Inhibition of Angiogenesis and Tumor Metastasis by Targeting a Matrix Immobilized Cryptic Extracellular Matrix Epitope in Laminin

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Abstract
Angiogenesis and tumor metastasis depend on extracellular matrix (ECM) remodeling and subsequent cellular interactions with these modified proteins. An in-depth understanding of how both endothelial and tumor cells use matrix-immobilized cryptic ECM epitopes to regulate invasive cell behavior may lead to the development of novel strategies for the treatment of human tumors. However, little is known concerning the existence and the functional significance of cryptic laminin epitopes in regulating angiogenesis and tumor cell metastasis. Here, we report the isolation and characterization of a synthetic peptide that binds to a cryptic epitope in laminin. The STQ peptide selectively bound denatured and proteolyzed laminin but showed little interaction with native laminin. The cryptic laminin epitope recognized by this peptide was selectively exposed within malignant melanoma in vivo, whereas little if any was detected in normal mouse skin. Moreover, the STQ peptide selectively inhibited endothelial and tumor cell adhesion, migration, and proliferation in vivo and inhibited angiogenesis, tumor growth, and experimental metastasis in vivo. This inhibitory activity was associated with a selective up-regulation of the cyclin-dependent kinase inhibitor P27KIP1 and induction of cellular senescence. These novel findings suggest the existence of a functionally relevant cryptic laminin epitope in vivo and that selective targeting of these laminin epitopes may represent an effective new strategy for the treatment of malignant tumors by affecting both the endothelial and tumor cell compartments. [Cancer Res 2007;67(9):4353–63]

Introduction
The importance of extracellular matrix (ECM) remodeling in regulating invasive cellular behavior is well known. Experimental evidence is accumulating that proteolytic remodeling of ECM proteins is not only a mechanism to facilitate destruction of physical barriers but also an active process required to expose cryptic regulatory information that can be selectively transferred to cells to help control complex cellular behavior. Recent studies have provided evidence that posttranslational modification of proteins as well as proteolytic release of fragments of larger molecules can result in the acquisition of unique functional properties (1–3). In fact, reports have documented new regulatory functions for noncollagenous domains of the basement membrane protein collagen type IV (4, 5). Studies have provided examples of this concept from other ECM molecules, including collagens VII, XV, and XVIII; thrombospondin; vitronectin; fibronectin; and perlecans (6–10). In addition, many non-ECM molecules also contain cryptic regulatory sites including matrix metalloproteinase-2 (MMP-2; PEX), plasminogen (angiostatin), and calreticulin (vasostatin; refs. 11–14). Thus, a more complete understanding of the molecular mechanisms by which these cryptic elements function in vivo will provide important new insight into the regulation of tissue homeostasis and disease.

In addition to collagen type IV, a second component of basement membrane is laminin. Laminin is a large heterotrimeric molecule composed of three chains designated as α, β, and γ. At least five α, three β, and three γ chains have been identified. Distinct combinations of these three chains result in at least 12 different laminin isoforms (15, 16). Cellular interactions with laminin regulates cellular and molecular processes, including adhesion, migration, cell cycle control, proliferation, differentiation, and gene expression (15–18). Although a wealth of information is currently available concerning how interactions with native laminin regulates cellular behavior, relatively little is known concerning the response of cells to interactions with structurally modified laminin. Given the facts that proteases known to cleave laminin are thought to contribute to angiogenesis and tumor metastasis, the possibility exists that structural remodeling of laminin may facilitate exposure of cryptic sites that regulate both endothelial and tumor cell behavior (19–24).

Here, we provide evidence for a cryptic epitope within laminin 1 that selectively regulates endothelial and tumor cell behavior. Exposure of this cryptic site was restricted to tissue microenvironments associated with ECM remodeling. A synthetic peptide directed to this cryptic epitope specifically blocked endothelial and tumor cell adhesion, migration, and proliferation on denatured but not native laminin and inhibited angiogenesis, tumor growth, and experimental metastasis in vivo. Moreover, this cryptic laminin site may play a role in selectively controlling expression of the cyclin-dependent kinase (CDK) inhibitor P27KIP1 and cellular senescence. These novel findings suggest the existence of a functionally relevant cryptic laminin epitope in vivo and that the selective targeting of this cryptic site may represent a new strategy for the treatment of angiogenesis and tumor metastasis.

