

Inhibition of Angiogenesis and Tumor Growth by a Synthetic Laminin Peptide, CDPGYIGSR-NH₂

Noritsugu Sakamoto, Michio Iwahana, Noriko G. Tanaka,¹ and Yasuaki Osada

Research Institute, Daiichi Pharmaceutical Co., Ltd., 16-13, Kitakasai 1-chome, Edogawa-ku, Tokyo 134, Japan

ABSTRACT

A laminin-derived synthetic peptide, Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂ (CDPGYIGSR-NH₂), containing an active site for cell binding inhibited both angiogenesis and solid tumor growth. It potently suppressed both embryonic angiogenesis of the chick chorioallantoic membrane and migration of vascular endothelial cells induced by a tumor-conditioned medium but neither the *in vitro* proliferation of endothelial cells nor that of tumor cells. Additionally, in *in vivo* tests, CDPGYIGSR-NH₂ markedly inhibited both the growth of s.c. solid tumor of Sarcoma 180 and that of Lewis lung carcinoma (3LL) in the lungs. On the contrary, ascitic tumor growth of Sarcoma 180 was not affected by this peptide, even though the same cell source was used. It was concluded that solid tumor growth inhibition by CDPGYIGSR-NH₂ was due not to a direct effect on cell growth but to an antiangiogenic effect mediated by the inhibition of endothelial cell migration.

INTRODUCTION

Angiogenesis, the formation of new blood vessels, is observed in physiological processes such as the development of embryos and in pathological processes such as wound healing, chronic inflammation, certain immune responses (1), and solid tumors (2). In solid malignant tumors, angiogenesis is necessary for their continued growth (3). Folkman (4) has hypothesized that the inhibition of angiogenesis might control tumor growth and obtained the following results. Until a tumor nodule was penetrated by new capillaries, the limitations imposed by diffusion of oxygen and nutrients prevented the tumor nodule from growing beyond a few mm in diameter (5).

Some studies have shown that basement membranes play important roles in angiogenesis (6-8). Basement membranes are mainly composed of collagen IV, fibronectin, heparin sulfate proteoglycan, and laminin. Of these, laminin has been demonstrated to have potent actions on the cells: promoting cell adhesion; migration; differentiation; and growth (9, 10). In 1987, it was demonstrated that an active site of laminin for some of these activities is composed of nine sequential amino acids: Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg (CDPGYIGSR) (11). CDPGYIGSR has been demonstrated to inhibit several biological activities of laminin by competing with laminin for cell surface receptors (11). Subsequent analysis of constituent peptides identified a pentapeptide, YIGSR (12), which was also active but to a lesser degree. On the basis of these findings, it has been shown that a synthetic laminin peptide prevents both the formation of lung tumor colonies by blocking the binding of tumor cells to basement membranes (12) and the morphological differentiation of endothelial cells into capillary-like structures (13, 14).

In the present study, we examined the inhibitory effects of a synthetic laminin peptide (CDPGYIGSR-NH₂) on angiogenesis

and tumor growth: (a) it was determined that this peptide might inhibit embryonic angiogenesis of the chick chorioallantoic membrane; (b) to clarify the mechanism of angiogenesis inhibition by CDPGYIGSR-NH₂, the effects of this peptide on the proliferation of cultured tumor cells and endothelial cells and on the migration of endothelial cells were examined; (c) the effects of CDPGYIGSR-NH₂ on the growth of both solid and ascitic tumors of Sarcoma 180 from the same cell source were compared, because angiogenesis is necessary for the continued growth of solid tumors (2) but unnecessary for the growth of ascitic tumors; (d) we compared the inhibitory effect of CDPGYIGSR-NH₂ on the lung colonization of Lewis lung carcinoma (3LL) mediated by angiogenesis inhibition with that mediated by blocking the binding of tumor cells to basement membranes.

MATERIALS AND METHODS

Agents. A synthetic laminin peptide, Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH₂ (CDPGYIGSR-NH₂), was purchased from Iwaki Glass (Tokyo, Japan). Before administration, it was diluted with PBS² to the appropriate concentration.

