Identification of a Potent Peptide Antagonist to an Active Laminin-1 Sequence That Blocks Angiogenesis and Tumor Growth

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ABSTRACT

The extracellular matrix plays an important role in many physiological processes. We have identified >20 angiogenic sites in the extracellular matrix protein laminin-1. The most potent sites are A13 (RQVFQVAYH-IKA) and C16 (KAFDITYVRLKF), which are present in homologous NH2-terminal domains of the α1 and γ1 chains, respectively. We reported recently that a scrambled C16 sequence, C16S (DFKLFAVTKYR), acts as an antagonist to both peptides. Here, we have identified a stronger antiangiogenic peptide, C16Y (C16S with a T to Y substitution), with potent activity in several biological assays including tumor growth. C16Y is more potent in promoting endothelial cell attachment and inhibiting attachment to laminin-1 than either C16 or C16Y. Disruption of tube formation by C16Y is also observed at concentrations at least five times lower than C16. The minimal active sequence was found to be DFKLFAYVY. C16Y is more potent in blocking C16-induced chick chorioallantoic membrane angiogenesis than C16S. Tumor growth studies on the chick chorioallantoic membrane showed that C16Y reduces breast cancer cell growth without affecting cell proliferation. This result suggests that angiogenesis is being inhibited by the peptide. In vivo animal studies demonstrated that C16Y treatment significantly reduced tumor growth and decreased tumor vessel number, as compared with controls, additionally suggesting that angiogenesis was affected. These results indicate that we have identified a more potent antiangiogenesis inhibitor peptide that may be used as a therapeutic to treat cancer.

INTRODUCTION

The ECM is vital for the maintenance and differentiation of many cell types including the endothelium, and it plays a role during the formation of new vessels from pre-existing ones in a process known as angiogenesis (1, 2). The endothelial cells of blood vessels generally remain in a quiescent state until they receive an angiogenic signal from their microenvironment that is triggered by a wound, inflammation, or disease, such as rheumatoid arthritis or cancer. The angiogenic signal activates the endothelium and elicits a cascade of events that leads to the formation of new vessels. One of the first steps of this cascade is the degradation of the ECM, which is composed of large proteins, including collagen, laminin, proteoglycans, and glycosaminoglycans, among others (3). In addition, basement membranes also serve as storage depots for many angiogenic growth factors, such as bFGF and epidermal growth factor. Therefore, the degradation of the matrix leads to the release of angiogenic factors that can, in turn, propagate this cascade. Several ECM protein fragments have been identified to modulate angiogenesis. For instance, a Mr 25,000 thrombospondin fragment has been shown to promote angiogenesis, whereas several other ECM fragments can inhibit it, including en-dostatin (derived from collagen XVIII), angiotatin (from plasminogen), the noncollagenous domains of collagen IV, and several thrombospondin peptides (4–8).

In our laboratory, we have also identified >20 peptides from laminin-1, a large ECM protein composed of three chains, α1, β1, and γ1, that can promote angiogenesis in vivo (9, 10). Two of the most potent sites, A13 and C16, are redundant angiogenic sites present in homologous regions of the α1 and γ1 chain, respectively (11–13). These sequences bind to the endothelial cell integrins αvβ3 and α5β1 (14). These peptides have also been shown to promote adhesion, tube formation, and angiogenesis in the chick CAM assay. Although these sequences bind to integrins, they do not seem to signal through mitogen-activated protein kinase or several serine or threonine kinases, and their mechanism of action has not yet been identified. The γ1 chain is present in 11 of the laminins; therefore, C16 is present in at least 11 of the 13 laminins (15). The A13 sequence is also highly conserved in the α chains; therefore, this sequence is present twice in some of the laminins including laminin-1 and -3 (16). Because of the putative significance of this site in angiogenesis and its related diseases, it has been important to identify an antagonist that could block its activity. We have reported previously a scrambled peptide sequence, C16S, that can inhibit C16 and bFGF-induced angiogenesis in the CAM assay (14). Here, we have identified a modified, and at least five times more potent sequence, C16Y, that can inhibit in vivo angiogenesis and tumor growth in mice. Furthermore, we have identified its minimum active sequence and have determined that it has homology to fibronectin.