Materials and Methods
Antibodies, peptides, and reagents. Antibodies directed to the M13 phage was obtained from Pharmacia. Antilaminin antibody was obtained from Chemicon International. Horseradish peroxidase (HRP)–labeled
Neur-Avidin was obtained from Pierce. Rhodamine-labeled Lycopersicon esculentum lectin was obtained from Vector Laboratories. Elastase, MMP-2, and laminin 5 were obtained from Chemicon International. Laminin 1, fibronectin, and collagen type IV were obtained from Sigma. Matrigel was obtained from Calbiochem. Bovine serum albumin (BSA), methanol, ethanol, acetone, and optimum cutting temperature (OCT) embedding compound were obtained from Sigma. STQ peptide (STQNASLLSTV), peptide 1 (STQNA), peptide 2 (SLLSL), and peptide 3 (LSLTV), and an inactive laminin-binding peptide (QHANHQAWPNLR) were obtained from QED BioSciences. An NH₂-terminal cysteine was added to the STQ peptide for immobilization and used throughout the studies. All peptides were purified to >90% by high-performance liquid chromatography and subjected to analysis by mass spectrometry. The peptides were reconstituted in 100% DMSO and adjusted to 10 to 20 mg/mL in sterile water.

**Cells and cell culture.** Melanoma cell lines M21 and CS-1 was a gift from Dr. David Cheresh (Scripps Research Institute, La Jolla, CA). Murine B16F10 melanoma, 67NR mammary carcinoma, and human umbilical vein endothelial cells (HUEVEC) were obtained from American Type Culture Collection. Tumor cells were maintained in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS), glutamine, and penicillin/streptomycin (100 μg/mL) at 37°C with 5% CO₂. HUEVECs were maintained in endothelial cell growth medium from Clontech supplemented with 20% FBS, endothelial cell growth supplements, and glutamine and penicillin/streptomycin at 37°C with 5% CO₂. All cells were maintained as subconfluent cultures and split 1:3 24 h before use.

**Subtractive screening of M13 phage display library.** Microtiter wells were coated (20 μg/mL) for 18 h at 4°C with native human laminin 1 or thermally denatured laminin 1. To deplete phage capable of binding native laminin, phages from the M13 phage display library (Ph.D.-12, New England Biolabs) were allowed to bind native laminin for 1 h. Nonbound phage were sequentially transferred to new laminin-coated wells. The sequential transfer was completed thrice. The negatively selected phages were transferred to wells coated with denatured laminin. Nonbound phages were discarded and the bound phage were eluted with glycine (100 mM/L; pH 2.2). The subtractive panning procedure was completed a total of three times, and selected phages were titrated and plaques were picked at random for expansion. To analyze the binding specificity, Escherichia coli ER2537 was infected with 50 μL phage stock and grown for 4 h at 37°C. The supernatant containing phage particles was harvested and DNA sequencing was carried out to determine the peptide insert.

**Solid-phase ELISA.** Briefly, 96-well plates were coated (10 μL/well) with native or denatured laminin, fibronectin, or collagen type IV (5–10 μg/mL in PBS) for 18 h at 4°C. Plates were blocked for 30 min with 0.1% Tween 20, 2% nonfat dry milk in PBS. Phage clones (10⁸ phages/well) were incubated for 1 h. After washing, plates were incubated with HRP-conjugated anti-M13 antibody (diluted 1:1,000 in PBS; Pharmacia). Plates were washed and substrate was added (0.25 mg/mL O-phenylenediamine/H₂O₂ in citrate buffer (pH 5.0). Absorbances were measured at 490 nm. For biotin-labeled peptide ELISAs, wells were coated with native, denatured, or proteolyzed laminin. To prepare elastase proteolyzed laminin, lyophilized neutrophil elastase was dissolved in 200 μL of PBS and laminin digestion was done overnight at an enzyme to substrate ratio of 1:100 (w/w) at 37°C. Reactions were terminated by adding phenylmethylsulfonyl fluoride. Elastase was inactivated by boiling before addition in control experiments. Biotin-labeled peptides (50–100 ng/mL) were added to the wells and allowed to bind for 1 h. Plates were washed and 100 μL of Neutr-Avidin-HRP conjugate (1:1,000) was incubated at 37°C for 1 h.

**Cell adhesion assays.** Cell adhesion assays were done as described (25). Nontissue culture 48-well plates were coated with native or denatured laminin (50 μg/mL) at 4°C for 18 h. M21 melanoma and HUEVECs (1 x 10⁶) were harvested and suspended in adhesion buffer (RPMI 1640 containing 1 mmol/L MgCl₂, 0.2 mmol/L MnCl₂, and 0.5% BSA) in the presence or absence of STQ or control peptides (0–20 μg/mL) and added to the upper chamber and allowed to migrate for 3 to 4 h at 37°C (26). Cells remaining on the topside were removed and cells that had migrated to the underside were stained with crystal violet. Cell migration was quantified by counting the number of cells per 200 microscopic fields with five fields per condition (26).

**Cell proliferation assay.** Plates were coated with native or denatured laminin (50 μg/mL) at 4°C for 18 h. Plates were blocked with 1% BSA in PBS. M21 melanoma and HUEVECs were suspended in proliferation buffer containing 1% serum. Cells (5 x 10⁵ per well) were added to the wells in the presence or absence of STQ or control peptide (0–50 μg/mL) and incubated at 37°C for 24 h. Cell proliferation was quantified using real-time cell electronic sensing (RT-CES; 27) and WST proliferation assay kits according to manufacturer's instructions.