Mice and Tumors. Male ICR mice, 4 weeks of age, or male C57BL/6 mice, 4 weeks of age, were purchased from Japan SLC, Inc. (Shizuoka, Japan). Sarcoma 180 was obtained from the National Cancer Center, Tokyo, Japan, and was maintained in an ICR mouse by weekly i.p. inoculation. The origin and maintenance of Lewis lung carcinoma (3LL) have been described elsewhere (15).

CAM Assay for Angiogenesis Inhibition. The details of the CAM assay method were mentioned elsewhere (16). Fertilized Norin-cross chicken eggs (Funabashi Farm, Funabashi, Japan) were used. A group of 5 eggs was used for each dose. Two days after the addition of CDPGYIGSR-NH₂ (0.1, 1, 10 μg), angiogenesis of treated CAM was compared with that of the control. The doses required to inhibit 50% of CAM vascularization were calculated by probit analysis on the basis of

$$\frac{\text{Data of treatment group}}{\text{Data of control group}} \times 100\%$$

Assay for Inhibition of Endothelial Cell and Tumor Cell Growth. Cultured human umbilical vein endothelial cells (the third passages; Kurabo Co., Osaka, Japan) were seeded on 96-well plates at 10³ cells/well in modified MCDB131 medium (Kurabo Co.) containing 2% FCS and 4 ng/ml of bovine recombinant basic fibroblast growth factor (Amersham International Plc., Amersham, United Kingdom). After attachment of the cells, the medium was discarded and fresh medium containing various concentrations of CDPGYIGSR-NH₂ (10⁻⁹-10⁻² M) and 4 ng/ml basic fibroblast growth factor was added to the culture. On days 2 and 5, the culture medium was discarded and the same fresh medium with CDPGYIGSR-NH₂ as above was added. On day 7, the cells were treated with trypsin and counted in a cell counter (Coulter Electronics Ltd., Luton, United Kingdom). In an assay for growth inhibition of Sarcoma 180 or 3LL cells, 100 μl of cell suspension (1 × 10⁴ cells/ml) in RPMI 1640 (Nissui Seiyaku, Tokyo, Japan) or Eagle's minimal essential medium (Nissui Seiyaku), respec-

² The abbreviations used are: PBS, phosphate-buffered saline; CAM, chorioallantoic membrane; FCS, fetal calf serum.

Received 7/31/90; accepted 11/20/90.

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¹ To whom requests for reprints should be addressed.

tively, both containing 10% FCS, was added to each well of 96-well plates. On days 2 and 5, the culture medium was discarded and then 100 μ l of the medium containing CDPGYIGSR-NH₂ (10^{-9} – 10^{-2} M) were added. On day 7, the cells were counted in a cell counter. All experimental measurements were run in triplicate.

Assay for Inhibition of Endothelial Cell Migration. Migration of endothelial cells was assayed using Boyden chambers (Neuroprobe, Inc., Bethesda, MD). Sarcoma 180-conditioned medium was prepared according to the method of Folkman and Haudenschild (17). Test samples of various concentrations of CDPGYIGSR-NH₂ (10^{-9} – 10^{-2} M) in a modified MCDB131 medium containing 2% FCS with 50% tumor-conditioned medium were loaded into each lower well of Boyden chambers. Trypsin-treated endothelial cells were collected by centrifugation and resuspended in 2% FCS/modified MCDB131 medium. Cell suspensions (3×10^5 cells/ml) were loaded into each upper well of the chambers. The upper and lower wells were separated by a polyvinylpyrrolidone nucleopore filter (8- μ m pores; Nucleopore Corp., Pleasanton, CA) precoated with gelatin. The chambers were incubated at 37°C with 5% CO₂ for 5 h. At the end of incubation, the cells which had migrated through the pores to the lower filter surface were fixed in methanol, stained with Giemsa's solution (Merck, Darmstadt, West Germany), and counted in 100 \times microscope fields. Each determination was done in triplicate, and 5 fields of each filter were counted. Checkerboard analysis (18) was performed to study the nature of cell migration in response to a Sarcoma 180-conditioned medium. The assay was constructed by examining the migration of cells in the presence of increasing levels of attractant above and/or below. The efficacy of CDPGYIGSR-NH₂ was expressed as the concentration including 50% inhibition in comparison with the control.