MATERIALS AND METHODS

Peptides. All of the peptides were synthesized by the CBER Facility for Biotechnology Resources (Food and Drug Administration, Bethesda, MD) or at Hokkaido University as described previously (16). The peptides contained an NH2-terminal amide and included C16, C16S, C16D, C16L, and C16Y, and C16-3 (Table 1). C16S, C16D, C16L, and C16-3 are scrambled peptides of C16, and C16Y is identical to C16S except that it has a threonine to tyrosine substitution. Various truncated peptides were also similarly prepared for determination of the minimal active sequence. Despite hydrophobic amino acids, all of the peptides were water-soluble. Laminin-1 was obtained from Collaborative Research (Bedford, MA).

Isolation and Culture of Endothelial Cells. Endothelial cells were isolated by collagenase treatment from HUVECs and were cultured as described previously (17). Only those cells from passages 3–5 were used.

Cell Adhesion and Competition Experiments. Cell adhesion assays were performed on 96-well plates coated overnight with either 0.5 μg of laminin-1 or synthetic laminin peptides (0–5 μg) as described previously (9). Wells were rinsed with PBS, blocked with 2 mg/ml of BSA, and rinsed again with PBS. Cell adhesion was performed in 0.1 ml of RPMI 1640 containing 35,000 HUVECs. Competition experiments were done in laminin-1-coated plates in the presence of 0–100 μg/ml of competing peptide as specified. Controls were wells coated only with BSA. After a 1.5-h incubation at 37°C, unbound cells were decanted, and attached cells were fixed and stained with 20% methanol/0.2% crystal violet. Dishes were extensively rinsed, and bound dye was solubilized in 2% SDS and quantitated at 600 nm. Assays were done in triplicate at least three times.
Endothelial Cell Tube Formation. Tube-forming assays were performed as described previously (14). Matrigel (200 μg/well) was used to coat 48-well dishes. HUVECs (24,000 cells/well) were plated in RPMI 1640 containing 10% bovine calf serum, defined and supplemented (HyClone Laboratories, Inc., Logan, UT), and 100 μg/liter of endothelial cell growth factor (Collaborative Biomedical). Tube assays were performed using 0–75 μg/ml of peptide as specified. Controls contained only medium. Cells were fixed and stained after 16 h with Diff-Quick fixative (methanol) and solution II [6.25% (w/v) each of azure A and methylene blue; Dade AG, Dadingen, Switzerland], and the tubes were scored by a blinded observer. Peptides were tested at least in triplicate and the assays were repeated a minimum of three times.

Chick CAM Assay. The CAM assay was performed using 10-day-old embryonated eggs (CBT, Charlestown, MD) as described previously (18). On embryonal day 3, ~4 ml of ovalbumin was removed from each egg. After opening windows on embryonal day 10, the angiogenic stimulus, C16, and test competitor peptides in 5 μl of distilled water were applied to the CAM after drying on 13-mm diameter quartered plastic coverslips (Thermanox; Nalge, NUNC International, Naperville, IL). Three days later, the eggs were scored for a positive response and photographed. The positive control was bFGF, and the negative control was the vehicle water. Experiments were repeated twice using a minimum of 11 eggs for each data point.

Tumor Growth. MDA-231 breast cancer cells (5 × 10^3 cells; a gift of Dan Welch, Pennsylvania State University, University Park, PA) mixed (1:4) with Matrigel were injected s.c. into nude mice. Daily peptide i.p. injections were started when the tumors reached a volume of 200–300 mm^3 (days 7–9 after initial tumor injection). Peptides injected included C16 (1 mg/day) and C16Y at 0.2, 0.5, and 1.0 mg/day. Controls were mice injected daily with vehicle (water). Tumor growth was monitored with a caliper, and volume was determined by using the formula width^2 × length × 0.52. Tumors were excised, weighed, and fixed with formalin at the end of the experiment. Vessel number was determined by staining sections with a CD-31 antibody (16) and counting 6 fields per section per mouse (n = 2 sections/mouse; total 3 mice).

We also investigated the growth of MDA-MB-231 tumor cells using the chick CAM (19). Tumor cells (1 × 10^6 cells/0.1 ml) in RPMI 1640 were mixed with the indicated amount of peptide, and then mixed 1:1 with a neutralized collagen I solution (4.6 mg/ml collagen type I) and added directly onto the CAM of a 7-day-old embryo. After a 7-day incubation, tumors were excised and weighed. This experiment was repeated twice with each data point tested in at least six replicates.

