), 5-S-GAD may have an effect on the signal transduction pathways that induce angiogenesis. In addition, we showed that 5-S-GAD inhibits VEGF-induced angiogenesis (Fig. 2). VEGF stimulates angiogenesis through the activation of its specific tyrosine kinase receptor.

Therefore, we investigated the effect of 5-S-GAD on the autophosphorylation of the VEGF receptor Flt-1. Fig. 4 shows that 5-S-GAD inhibited the tyrosine kinase activity of Flt-1 in a dose-dependent manner. However, the effective concentration of 5-S-GAD was 100 μg/ml (174 μM), and the effect was 4 times less than that of the positive control SU5416 at 10 μg/ml (42 μM).

4. Discussion

Our results provide the first evidence that 5-S-GAD inhibits embryonic and tumor cell-induced angiogenesis. Previously, we showed that 5-S-GAD is cytotoxic to some tumor cell lines and inhibits their growth in vivo (Akiyama et al., 2000). This compound possibly inhibits tumor growth in vivo through dual effects, namely, the cytotoxic and antiangiogenic effects.

In vitro experiments showed that 5-S-GAD did not suppress the proliferation, migration, and tube formation of endothelial cells but had a weak inhibitory effect on VEGF receptor autophosphorylation. However, it is difficult to explain the in vivo antiangiogenic activity of 5-S-GAD based on this inhibitory effect alone. The effective dose of 5-S-GAD for inhibiting VEGF-induced angiogenesis in vivo was 40 μM; this dose was 4 times less than that in the VEGF receptor tyrosine kinase assay (174 μM). In addition to the weak inhibition of Flt-1 autophosphorylation, other mechanisms may also contribute to the antiangiogenic activity of 5-S-GAD. 5-S-GAD may affect the activities of other VEGF receptors (Bikfalvi and Bicknell, 2002) or other receptor tyrosine kinases that promote angiogenesis (Mazitschek and Giannis, 2004).

In the mouse dorsal air sac assay, the neovessels observed in the 5-S-GAD-treated group appeared to be thinner than those in the control group. Since large blood vessels are surrounded by mural cells, we speculate that 5-S-GAD might inhibit the formation of larger blood vessels by affecting the mural cells but not that of small blood vessels composed of only endothelial cells. This may also be one of the possible reasons why dose-dependent inhibition of 5-S-GAD was not observed in this assay. To assess the antiangiogenic effects of this compound, another indicator, e.g., the vascular structure, may be required in addition to the number of blood vessels.

Finally, 5-S-GAD has the advantage of low toxicity. 5-S-GAD administered at 12.5 mg/kg/day i.p. suppressed the tumor cell-induced angiogenesis. This dose is much lower than the toxic dose of 5-S-GAD since a single i.p. administration of 5-S-GAD up to 8 g/kg does not result in toxic death. 5-S-GAD could be useful for the treatment of diseases caused by excessive angiogenesis and has fewer potential side effects. The mechanisms of the antiangiogenic effect exhibited by 5-S-GAD could not be explained; however, understanding the mechanisms may provide an insight into potential therapeutic targets for the treatment of angiogenesis-dependent diseases.

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