Assay for Inhibition of Endothelial Cell Adhesion. Cell adhesion assays were carried out as described previously (11). To test the inhibition of endothelial cell attachment to basement membrane constituents (laminin, fibronectin, collagen IV) and to gelatin by CDPGYIGSR-NH₂, 24-well plates were coated with 5 μ g of each protein and dried. Prior to the addition of endothelial cells, CDPGYIGSR-NH₂ at various concentrations (25, 50, 100, 200 μ g/ml) in modified MCDB131 medium containing 2% FCS were added to each well. Then, endothelial cells in the same medium were added (10^4 /well), and incubated for 2 h at 37°C in 5% CO₂. At the end of this period, plates were gently washed three times with PBS to remove unattached cells. Attached cells were trypsinized and counted. All experimental measurements were run in triplicate.

Treatment of Mice Bearing Solid or Ascitic Tumor of Sarcoma 180. Sarcoma 180 cells were suspended in PBS at a rate of 5×10^7 cells/ml. A 0.1 ml of the suspension was inoculated s.c. into the right flank of the mice or inoculated i.p. into a mouse. Three days after inoculation, the mice with tumors were divided into groups of 6, and treatments with CDPGYIGSR-NH₂ were begun. CDPGYIGSR-NH₂ was administered i.v. once a day at a dose of 3 mg/head for 4 days. All mice were killed on day 7 after cell inoculation. In mice bearing solid tumor, the tumors were removed and weighed immediately after death. In mice bearing ascitic tumor, the ascites were collected and the number of ascitic tumor cells was counted.

Pretreatment of 3LL Cells with CDPGYIGSR-NH₂. A single cell suspension from solid tumors was prepared through enzymatic treatment using 0.14% collagenase and 0.03% DNase (Sigma Chemical Co.) as described previously (19), and pretreated with CDPGYIGSR-NH₂ according to the method of Iwamoto *et al.* (12). A 0.1-ml sample of the suspension containing 5×10^6 cells and 3 mg of the peptide was inoculated into the tail vein of mice. Eight mice in each group were autopsied on the 14th day after implantation and their lung weights were measured.

Posttreatment of Mice Inoculated with 3LL Cells. A single cell suspension was prepared by the same method as above. Tumor cells were suspended in PBS at a rate of 5×10^6 cells/ml. A 0.1-ml sample of the suspension was inoculated i.v. into the tail vein of mice. Four days after inoculation, treatments with CDPGYIGSR-NH₂ started. CDPGYIGSR-NH₂ was administered i.v. at a dose of 3 mg/head/day

for 10 days. Eight mice in each group were autopsied on the 14th day after implantation and their lung weights were measured.

RESULTS

Effect of CDPGYIGSR-NH₂ on Embryonic Angiogenesis. CDPGYIGSR-NH₂ suppressed embryonic CAM angiogenesis dose dependently (Fig. 1). As shown in Fig. 1, 1.13 μ g of this peptide were enough to inhibit 50% of CAM capillarization.

In Vitro Effects of CDPGYIGSR-NH₂ on Growth of Endothelial Cells or Tumor Cells. CDPGYIGSR-NH₂ affected neither the *in vitro* proliferation of endothelial cells nor that of tumor cells at the doses given (10^{-9} – 10^{-2} M) (Fig. 2).

Inhibition of Endothelial Cell Migration by CDPGYIGSR-NH₂. Checkerboard analysis revealed that the endothelial cell migration induced by Sarcoma 180-conditioned medium was due to chemotaxis and not to chemokinesis (data not shown). About 6-fold increase of the migration of endothelial cells was observed in the presence of 50% Sarcoma 180-conditioned medium. CDPGYIGSR-NH₂ suppressed endothelial cell chemotaxis dose dependently, giving an ID₅₀ of 5×10^{-7} M (Fig. 3).