Cell Proliferation. Proliferation of MDA-MB-231 cells was quantified using a Cell Titer 96 Aqueous Cell Proliferation assay kit (Promega, Madison, WI). Cells were plated on four 96-well dishes at 5 × 10^3 cells/well and cultured in AIM-V serum-free medium (Life Technologies Inc., Gaithersburg, MD). After 1 h, peptides C16 and C16Y were added at a final concentration of 100 μg/ml. A separate dish was used to quantitate proliferation at 2, 24, 48, and 72 h by reading absorbance at 490 nm on an Emax plate reader. Each experiment was repeated in triplicate two times.

RESULTS

Identification of Peptides That Promote Endothelial Cell Adhesion and Inhibit Attachment to Laminin-1. We found previously that a laminin γ1 chain-derived peptide, C16, was angiogenic in vivo and that a scrambled version of this peptide, C16S, had strong antiangiogenic activity (10, 11). Here, several scrambled C16 peptide sequences were synthesized to determine whether we could identify another sequence that would possess a more potent antiangiogenic activity than that observed with C16S (Table 1). We first investigated the cell attachment activity of the new C16 scrambled peptides. Different peptide amounts were coated on 96-well dishes ranging from 1 ng to 3 μg/well, and their activities were compared. Cells attached to C16, the parent peptide, and to C16S at amounts >0.1 μg (Fig. 1A). Although C16Y showed a similar attachment pattern to that of C16 and C16S, more cells adhered to it than to any other peptide at all of the amounts tested. Endothelial cells did not attach to C16L at any dose, and the cells only weakly bound to those wells coated with the highest amounts of C16J.

The peptides were also used in competition experiments to determine whether soluble peptide could inhibit cell attachment to laminin-1. Once again, it was found that C16Y had the strongest inhibitory activity at all concentrations tested (Fig. 1B). The most significant differences were observed at 50 μg/ml where C16Y inhibited attachment by >70%, whereas C16 and C16S inhibited by <40%. C16J and C16L showed weak activity at this concentration. Furthermore, C16J had little activity at all of the doses used, and C16L showed some inhibition at doses >75 μg/ml. The results of these experiments suggest that C16Y can promote cell attachment and can more strongly compete with laminin-1 for binding than C16, C16S, or other scrambled peptides.

C16Y Most Actively Disrupts Tube Formation. Endothelial cells form capillary-like structures when placed on Matrigel, a basement membrane matrix (19, 20). Disruption of endothelial cell tube formation is highly suggestive of the ability of a compound to either promote or inhibit angiogenesis (10, 11). We had determined previously that peptide C16, which is angiogenic, and C16S, its antagonist, were active in this assay at doses ≥50 μg/ml (14). To identify other
peptides that might possess a stronger tube-disrupting activity, a tube-forming assay was used in which soluble peptide was added at doses that ranged between 10 and 75 μg/ml. As expected, C16 and C16S disrupted tube formation at 50 and 75 μg/ml, with little or no activity observed at lower doses (Fig. 2). In contrast, C16Y strongly disrupted tube formation at all of the doses tested, even as low as 10 μg/ml, a dose five times lower than that at which C16 or C16S are active. The other peptides, C16J, C16-3 (not shown), and C16L, showed no activity at the lower doses and only slight tube disruption at higher concentrations.

Truncated peptides were tested in the tube assay for determination of the minimal active sequence (Table 2). The carboxyl end of the peptide was not necessary for activity, whereas the amino terminal aspartic acid was required, yielding a minimal active sequence of DFKLFAVY.

