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

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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 vitro 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 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 ng), 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

\[
\text{Data of treatment group} \times 100\% \quad \text{Data of control group}
\]

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 (10⁻⁵–10⁻² 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.

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Antitumor Effect of a Synthetic Laminin Peptide

Effect of CDPGYIGSR-NH$_2$ on Embryonic Angiogenesis. CDPGYIGSR-NH$_2$ suppressed embryonic CAM angiogenesis dose dependently (Fig. 1). As shown in Fig. 1, 1.13 $\mu$g of this peptide were enough to inhibit 50% of CAM capillarization.

In Vitro Effects of CDPGYIGSR-NH$_2$ on Growth of Endothelial Cells or Tumor Cells. CDPGYIGSR-NH$_2$ 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-NH$_2$. 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-NH$_2$ suppressed endothelial cell chemotaxis dose dependently, giving an ID$_{50}$ of $5 \times 10^{-7}$ M (Fig. 3).

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Fig. 2. In vitro effects of CDPGYIGSR-NH$_2$ on the growth of endothelial cells and tumor cells. Cell suspension (100 $\mu$l; $1 \times 10^6$ cells/ml of endothelial cells or tumor cells) were added to each well of 96-well plates. On days 2 and 5, 100 $\mu$l of each of medium containing CDPGYIGSR-NH$_2$ ($10^{-9}$-$10^{-2}$ M) were added to the culture. On day 7, the cells were treated with trypsin and counted.

Fig. 3. CAM angiogenesis inhibition by CDPGYIGSR-NH$_2$. Ten $\mu$l of solution containing CDPGYIGSR-NH$_2$ (0.1, 1, 10 $\mu$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 (ID$_{50}$) were calculated by probit analysis on the basis of

Data of treatment group

Data of control group

No. of cells (X 10$^3$/well)

CDPGYIGSR-NH$_2$ (M)

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

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-NH$_2$, 24-well plates were coated with 5 $\mu$g of each protein and dried. Prior to the addition of endothelial cells, CDPGYIGSR-NH$_2$, at various concentrations (25, 50, 100, 200 $\mu$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% CO$_2$. 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 X 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-NH$_2$ were begun. CDPGYIGSR-NH$_2$ 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.

Pre-treatment of 3LL Cells with CDPGYIGSR-NH$_2$. 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-NH$_2$ according to the method of Iwamoto et al. (12). A 0.1 ml sample of the suspension containing $5 \times 10^3$ 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.

Post-treatment 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 $5 \times 10^6$ 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-NH$_2$ started. CDPGYIGSR-NH$_2$ 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.

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Data of treatment group

Data of control group

No. of cells (X 10$^3$/well)

CDPGYIGSR-NH$_2$ (M)

For 10 days. Eight mice in each group were autopsied on the 14th day after implantation and their lung weights were measured.
Tumors of Sarcoma 180. CDPGYIGSR-NH2 significantly inhibited the growth 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 ascitic tumors of Sarcoma 180**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ascitic tumor [no. of ascitic S180 cells (×10^3)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>413 ± 30</td>
</tr>
<tr>
<td>CDPGYIGSR-NH2</td>
<td>8 ± 6†</td>
</tr>
</tbody>
</table>

"Each numerical expression represents the mean ± SE. The statistical significance was evaluated by Student’s t test.

"p < 0.001 versus controls.

**Table 2 Effects of CDPGYIGSR-NH2 on pulmonary metastasis**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumor wt in the lungs (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>413 ± 30</td>
</tr>
<tr>
<td>Pretreatment†</td>
<td>8 ± 6†</td>
</tr>
<tr>
<td>Posttreatment‡</td>
<td>77 ± 17‡</td>
</tr>
</tbody>
</table>

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

The statistical significance was evaluated by Student’s t test.

† Eight C57BL/6 mice in each group were inoculated i.v. with 5 × 10^6 3LL cells and 3 mg of CDPGYIGSR-NH2. All mice were autopsied on the 14th day after implantation and their lung weights were measured.

‡ p < 0.001 versus controls.

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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.

REFERENCES

Inhibition of Angiogenesis and Tumor Growth by a Synthetic Laminin Peptide, CDPGYIGSR-NH$_2$

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