**Immunohistochemistry.** Melanoma tumors growing in nude mice and normal mouse skin were resected, washed, embedded in OCT, and snap frozen at −70°C. Frozen sections (4 μm) of mouse skin, M21, or CS-1 melanoma tumors were fixed by incubation for 30 s in 50% methanol 30% aceton (28). Tissues were blocked by incubation in 1% BSA followed by incubation with biotinylated STQ or control (50 μg/mL) in 1% BSA for 2 h at 37°C. Tissues were washed and incubated with HRP-labeled Neur-Avidin. Tissues were washed and visualized by incubation with 3,3'-diaminobenzidine hydrochloride (Vector Laboratories). Photomicrographs were taken at a magnification of ×200. For immunofluorescence staining, tissues were prepared as described and incubated with biotin-labeled STQ peptide (50 μg/mL) for 2 h at 37°C. The sections were washed and incubated with FITC-labeled Neur-Avidin and rhodamine-labeled L. esculentum lectin for detection of blood vessels. For staining of STQ epitope and laminin, tumor tissues were incubated with antilaminin-specific antibody (1:50 dilution) followed by incubation with rhodamine-labeled goat anti-rat secondary antibody (1:400 dilution). Photomicrographs were taken at a magnification of either ×400 or ×630 under oil immersion.

**Matrigel endothelial cord formation assay.** Growth factor-reduced Matrigel (100 μL) was added to wells of a 96-well plate and allowed to polymerize for 1 h at 37°C. Subconfluent HUEVECs were resuspended in 5% FBS containing medium and added to the Matrigel in the presence or absence of STQ or inactive control (0–100 μg/mL). HUEVECs were allowed to form cords for 24 h at 37°C (29). Endothelial cord formation was monitored at 24 h by light microscopy. Photographs were taken using a Sony digital camera with an inverted microscope.

**Chick embryo angiogenesis assay.** The cell adhesion molecules (CAM) of 10-day-old chick embryos were separated from the shell (30). Filters containing buffer only or basic fibroblast growth factor (bFGF, 40 ng) were placed on the CAMs to induce angiogenesis. Twenty-four hours later, the filter discs and CAM tissue were harvested. Angiogenesis was quantified by counting branching blood vessels within the filter disc. Seven to 10 embryos were used per condition.

**Chick embryo B16F10 melanoma experimental metastasis assay.** Twelve-day-old chick eggs were obtained from SPAFAS (North Franklin, CT; refs. 26, 31). Tumor cells (B16F10) were washed and resuspended in PBS at concentrations of 0.5 x 10⁶/mL in the presence or absence of STQ or control peptides (100 μg/embryo). Cells suspensions were injected in a total volume of 100 μL per embryo. To quantify experimental lung metastasis, embryos were sacrificed at day 19 and both lobes of the chick lungs were dissected. The total number of lung lesions was counted on each side of each lobe for each embryo. The extent of B16F10 lung colonization was confirmed by histologic analysis (32). Experimental metastasis was described as the mean number of surface B16F10 lesions per lung. At least 8 to 10 embryos were used per condition.

**Tumor growth assay.** Subconfluent tumor cells (1 x 10⁶ B16F10 melanoma and 1 x 10⁶ 67NR mammary carcinoma) were injected s.c. into...
nude mice in a total volume of 100 μL. Four days later, when palpable tumors were detected, mice were injected daily with either STQ peptide (0–100 μg/d) or controls (normal mouse IgG or QHA-peptide) for 14 (B16F10) or 15 (67NR) days. Tumor size was monitored by caliper measurements and volumes were estimated using the formula \( V = \frac{L^2 \times W}{2} \), were \( V \) is volume, \( L \) is length, and \( W \) is width. Four to six mice were used per experimental condition.

**Real-time quantitative reverse transcription-PCR**. Plates were coated with denatured laminin (50.0 μg/mL). Equal numbers of cells from subconfluent cultures were harvested, washed, and added to the coated plates in the presence or absence of STQ or control peptides (100 μg/mL) and incubated for 12 h. Total RNA was isolated with RNeasy miniprep columns (Qiagen). Total RNA (1 μg) was reverse transcribed using 1 × reverse transcriptase buffer, MgCl₂ (3 mmol/L), deoxynucleotide triphosphate (2.0 mmol/L), RNase inhibitor (0.2 unit/μL), random hexamer primers (0.5 mmol/L), and Moloney murine leukemia virus reverse transcriptase (0.3 unit/μL) in 20 μL reactions using a three-step cycle (Promega). Real-time fluorescence detection was carried out using an ABI Prism 7900 Sequence Detection System. All primers and probes were designed using Primer3 version 2 and Ensembl. P27KIP1 primer sets include CTTGTCGCTGTCTTGCACTC (forward) and AATCTGTCAGGCTGGTCTGC (reverse), and \( \beta \)-microglobulin AAAGATGATGATGCTGCG (forward) and CCTCCATGATGATGCTGCTTACA (reverse). Samples were analyzed in triplicate. \( \beta \)-microglobulin was used as an internal control (33).