**C16Y Is a Strong Angiogenesis Inhibitor.** The ability of C16Y to disrupt tube formation at a concentration five times lower than C16S strongly suggested that this peptide might be a more potent angiogenesis inhibitor. We used the CAM assay as an *in vivo* animal system to determine the relative angiogenic activity of C16Y (Fig. 3). As shown previously, 0.5 μg of C16 was able to induce angiogenesis in the CAM (Fig. 3A, panel d). When a mixture containing 0.5 μg of C16 and either 0.2 μg of C16S (Fig. 3A, panel b) or C16Y (panels e, 0.2 μg and f, 0.05 μg) were mixed with C16. Panel a is control vehicle and d is C16 alone. B, dose response of angiogenic CAMs in the presence of bFGF and peptides. Control is the number of angiogenic CAMs induced by bFGF alone (□). □ are a mixture of C16S (0.05, 0.1, and 0.2 μg) and 50 ng of bFGF; □ are a mixture of C16Y (0.05, 0.1, and 0.2 μg) and 50 ng of bFGF; bars, ±SD.

![Fig. 2. Effect of C16 and various scrambled C16 peptides on endothelial cell tube formation on Matrigel. Tube formation was assessed after an 18-h incubation. All peptides were tested at ranges varying between 10 and 75 μg/ml. C16S shows tube disruption at concentrations higher than 50 μg/ml. C16Y disrupted tube formation at all concentrations shown, lower concentrations had no effect on tubes. C16J did not affect tube formation at any concentration. Peptide activity of C16L and C13 are shown on the table.]

**Table 2.** Tube-disrupting activity of truncated C16Y peptides for determination of minimal active sequence as DFKLFAVY

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<th>Sequence</th>
<th>Activity</th>
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<tr>
<td>FKLFAYVYIKY</td>
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<tr>
<td>KLFAYVYIKY</td>
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<td>LFAYVYIKY</td>
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<td>YIKY</td>
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<td>DFKLFAVYIKY</td>
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As expected, C16Y (Fig. 3A, panel e) or C16S alone did not stimulate angiogenesis (data not shown) similar to the control vehicle (Fig. 3A, panel a).
C16Y Inhibits CAM Tumor Growth but not Tumor Cell Proliferation in Vitro. Because peptide C16Y inhibited C16-induced angiogenesis in the CAM assay, we investigated whether tumor growth could also be affected by C16Y using two different in vivo systems. First, we used the chick CAM to overlay a collagen I gel containing MDA-MB 231 tumor breast cells mixed with 0, 50, or 100 μg/ml of peptides C16 or C16Y. After 7 days, the membrane was fixed, and the tumor cells were removed and weighed (Fig. 4). In the absence of peptide, the tumor grew to an average weight of 5.40 ± 0.59 mg. The presence of either 50 or 100 μg/ml of the angiogenic peptide C16 did not considerably affect its growth, 5.45 ± 0.84 mg and 5.85 ± 0.54 mg, respectively. At both concentrations, C16Y inhibited tumor growth by ~40% of its control C16 (P < 0.05). More vessels were observed in the untreated tumors and in those tumors treated with C16 than in the tumors treated with C16Y (7.5 ± 0.45 versus 9.6 ± 0.23 versus 5.1 ± 0.16; P ≤ 0.027). Whether the difference in tumor growth was because of inhibition of angiogenesis or because of an effect on cell proliferation was still uncertain.

We performed a proliferation assay in the presence of 100 μg/ml of soluble peptides to determine whether these peptides directly affected tumor cell proliferation (data not shown). Neither C16 nor C16S altered MDA-MB 231 cell growth, suggesting that inhibition in tumor growth is because of an effect on angiogenesis rather than on cell proliferation. Lower doses of peptides were also without effect on cell proliferation (data not shown).

C16Y Inhibits the Growth of Primary Tumors. To additionally confirm the antiangiogenic effect of C16Y, we used a second in vivo system in which nude mice bearing a pre-established 200–300 mm³ breast carcinoma tumor were treated for 7–10 days with peptide. Tumor growth was either significantly (P < 0.028) inhibited (Fig. 5A) or its size was reduced to ~33% after a week of treatment with 1 mg of C16Y (Fig. 5B). In contrast, during the same period, control tumors and those of animals treated with C16 grew 1.7–2.5 times their initial size. Although we had expected C16 animal tumors to grow more rapidly than the control, it was interesting to note that C16 treatment did not significantly affect tumor growth (Fig. 5, A and B). This could be explained by the production of high levels of growth factors that are endogenously secreted by the tumor itself. Lower C16Y doses (0.2 and 0.5 mg) also led to a dose-dependent reduction of initial tumor burden (Fig. 5B). However, when daily administration of the antiangiogenic peptide was stopped on day 7, the tumors began to grow rapidly (Fig. 5B). The tumors of those animals treated with the lowest C16Y dose (0.2 mg) grew the fastest, approaching control tumor volume within 3 days. Tumors of animals that received 0.5 mg of C16Y daily grew more slowly, and those that received the highest doses reached initial tumor size ~6 days after peptide treatment was stopped. These results strongly suggest that C16Y can reduce tumor growth via its antiangiogenic activity.