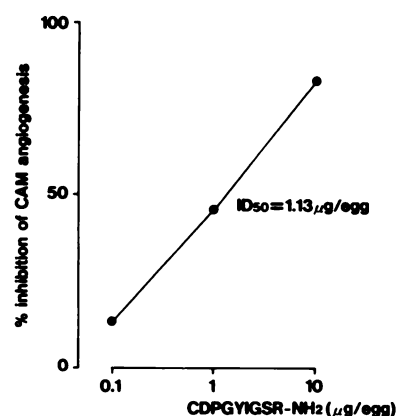


Fig. 1. CAM angiogenesis inhibition by CDPGYIGSR-NH₂. Ten μ l of solution containing CDPGYIGSR-NH₂ (0.1, 1, 10 μ g) were added to CAM. Two days after the addition of agents, angiogenesis of treated CAM was compared with that of the control. The doses required to inhibit 50% of CAM vascularization (ID₅₀) were calculated by probit analysis on the basis of

$$\frac{\text{Data of treatment group}}{\text{Data of control group}} \times 100\%$$

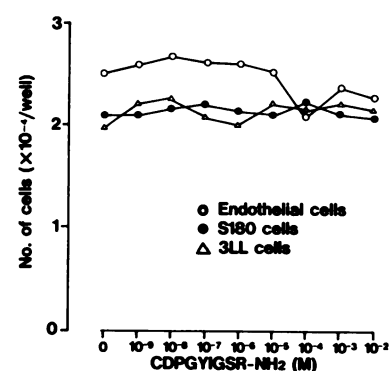


Fig. 2. *In vitro* effects of CDPGYIGSR-NH₂ on the growth of endothelial cells and tumor cells. Cell suspension (100 μ l; 1×10^4 cells/ml of endothelial cells or tumor cells) were added to each well of 96-well plates. On days 2 and 5, 100 μ l of each of medium containing CDPGYIGSR-NH₂ (10^{-9} – 10^{-2} M) were added to the culture. On day 7, the cells were treated with trypsin and counted.

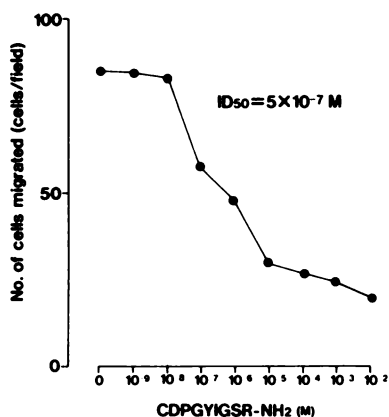


Fig. 3. Inhibition of endothelial cell migration by CDPGYIGSR-NH₂. Migration of endothelial cells was assayed using Boyden chambers. Test samples of various concentrations of CDPGYIGSR-NH₂ (10⁻⁹-10⁻² M) in modified MCDB131 medium containing 2% FCS with Sarcoma 180 conditioned medium were loaded into each lower well of Boyden chambers. Cell suspensions (3 × 10⁵ cells/ml) were loaded into each upper well of the chambers. The upper and lower wells were separated by a polyvinylpyrrolidone nucleopore filter (8-μm pores) precoated with gelatin. Five h after incubation, the cells which had migrated to the lower filter surface were counted in 100× microscope fields. Each determination was done in triplicate, and 5 fields of each filter were counted. ID₅₀, 50% inhibitory concentration.

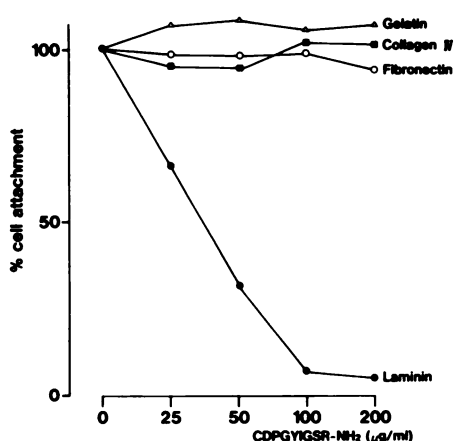


Fig. 4. Effect of CDPGYIGSR-NH₂ on endothelial cell attachment. To test the inhibition of endothelial cell attachment to laminin, fibronectin, collagen IV, or gelatin by CDPGYIGSR-NH₂, 24-well plates were coated with 5 μg of each protein and dried. Prior to the addition of endothelial cells, CDPGYIGSR-NH₂ at various concentrations (25, 50, 100, 200 μg/ml) in modified MCDB131 medium containing 2% FCS was added to each well. Then, endothelial cells in the same medium were added (10⁶/well). Two h after incubation, plates were washed with PBS to remove unattached cells. Attached cells were trypsinized and counted.