**Western blot analysis**. Plates were coated with denatured laminin (150 μg/mL). Equal numbers of cells from subconfluent cultures were added to the coated plates in the presence or absence of STQ or control peptides (100 μg/mL) and incubated for 12 h. Cells were harvested, washed, and lysed in 1.0% Triton X-100 buffer containing 300 mmol/L NaCl, 50 mmol/L Tris (pH 7.0), and 1 × protease inhibitors. Equal amounts of cell lysate (25 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with polyclonal antibodies directed to P27\(^{\text{kip1}}\) or Actin. Western blots were visualized by chemiluminescence.

**Analysis of cellular senescence**. To examine the levels of cellular senescence, senescence-associated \( \beta \)-galactosidase (SA-\( \beta \)-Gal) was monitored (25, 34). Plates were coated with native or denatured laminin (50.0 μg/mL). Equal numbers (\( 5 \times 10^4 \)) of M21 cells were resuspended in the presence or absence of STQ or control peptides (100 μg/mL). Cells were washed and fixed with 0.2% formaldehyde, 0.2% glutaraldehyde for 2 min. The cells were washed and incubated in the SA-\( \beta \)-Gal staining solution [1.0 mg/mL X-gal in 20 mmol/L sodium phosphate, 20 mmol/L citric acid, 5 mmol/L potassium ferricyanide, 2 mmol/L magnesium hexacyanoferrate, and 150 mmol/L sodium chloride (pH 6.0) without CO\(_2\)]. The cells were photographed at \( \times 100 \) magnification.

**Statistical analysis**. Statistical analysis was done using the InStat statistical program for Macintosh computers. Data were analyzed for statistical significance using Student’s t test. \( P \) values <0.05 were considered significant.

**Results**

**Isolation of M13 phage exhibiting selective binding to denatured laminin**. To gain a more complete understanding of the roles that cryptic ECM sites play in invasive cellular events, we sought to generate reagents that would selectively bind to cryptic sites that are normally inaccessible within laminin. We chose a strategy of subtractive panning using a peptide phage display library. Native human laminin 1 was coated on microtiter wells and phage from the peptide library was allowed to interact over several binding cycles. Nonbound phage were recovered and allowed to bind to thermally denatured laminin. Phage specifically bound to denatured laminin were eluted and subjected to repeated cycles of subtractive panning. Following four rounds of negative selection, 29 random clones were selected, amplified, and sequenced. Of the 29 clones selected for binding to denatured laminin, 20 (69%) were identical in sequence to clone 1, whereas 6 (20%) were identical to the sequence in clone 2 (Table 1). The selected laminin-binding clones exhibited no homology to each other and contained no specific functional motifs, as assessed by alignment analysis by the MacVector 6.5 (Oxford Molecular, Ltd.) software.

**Phage clone 1 exhibits selective binding to denatured laminin**. To examine the specificity of the laminin-binding clones, solid-phase ELISAs were carried out using antibodies directed to the M13 phage particle (35). As shown in Fig. 1A, clones 3 and 4 showed little binding to denatured or native laminin. In contrast, clone 1 exhibited an 8-fold increase in binding to denatured laminin compared with native laminin. Although clone 2 bound to denatured laminin, it also bound to native laminin. Given the selectivity of clone 1 for binding to denatured laminin, we examined the ability of this clone to bind to other ECM components, including fibronectin and collagen type IV. As shown in Fig. 1B, clone 1 bound to denatured laminin while exhibiting little interactions with native laminin. Clone 1 exhibited no interactions with fibronectin or collagen IV in either their native of thermally denatured forms (Fig. 1B).

A cryptic laminin epitope defined by STQ peptide is expressed after proteolysis in vitro and within melanoma tumors in vivo. To study the cryptic epitope defined by clone 1, we synthesized a peptide (STQ peptide) that corresponds to the amino acid insert found in clone 1 (Table 1). To examine the relative exposure of the cryptic laminin epitope, we assessed the ability of a biotin-labeled STQ peptide to bind native or thermally denatured laminin. As shown in Fig. 2A, STQ peptide showed little binding to native laminin while exhibiting a dose-dependent increase in binding to denatured laminin. Moreover, although STQ peptide bound to denatured laminin 1, little binding was observed with native or denatured laminin 5 (Fig. 2B). Proteolytic enzymes, including MMPs and elastase, have been reported to cleave laminin (36, 37). In this regard, we examined whether the cryptic laminin epitope could be expressed after proteolysis. As shown in Fig. 2C, incubation of intact laminin with elastase (proteolyzed) resulted in exposure of the cryptic epitope as indicated by binding of STQ peptide. Similar results were also observed after MMP-2 digestion of laminin (data not shown). Incubation of laminin with heat-inactivated (control) elastase (Fig. 2C) resulted in little exposure of the cryptic site.