DISCUSSION

The basement membrane-derived glycoprotein laminin-1 is very biologically active (21). It increases cell adhesion, migration, differentiation, tumor growth, and angiogenesis via multiple active sites. Many cellular receptors have been identified (22). Using systematic peptide screening, we have found several active sites on laminin-1, with peptide C16 being the most active on the γ1 chain (10–12). This site recognizes integrins α5β1 and αvβ3. A scrambled version of this peptide, C16S, was created as a negative control but had unexpected antagonist activity (14). The scrambled peptide bound to the receptor and promoted cell adhesion, but blocked adhesion to the parent peptide C16 and to laminin-1. This peptide also blocked peptidase- and fibroblast growth factor-mediated angiogenesis in the chick CAM assay. Here, we have searched for a more potent antagonist using the sequence present in C16S and making different amino acid substitutions. One peptide, C16Y, which contains a single amino acid substitution of a T to a Y, is 5–10-fold more potent in its antiangiogenic activity than C16S. This peptide is also active in vivo and can inhibit tumor growth in a dose-dependent manner when given daily as an i.p.
injection. C16Y likely acts on tumor growth by blocking angiogenesis, because it had no effect on tumor cell proliferation in vitro.

Peptide C16Y is not toxic and may interfere with the normal functions of laminin-1, as well as other molecules that use the integrin α5β1 and αvβ3 receptors. Angiogenesis has been shown to require the activity of integrin αvβ3 (23, 24). Laminin-1 has many biological activities that would promote tumor growth and angiogenesis (25).

Laminin-1 promotes cell adhesion, migration, invasion, and protease activity, and laminin-1-adherent cells are more malignant than non-adherent cells or fibronectin-adherent cells. Because several laminin-1 peptides can modulate tumor growth and angiogenesis in vivo, endogenous laminin-1 is likely physiologically active in vivo during tumor growth and angiogenesis, although this has not yet been demonstrated directly in vivo. We believe that the C16 site on laminin-1 is functionally important in vivo in both tumor growth and angiogenesis, and that this active site on laminin-1 is blocked by the C16Y peptide. In our previous screens for finding active sites for malignancy on laminin-1, C16 and its homologue on the α chain, A13, were identified as being the most potent on their respective chains (9, 10, 13, 16, 26). Furthermore, either or both of these redundant active sites are present in all of the laminin-1 isoforms.

When initially preparing control peptides for C16, many different randomly scrambled peptides were prepared. Most were inactive, but some had low activity, and C16S had the highest activity (12). A comparison study of the C16Y sequence (DFKLFAVYIKYR) was made for amino acid homology to other short sequences using National Center for Biotechnology Information Protein BLAST search, and the results indicate that there was 66% (8 of 12) identity to the fibronectin sequence (residues 1023–1033) in c-elegans (DYKY-FAVYVR; Ref. 27). We have prepared this peptide and find that it has no activity for blocking endothelial cell tube formation.3 It had already been established that VAYI was the minimal active sequence for A13 and that this sequence is highly conserved among the α chains (16). With C16, the minimal active sequence was found previously to be YYRL (26). Although different in sequence, these peptides are located in homologous sites on laminin-1 and recognize the same cellular receptors. The minimal active sequence for C16Y is DFKLFAVYVR. All of these peptides regulate angiogenesis and recognize the promiscuous integrin αvβ3 (28, 29).

Many sites for angiogenesis have been identified on laminin-1, but only one site has been found to be an inhibitor of angiogenesis, YIGSR, which is located on the β1 chain (residues 929–933; Refs. 30, 31). This peptide also blocks tumor growth and lung colonization but is much less active (~10-fold) than the C16Y peptide described here (32, 33). C16Y is the most potent peptide described to date and may function as an antagonist to integrins during angiogenesis.

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3 Unpublished observations.
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