Effect of CDPGYIGSR-NH₂ on Endothelial Cell Attachment. Endothelial cells adhered equally well to each basement membrane constituent (laminin, fibronectin, collagen IV, or gelatin) and CDPGYIGSR-NH₂ effectively prevented attachment to laminin but not to other constituents (Fig. 4). This pattern of inhibition by the peptide is in agreement with the results of Grant *et al.* (13).

Effects of CDPGYIGSR-NH₂ on Growth of Solid and Ascitic Tumors of Sarcoma 180. CDPGYIGSR-NH₂ significantly inhibited the growth of solid tumor of Sarcoma 180 (*P* < 0.001 *versus* control by Student's *t* test) but not that of ascitic tumor of Sarcoma 180 (Table 1). The mean body weight of the treated mice was almost equal to that of the control mice (data not shown).

Table 1 Effects of CDPGYIGSR-NH₂ on growth of both solid and ascitic tumors of Sarcoma 180^a

Treatment	Solid tumor ^b [tumor wt (mg)]	Ascitic tumor ^c [no. of ascitic S180 cells (×10 ⁶)]
Controls	438 ± 44	6.04 ± 0.20
CDPGYIGSR-NH ₂	187 ± 25 ^d	6.07 ± 0.17

^a Each numerical expression represents the mean ± SE. The statistical significance was evaluated by Student's *t* test.

^b Groups of 6 ICR mice were inoculated s.c. with 5 × 10⁶ Sarcoma 180 cells. CDPGYIGSR-NH₂ was administered i.v. once a day at a dose of 3 mg/head for 4 days. All mice were killed on day 7 after cell inoculation, and the tumors were removed and weighed.

^c Six ICR mice in each group were inoculated i.p. with 5 × 10⁶ Sarcoma 180 cells. CDPGYIGSR-NH₂ was administered i.v. once a day at a dose of 3 mg/head for 4 days. After all mice were killed on day 7 after cell inoculation, the ascites was collected and the number of ascitic tumor cells was counted.

^d *p* < 0.001 *versus* controls.

Table 2 Effects of CDPGYIGSR-NH₂ on pulmonary metastasis

Treatment	Tumor wt in the lungs (mg) ^a
Controls	413 ± 30
Pretreatment ^b	8 ± 6 ^c
Posttreatment ^d	77 ± 17 ^c

^a Each numerical expression represents the mean ± SE of the tumor weight in the lungs.

Tumor wt in the lungs

$$= \text{Lung wt of a tumor-bearing mouse} - \text{normal lung wt}$$

The statistical significance was evaluated by Student's *t* test.

^b Eight C57BL/6 mice in each group were inoculated i.v. with a cell suspension containing 5 × 10⁶ 3LL cells and 3 mg of CDPGYIGSR-NH₂. All mice were autopsied on the 14th day after implantation and their lung weights were measured.

^c *P* < 0.001 *versus* controls.

^d Eight C57BL/6 mice in each group were inoculated i.v. with 5 × 10⁶ 3LL cells. Four days after inoculation, treatments with CDPGYIGSR-NH₂ started. CDPGYIGSR-NH₂ was administered i.v. at a dose of 3 mg/head/day for 10 days. All mice were autopsied on the 14th day after implantation and their lung weights were measured.

Inhibitory Effects of CDPGYIGSR-NH₂ on the Formation of Lung Tumor Colonies. CDPGYIGSR-NH₂ showed outstanding decreases in the weight of lung colonies by 98% when administered with pretreated 3LL cells (Table 2). Also posttreatment of mice with the peptide prevented tumor growth in the lungs to a significant degree (Table 2).

DISCUSSION

Kubota *et al.* (14) found that a synthetic laminin peptide corresponding to the receptor binding-cell attachment site (YIGSR) blocked the morphological differentiation of endothelial cells into capillary-like structures. Grant *et al.* (13) then confirmed the inhibitory effect of YIGSR on tube formation. In the present study, which used a laminin-derived synthetic peptide, CDPGYIGSR-NH₂, it was confirmed that this peptide markedly inhibited embryonic angiogenesis of the chick chorioallantoic membrane (Fig. 1).