Next, we sought to determine whether this cryptic epitope could also be expressed in vivo. Frozen sections of melanoma (M21) tumors grown in mice or normal mouse skin were stained with biotin-labeled STQ peptide followed by incubation with HRP-labeled Neutr-Avidin. As shown in Fig. 2D (left top), STQ peptide specifically reacted with human melanoma tumors, whereas little binding of the STQ peptide was observed in normal mouse skin (Fig. 2D, bottom left). STQ peptide binding seemed to be localized to basement membranes structures. In addition, some staining was

**Table 1. Amino acid sequence inserts of four selected clones and their frequency of occurrence in 29 randomly selected phage**

<table>
<thead>
<tr>
<th>Phage clone number</th>
<th>Sequence</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>1</td>
<td>STQNASLLSLTV</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>QHANHQAWPNLR</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>NNSPAWLQLDF</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>QAPFAA5PSLVQY</td>
<td>1</td>
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also observed surrounding some individual cells. In control experiments, Neutr-Avidin alone (top and bottom right) showed no specific reactivity. To examine whether the STQ peptide binding was associated with tumor blood vessels, colocalization studies were carried out. Tumors including hamster CS-1 (Fig. 2E) melanoma sections were costained with rhodamine-labeled tomato lectin (L. esculentum) known to bind to blood vessels as well as biotin-labeled STQ peptide followed by incubation with FITC-labeled Neutr-Avidin. As shown in Fig. 2E, the cryptic laminin epitope colocalized with tumor-associated blood vessels, suggesting that the cryptic epitope can be exposed in association with tumor vessels. In similar studies, tumor tissues (M21 melanoma) were costained with a monoclonal antibody specifically directed to laminin as well as biotin-labeled STQ peptide. As shown in Fig. 2F, the STQ cryptic epitope could be detected colocalizing (yellow) in discrete regions with laminin (red), a major component of basement membranes. However, the regions of colocalization with laminin were few, which may have resulted from competition for binding between the STQ peptide and the anti-laminin antibody. Taken together, these data are consistent with the notion that the STQ cryptic epitope can be localized in close association with tumor vessels, the basement membrane component laminin, as well as surrounding some cells in vivo. Further detailed expression studies will be necessary to determine the differential expression of this cryptic epitope within other malignant tissues as well as the possibility of cell surface association of denatured forms of laminin.

Blocking cellular interactions with a cryptic laminin epitope selectively inhibits tumor and endothelial cell adhesion. Studies have suggested that cellular interactions with the ECM plays key roles in regulating tumor invasion and angiogenesis. Therefore, we assessed the effects of the STQ peptide on tumor cell adhesion to native or denatured laminin. M21 melanoma cells were resuspended in the presence or absence of STQ or a control peptide corresponding to the original clone 2, which exhibited binding activity to both native and denatured laminin. As shown in Fig. 3A, STQ and control peptides at concentrations up to 100 μg/mL had no effect on M21 cell adhesion to native laminin or other ECM components (data not shown). In contrast, STQ peptide caused a dose-dependent inhibition of M21 cell adhesion to denatured laminin, with maximum inhibition (80%) observed at a concentration of 100 μg/mL (Fig. 3B). Importantly, the control peptide that can bind to either native or denatured laminin exhibited little effect. Moreover, the ability of STQ peptide to selectively inhibit cell adhesion was not restricted to tumor cells because STQ peptide also dose-dependently inhibited HUVEC adhesion to denatured but not native laminin compared with controls (Fig. 3C and D). In addition, no difference in function-blocking ability was observed between unlabeled and biotin-labeled STQ peptide (data not shown).

To further evaluate the ability of the STQ peptide to inhibit cell adhesion and to determine potential functional regions of the peptide, we examined the effects of truncated versions of the STQ peptide on cell adhesion. Synthetic 5-mer peptides corresponding to the NH2-terminal (peptide 1), middle (peptide 2), and COOH-terminal (peptide 3) regions of STQ peptide were resuspended with HUVEC cells and adhesion was assessed. The NH2-terminal truncated versions of STQ peptide exhibited little effect on cell adhesion, whereas the COOH-terminal peptide corresponding to the amino acid sequence (LSTLTV) exhibited some inhibitory activity (35%) compared with controls (data not shown). Given the minimal effects observed, a more detailed experimental analysis with additional overlapping peptides will be required to define the exact functional amino acids. These data are consistent with the possibility that the entire peptide may be required for maximal effect.

