Peptides selected for binding to clotted plasma accumulate in tumor stroma and wounds

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Contributed by Erkki Ruoslahti, December 27, 2005

Screening of a phage library for peptides that bind to clotted plasma in the presence of liquid plasma yielded two cyclic decapeptides, CGLLIQQKNKC (CLT1) and CNAGESSSKNC (CLT2). When injected intravenously into mice bearing various types of tumors, fluorescein-conjugated CLT peptides accumulated in a fibrillar meshwork in the extracellular compartment of the tumors, but were not detectable in other tissues of the tumor-bearing mice. The tumor homing of both peptides was strongly reduced after coinjection with unlabeled CLT2, indicating that the two peptides recognize the same binding site. The CLT peptide fluorescence colocalized with staining for fibrin(ogen) present in the extracellular compartment of tumors, but not in other tissues. The CLT peptides did not home to tumors grown in fibrinogen-null mice or in mice that lack plasma fibronectin. The CLT peptides also accumulated at the sites of injury in arteries, skeletal muscle, and skin. We conclude that the CLT peptides recognize fibrin–fibronectin complexes formed by clotting of plasma proteins that have leaked into the extravascular space in tumors and other lesions. These peptides may be useful in targeting diagnostic and therapeutic materials into tumors and injured tissues.

fibronectin | imaging | phage display | tumor targeting | fibrin

The connective tissue (stroma) in and around malignancies is a complex and dynamic structure that integrates the tumor into the host tissue by providing a functional matrix for angiogenesis and lymphangiogenesis, for migrating bystander cells, and for invading tumor cells (1). Normal connective tissue and its extracellular matrix create an antiproliferative environment, whereas tumor extracellular matrix promotes cell migration, survival, and proliferation by providing adhesion proteins, pro- teases, and growth factors critical for these processes (2).

The distinctiveness of tumor extracellular matrix is reflected in its content of specific markers, such as tenascin (3) and oncostatin fibronectin (4). Moreover, high levels of collagen expression (5) and the presence of an alternatively spliced form of fibronectin (6) set the extracellular matrix of tumor blood vessels apart from that of normal vessels. The interstitial spaces of tumors also contain fibrin, presumably as a result of VEGF-induced leakage of plasma proteins into the tumor tissue (7). In a manner similar to wounds, the leaked fibrinogen becomes converted to fibrin by tissue procollagen factors (8, 9). In the present study, we sought to determine whether the interstitial clotting could be used in tumor targeting.

Our laboratory has successfully used peptide libraries displayed on phage as a tool to identify peptides that selectively home to tumors in vivo (10). Homing peptides that are selected by this method have shown promise as carriers of drugs and imaging agents (11, 12). In the present study, we have used the phage methodology to identify peptides that recognize clotted plasma proteins in tumors. We screened a phage library on plasma clots in vitro and derived two cyclic decapeptides (CLT1 and CLT2) that specifically home to tumors and wounds in vivo and bind to tumor tissue in overlay of tissue sections. Tissue specificity of the peptides appears to be associated with plasma fibronectin, which becomes bound to fibrin clots. The peptides may be useful in targeting of diagnostic and therapeutic agents into tumors and tissue lesions.

Results

Peptides Selected from Plasma Clot Bind to Tumor Stroma. The phage selection scheme was designed to amplify phage that bind to plasma clots but not to anticoagulated plasma. Three rounds of selection produced a pool with 7-fold increased binding to plasma clots. Additional selection rounds did not increase the binding. Sequencing showed that the phage encoding the peptide insert CGLLIQQKNKC (CLT1 peptide) and CNAGESSSKNC (CLT2 peptide) were highly enriched among the clot-binding phage. Three of 24 clones sequenced encoded CLT1, and two encoded CLT2; the rest of the clones were only represented once. The CLT phage clones bound to plasma clots with 3- to 4-fold efficiency compared to control phage. They did not significantly bind to clots made out of purified fibrin, or fibronectin immobilized on plastic (results not shown).

To determine whether the CLT peptides would recognize clotted plasma proteins in vivo, we synthesized the peptides as fluorescein conjugates and imaged their accumulation in tumors. Mice bearing orthotopic MDA-MB-435 xenograft tumors were intravenously injected with fluorescein-conjugated CLT1 (n = 6), CLT2 (n = 4), or a control peptide (n = 4) with the sequence KAREC. Examination of whole tissues from these mice under blue light 3 h after the peptide injection revealed intense fluorescence in the tumors of the mice injected with CLT1 and CLT2, whereas essentially no fluorescence was detected in tumors from the control peptide-injected mice (Fig. 1). We also detected no fluorescence in healthy organs of the CLT-injected animals.

Upon histological examination, CLT peptide fluorescence in tumors appeared in a network pattern within the tumors (Fig. 1 C and G). Tumor homing of both peptides was strongly reduced after coinjection of fluorescein-conjugated CLT1 or CLT2 in combination with unlabeled CLT2 in 5-fold excess (Fig. 1 D and H). This finding indicates that the CLT1 peptide recognizes the same binding site within the tumor as CLT2. Quantification of these results showed that the intensity of fluorescence in the MDA-MB-435 tumors of mice injected with the CLT peptides was 60–130 times stronger than in mice injected with the KAREC control peptide (Fig. 1M). Also shown in Fig. 1M is that the CLT peptides specifically accumulated in all of the various tumors we tested (n = 2–8). A fibrillar pattern similar to the one

Conflict of interest statement: No conflicts declared.