Angiogenesis (or neovascularization) denotes the formation of new blood vessels. It is now recognized that angiogenesis takes place as a series of sequential steps (20): (a) an early morphological step is the local degradation of the basement membrane of the parent vessel; (b) endothelial cells pseudopod, protrude through these holes in the basement membrane; (c) endothelial cells migrate toward an angiogenic stimulus; (d) the cells align in a bipolar configuration to form a sprout; (e) DNA

synthesis and mitosis of endothelial cells begin; (*f*) a lumen is then formed by each endothelial cell in the sprout and individual sprouts soon join with each other to form a loop through which blood begins to flow. An angiogenesis inhibitor is considered to inhibit one or more of these angiogenesis steps and consequently inhibits new vascular formation. To investigate which step in the angiogenic series was inhibited by CDPGYIGSR-NH₂, cultured vascular endothelial cells were used in the present study. CDPGYIGSR-NH₂ potently suppressed the migration of vascular endothelial cells induced by tumor-conditioned medium (Fig. 3) but not the proliferation of endothelial cells (Fig. 2). As shown in Fig. 4, CDPGYIGSR-NH₂ did not affect endothelial cell attachment to gelatin at all. Thus, the inhibitory effect of endothelial cell migration by the peptide was not caused by the inhibition of cell attachment to nucleopore filters precoated with gelatin. These results suggest that an antiangiogenic effect of CDPGYIGSR-NH₂ was caused by the inhibition of endothelial cell migration.

We next examined the effects of CDPGYIGSR-NH₂ on the growth of both solid and ascitic tumors of Sarcoma 180. The peptide significantly inhibited the growth of solid tumor of Sarcoma 180 (Table 1). However, this peptide did not affect ascitic tumor growth at all, even though the same cell source was used (Table 1). Solid tumors require angiogenesis for their continued growth (3), but ascitic tumors do not. Furthermore, CDPGYIGSR-NH₂ did not affect the *in vitro* growth of Sarcoma 180 cells at all (Fig. 2). Therefore, it was concluded that the inhibition of solid tumor growth was due not to a direct effect on cell growth but to the antiangiogenic effect of CDPGYIGSR-NH₂.

We further showed that CDPGYIGSR-NH₂ also inhibited pulmonary metastasis both when administered with pretreated 3LL cells and when the treatment started after *i.v.* injection with 3LL cells (Table 2). The former result is in agreement with the finding of Iwamoto *et al.* (12). They reported that the inhibition of lung tumor colonization by pretreatment of tumor cells with CDPGYIGSR-NH₂ was caused by the blocking of cell binding to basement membranes, followed by prevention of the invasion of tumor cells through basement membranes. On the other hand, the latter appears to result from a different effect of the peptide. The treatment started on the 4th day after *i.v.* injection of 3LL cells, when all tumor cells had probably been eliminated from the blood stream, because the previous study (21) showed that almost all pulmonary emboli and thrombi disappeared completely within 48 h after cell injection, and mitosis of extravasated tumor cells was observed as early as 24 h after 3LL cell inoculation. Therefore, the posttreatment must affect only secondary tumor growth in the process of pulmonary metastasis. In addition, angiogenesis is necessary for the growth of pulmonary metastatic tumors as well as for the growth of primary tumors (22), and CDPGYIGSR-NH₂ did not affect the *in vitro* growth of 3LL cells at all (Fig. 3). These findings suggest that CDPGYIGSR-NH₂ inhibited secondary tumor growth in the lungs mediated by angiogenesis inhibition, as well as *s.c.* tumor growth of Sarcoma 180 (Table 1).

In conclusion, a laminin-derived synthetic peptide, CDPGYIGSR-NH₂, neither affected tumor cells directly nor

prevented endothelial cells from growing but inhibited endothelial cell chemotaxis specifically. It was suggested that solid tumor growth inhibition by CDPGYIGSR-NH₂ was caused by an antiangiogenic effect mediated by the inhibition of endothelial cell migration. Further investigation will be required in order to clarify the inhibitory mechanisms of CDPGYIGSR-NH₂ against endothelial cell migration and to prove the inhibitory effect on endothelial cell migration in tumor masses *in situ*.

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