Disruption of cellular interactions with a cryptic laminin epitope inhibit tumor and endothelial cell migration. To examine the effect of the STQ peptide on cell motility, we examined M21 and HUVEC migration. M21 cells were resuspended in the presence or absence of STQ or control peptide. As shown in Fig. 4A and B, STQ peptide had little if any effects on M21 cell migration on intact laminin. In contrast, migration of M21 cells on denatured laminin was inhibited dose dependently compared with no treatment or control peptide (Fig. 4B). In similar studies, the STQ peptide failed to inhibit HUVEC migration on intact laminin (Fig. 4C) while exhibiting dose-dependent inhibition of HUVEC migration on denatured laminin (Fig. 4D).

Disruption of cellular interactions with a cryptic laminin epitope inhibits proliferation, up-regulates P27kip1, and induces cellular senescence. Little information is available concerning the roles of cryptic ECM epitopes in regulating cellular proliferation. To this end, M21 and HUVECs were resuspended in the presence or absence of STQ or a control peptide and proliferation was assessed (27, 38). As shown in Fig. 5A, M21 cell proliferation on denatured laminin was dose-dependently inhibited.
compared with no treatment or control peptide. In contrast, the STQ peptide had no effect on M21 cell proliferation on native laminin (data not shown). In similar studies, STQ peptide dose dependently inhibited HUVEC proliferation on denatured laminin (Fig. 5B), but not native laminin (data not shown), whereas a control peptide had little effect.

To assess potential mechanisms by which the STQ peptide may inhibit proliferation, we examined the effects of STQ peptide on the expression of the cryptic laminin epitope.

**Figure 2.** Exposure of a cryptic laminin epitope in vitro and in vivo. ELISA assays were carried out by coating microtiter plates (10 μg/mL) with native, denatured, or proteolyzed (elastase-treated) laminin. Biotin-labeled STQ peptide (1–50 ng/mL) was added to the coated wells and allowed to incubate for 1 h at room temperature. The plates were washed and incubated with 100 μL of HRP-labeled Neutr-Avidin and allowed to incubate for 1 h. A, reactivity of biotin-labeled STQ peptide to native or thermally denatured laminin 1. B, reactivity of biotin-labeled STQ peptide to native and thermally denatured laminin 1 and 5. C, reactivity of biotin-labeled STQ peptide to native (Control) or elastase-treated (Proteolyzed Laminin) laminin 1. All data were corrected for nonspecific binding. Columns, mean absorbance from triplicate wells; bars, SD. D, immunohistochemical analysis of the STQ cryptic laminin epitope (brown) in vivo. Top, representative examples of exposure of the STQ cryptic laminin epitope within human M21 melanoma tumors (left) or control (right). Bottom, representative examples of exposure of STQ cryptic laminin epitope within normal mouse skin. Stained with biotin-labeled STQ peptide (left) or control Neutr-Avidin (right). E, representative examples of colocalization of the STQ cryptic laminin epitope (green) and tumor-associated blood vessels (red) from CS-1 melanoma tumors. Magnification, ×630 under oil immersion. F, representative examples of colocalization (yellow) of the STQ cryptic epitope (green) and the basement membrane protein laminin (red) from M21 melanoma tumors. Bottom right, an example of H&E staining of the same tissue. Magnification, ×400.
peptide on apoptosis and cellular senescence. Interestingly, treatment of cells with STQ peptide had little capacity to directly induce tumor or endothelial cell apoptosis in vitro (data not shown). Thus, we sought to determine whether the ability of STQ peptide to inhibit proliferation might be associated, in part, with altered cellular senescence. M21 cells were incubated in the presence or absence of STQ and control peptides for 24 h and stained for SA-β-Gal, a well-documented marker of senescence (39, 40). As shown in Fig. 5C, untreated M21 cells (NT) exhibited a well-spread and flattened morphology on denatured laminin and exhibited little SA-β-Gal staining (blue). In contrast, cells treated with STQ peptide exhibited marked rounding and an increase in the number of granulated cells staining positive (blue) for SA-β-Gal (middle). M21 cells incubated with the control peptide did show a slightly rounded morphology (right) and exhibited little SA-β-Gal expression. Moreover, little change in levels of SA-β-Gal staining was observed among any of the experimental conditions when cells were cultured on native laminin (data not shown).

Induction of cellular senescence is thought to be associated with altered regulation of cell cycle progression and changes in expression of CDK inhibitors (41). To this end, we examined the effects of STQ peptide on expression of the CDK inhibitor P27KIP1 by real-time quantitative reverse transcription-PCR and Western blot analysis. As shown in Fig. 5D, incubation of M21 melanoma cells with STQ peptide resulted in an ~6-fold induction in the relative levels of P27KIP1 mRNA compared with control. Importantly, treatment of cells with STQ peptide also increased P27KIP1 protein expression as indicated by Western blot analysis (Fig. 5E).

STQ peptide inhibits endothelial cord formation in vitro and angiogenesis, tumor growth, and metastasis in vivo. We examined the effects of the STQ peptide on endothelial cord formation in Matrigel, a basement membrane preparation containing laminin. HUVECs were resuspended in the presence or absence of STQ or the inactive control. Endothelial cord formation was assessed 24 h later by light microscopy. As shown in Fig. 6A, treatment of endothelial cells with STQ peptide resulted in a dose-dependent disruption of endothelial cords compared with either no treatment or control peptide. Although tube formation may mimic certain events in the angiogenesis, it does not recapitulate the integrated events of angiogenesis in vivo. Therefore, angiogenesis was induced by bFGF in the CAMs of 10-day-old chicks. Twenty-four hours after stimulation, embryos were treated with STQ or control (100 μg/embryo). As shown in Fig. 6B, STQ peptide significantly (P < 0.05) inhibited angiogenesis by ~90% as indicated by a reduction in the number of branching blood vessels within the area of the filter discs. In contrast, the control peptide had little effect, if any.

We next examined the effects of STQ peptide on the growth of two distinct tumor types in vivo. Murine B16F10 melanoma and 67NR mammary carcinoma cells were injected s.c. in nude mice and cells were allowed to grow for 4 days until palpable tumors were detected. Mice were either untreated or treated (i.p.) with STQ peptide or controls over a dose range (0–100 μg/d). As shown in Fig. 6C and D, STQ peptide exhibited a dose-dependent inhibition of B16F10 and 67NR tumor growth. Significant (P < 0.05) inhibition was observed at 100 μg/d with a maximum inhibition of ~45% to 50% compared with controls.

**Figure 3.** Effects of STQ peptide on M21 melanoma and HUVEC adhesion. To examine the effects of the STQ peptide directed to a cryptic laminin site has on cell adhesion, in vitro adhesion assays were done. Nontissue culture 48-well plates were coated with native of denatured laminin 1 (50 μg/ml). Cells (M21 melanoma or HUVECs) were resuspended in adhesion buffer in the presence (0–100 μg/ml) or absence of STQ or a control laminin binding peptide and cell adhesion was quantitated. A, effects of STQ peptide on M21 melanoma cell adhesion to native laminin. B, effects of STQ peptide on M21 melanoma cell adhesion to denatured laminin. C, effects of STQ peptide on HUVEC adhesion to native laminin. D, effects of STQ peptide on HUVEC adhesion to denatured laminin. Columns, mean cell adhesion from triplicate wells; bars, SD. Experiments were completed twice to thrice with similar results.
Many of the same molecular mechanisms that regulate angiogenesis are also thought to contribute to tumor cell metastasis. Therefore, we sought to examine whether cellular interactions with the cryptic laminin epitope might play a role in experimental metastasis. B16F10 cells were resuspended in PBS in the presence or absence of STQ or the inactive control (100 μg/embryo) and injected into 12-day-old chick embryos. As shown in Fig. 6E, injections of B16F10 cells resulted in numerous well-defined melanotic tumor lesions. Histologic analysis of the chick lungs confirmed the extensive invasion of melanoma cells (data not shown). Importantly, lungs from embryos treated with the STQ peptide exhibited a reduction in the number of B16F10 lung lesions compared with controls. In fact, quantification of the number of lung tumor lesions indicated that STQ peptide significantly (P < 0.05) inhibited experimental metastasis by ~50%.

Discussion

Proteolytic remodeling of the ECM plays an important role in normal physiology and pathologic processes. Remodeling of ECM not only alters restrictive barriers that hinder cell motility, but can mobilize and activate growth factors and release a number of bioactive peptides (42). Evidence is accumulating that a cell response to structurally altered ECM proteins may result in activation of unique signaling pathways (43, 44). These signaling cascades may allow cells the capacity to modify their responses to the changing microenvironments associated angiogenesis and tumor invasion.

In our previous studies, we used the technique of subtractive immunization to develop a set of antibodies that bound cryptic sites within collagen (45–48). These cryptic epitopes were selectively exposed within the ECM of angiogenic blood vessels and surrounding invasive cells (45–48). Studies have documented the importance of laminin in regulating cellular events including, adhesion, migration, differentiation, and gene expression (1–5). An elegant set of studies have identified well over 20 peptide corresponding to regions of native laminin 1 that possess biological activity and regulate angiogenesis and tumor metastasis (20–24). In fact, the entire α1, β1, and γ1 chains of laminin 1 was examined for regulatory sites. Interestingly, the majority of peptides with bioactivity corresponded to regions of laminin thought to be normally exposed within its intact and native conformation (24). These studies show the importance of cellular communication with native forms of laminin in regulating invasive processes. In contrast, relatively little is known concerning the existence and biological relevance of cryptic elements in laminin. Interestingly, laminin 5 seems to contain a cryptic site because cleavage of the γ2 chain of laminin 5 resulted in enhanced migration of breast carcinoma cells in vitro (19). Moreover, expression of the γ2 chain of laminin 5 has been correlated with a more malignant tumor phenotype (49). Recently, new studies have shed light on novel mechanisms by which a distinct peptide from laminin α5 chain may inhibit metastasis and angiogenesis by disrupting FGF2 binding to heparan sulfate side chains of CD44 (50). These findings along with recent reports that deletion of laminin 8 may increase...
metastasis and neovascularization help confirm the importance of laminin in invasive cellular processes (51).

In the current report, we used a subtractive panning strategy to generate the STQ peptide that recognizes proteolyzed or denatured laminin 1, but not intact laminin 1 or native and denatured laminin 5. The cryptic epitope recognized by STQ peptide was exposed in the ECM of malignant melanomas in vivo, but little was detected in normal mouse skin. Endothelial and tumor cells can adhere and migrate on denatured laminin and these processes can be selectively inhibited by the STQ peptide. These

Figure 5. Effects of STQ peptide on proliferation, senescence, and P27KIP1 expression. To examine the effects of STQ peptide on proliferation, we used RT-CES. Microtiter wells were coated with denatured laminin (50 μg/mL). Cells (M21 melanoma and HUVECs) were harvested and resuspended in low serum medium in the presence or absence of STQ or control peptide (0–50 μg/mL). Cells were allowed to proliferate for 24 h. A, quantification of M21 melanoma cell proliferation on denatured laminin. B, quantification of HUVEC proliferation on denatured laminin. Columns, mean sweep number (RT-CES) from triplicate wells; bars, SD. Tumor cell senescence was assessed by monitoring the expression of SA-β-Gal. C, representative examples of SA-β-Gal expression (blue) within untreated, STQ, and control peptide–treated M21 melanoma cells culture on denatured laminin. Magnification, ×100. To examine the expression of P27KIP1, we used real-time reverse transcription-PCR (D) and Western blot analysis (E).
Figure 6. Effects of STQ peptide on angiogenesis, tumor growth, and experimental metastasis in vivo. A, representative examples of HUVEC cords formed after 24-h incubation in the presence or absence of STQ or control peptide (0–100 μg/mL). Magnification, ×100. B, quantification of the effects of STQ or control peptide (100 μg/mL) on bFGF-induced angiogenesis. Columns, mean number of angiogenic blood vessel branch points per CAM; bars, SE. To evaluate whether STQ peptide affects solid tumor growth, B16F10 melanoma (C) and 67NR mammary carcinoma (D) was injected into nude mice and the effects of STQ peptide or control (0–100 μg/d/mouse) was examined. C and D, quantification of the effects of STQ or controls (0–100 μg/d/mouse) on tumor growth. Columns, mean tumor volume for each condition; bars, SE. E and F, chick embryos were injected with B16F10 cells in the presence or absence of STQ or control peptide (100 μg/embryo). Chick lungs were removed and the number of pigmented lung tumor lesions was quantified. E, representative examples of 19-d-old chick lungs from each experimental condition. F, quantification of effects of STQ peptide on B16F10 experimental metastasis. Columns, mean number of tumor lesions per lung, per experimental condition; bars, SE.
findings are in good agreement with reports indicating a functional role for cryptic ECM elements in mediating cellular interactions (45–48). Although a cryptic epitope in laminin 5 was shown to promote breast tumor cell migration, it is likely that the cryptic epitope identified in our studies is distinct from that found in laminin 5 because laminin 1 does not share common chains and the STQ peptide exhibited little interaction with laminin 5.

Integrin interactions with basement membranes may regulate the expression of mitogenic growth factors such as vascular endothelial growth factor. Moreover, integrin interactions with ECM can differentially alter mitogen-stimulated cell cycle progression and proliferation (52). Our studies indicate that blocking either M1 or M6 melanoma or HUVEC cell interactions with a cryptic epitope in laminin can selectively inhibit proliferation. The STQ peptide up-regulated the CDK inhibitor p27kip1 and induced cellular senescence. These data are consistent with the possibility that the antiproliferative activity of the STQ peptide may be associated, in part, with the induction of senescence and altered expression of P27kip1. Importantly, administration of STQ peptide inhibited angiogenesis and growth of two distinct tumor types, including B16F10 melanoma and 67NR mammary carcinoma in vivo. Moreover, systemic administration of STQ peptide also inhibited experimental metastasis in vivo. Taken together, these findings suggest that the cryptic epitope recognized by STQ peptide may represent a functionally important therapeutic target. Importantly, the STQ peptide was produced synthetically and its continued use in animals may induce an immune response. Moreover, although small peptides may have enhanced penetration and distribution in tumors, their stability and half-life may be low. However, although the STQ peptide may not represent an optimal antagonist, these studies provide evidence that the cryptic epitope recognized by the STQ peptide may represent a highly selective therapeutic target. Collectively, our findings suggest that targeting unique cryptic epitopes within specific ECM proteins may represent a new and highly selective approach for controlling invasive cellular behavior by affecting both the tumor and endothelial cell compartments.

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Inhibition of Angiogenesis and Tumor Metastasis by Targeting a Matrix Immobilized Cryptic Extracellular Matrix Epitope in Laminin

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