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shown in Fig. 1 C and G was seen in each of these other tumors (data not shown). To study the ability of the CLT peptides to recognize human clinical cancers, we developed a peptide overlay assay. Overlay with the CLT1 peptide produced a fibrillar network in mouse Lewis lung carcinoma similar to that seen in the tumors after i.v. injection of the peptide (Fig. 2 A). The binding of the peptide was also inhibited by unlabeled CLT1 peptide (Fig. 2 B) but not by a control peptide (Fig. 2 C), showing the specificity of the binding. Sections from two clinical breast cancers (Fig. 2 D and E) and from PPC1 xenografts were positive in this overlay assay (Fig. 2 F).

**CLT Peptides Associate with Fibrin and Fibronectin in Tumor Stroma.** Staining of tumor sections with antibodies against fibrinogen produces a fibrillar staining that is not seen in normal tissues (8). Fluorescence from intravenously injected CLT peptide (CLT1; Fig. 3 A) and fibrin staining (Fig. 3 B) colocalized (Fig. 3 C) in tumor sections of MDA-MB-435 breast cancer xenografts. Plasma fibronectin and fibrin are deposited together into plasma clots by Factor XIII activity (13), prompting us to analyze the relationship of CLT peptide fluorescence and fibronectin staining in tumors. The results showed that CLT peptides also codistribute with fibronectin (not shown). We also studied the distribution of the CLT peptides, fibrin, and fibronectin in MMTV-PyMT transgenic breast tumors. The results (data not shown) were identical to those obtained with the MDA-MB-435 xenografts.

**CLT Peptide Tumor Homing Requires Fibrin and Plasma Fibronectin.** We next used knockout mice to study the role of fibrinogen and fibronectin in the homing of the CLT peptides to tumors. Fibrinogen knockout mice are viable (14) and could be used as tumor recipients. Complete absence of fibronectin is lethal, but mice that lack plasma fibronectin can be generated by postnatally deleting the fibronectin gene in the liver (15). The CLT peptides homed to B16F1 tumors grown in wild-type C57BL/6 mice (n = 10) producing a fibrillar meshwork (CLT1; Fig. 3 D). In contrast, only faint, evenly distributed fluorescence was present in tumors (n = 4) grown in the fibrinogen knockout mice (which lack the ability to produce fibrin) (Fig. 3 E). B16F1 tumors grown in wild-type littermates of plasma fibronectin-deficient mice also accumulated CLT peptides in a fibrillar matrix (Fig. 3 F), but mice lacking plasma fibronectin (n = 6) did not (Fig. 3 G). Unlike in the fibrinogen knockout mice, there was no residual peptide binding to the tumors in the plasma fibronectin-deficient mice (Fig. 3 H). These results show that both fibrin and fibronectin...
from plasma are needed for the CLT peptides to highlight a fibrillar matrix in tumors, but that some diffuse binding of the peptide persists in the absence of fibrin.

**CLT Binds Sites of Tissue Injury in Vivo.** Clotting is an important part of wound healing. Therefore, we tested CLT peptide homing to tissue injuries. Fluorescein-conjugated CLT peptides homed to deendothelialized femoral arteries \( n = 5 \), producing strong fluorescence in the vessel wall (Fig. 4A). CLT homing was also seen in crush injuries of the muscle \( n = 3 \); Fig. 4D) and in skin wounds resulting from incisions \( n = 4 \); Fig. 4E). No CLT peptide homing was observed in intact arteries, muscles, skin (Fig. 4B, F, and G), or other healthy tissues of the mice with the injuries, and a control peptide showed no homing to the injured tissues (data not shown).

**Discussion**

We report on two cyclic peptides that were isolated by screening phage libraries for peptides that bind to plasma clots. Fluorescein conjugates of these peptides, CLT1 and CLT2, specifically accumulate in tumor tissue after an i.v. injection in multiple cancer models. The peptides also homed to sites of tissue injury. We also show that the injected CLT peptides outline a meshwork that colocalizes with fibrin and fibronectin staining, and that peptides do not home to tumors grown in mice that lack fibrinogen or plasma fibronectin. Our results indicate that the CLT peptides bind to an epitope in a fibrin–fibronectin complex formed as a result of plasma clotting within tumors and at sites of tissue injury.

Our starting point in this work was the expectation that peptides that bind to plasma clots might specifically recognize interstitial clotting products known to be present in tumors (8). This expectation is borne out by our results. In vitro phage screening on plasma clots yielded two related peptide sequences, CLT1 and CLT2, that home to tumor stroma, while not being detectable in normal tissues. These peptides also homed to sites...
of tissue injury, where coagulation typically takes place (8, 16). The meshwork the CLT peptides produced in tumors colocalized with fibrinogen and fibronectin. Finally, the most direct evidence that the CLT peptides detect clotting products in tumors comes from our demonstration that homing of CLT peptides to tumors grown in mice that lack plasma fibronectin or fibrinogen is greatly reduced. Because the plasma fibronectin-deficient mice do have tissue fibronectin, these results show that the fibronectin required for the CLT binding site is derived from the blood. We conclude that the CLT peptides specifically home to tumors through binding to a binding site that depends on the incorporation of fibrin and fibronectin from plasma to tumor interstitium as a result of vascular leakage and tissue clotting activity.

We have not identified the binding site detected by the CLT peptides in plasma clots, but our results suggest that it may be on fibronectin. There was no homing of the peptides to tumors grown in mice that lack plasma fibronectin. In contrast, the CLT peptides homed to some degree to tumors grown in fibrinogen-null mice, but the peptide fluorescence was diffuse instead of forming the usual meshwork pattern. Fibronectin contains a number of cryptic binding sites, which become available when the molecule is subjected to tension or interacts with hydrophobic entities (17, 18). Cryptic epitopes uncovered in the clotting process have not been described so far, and a BLAST search with the CLT peptides did not produce leads for proteins that might bind to a cryptic site in fibronectin. Nonetheless, these results are compatible with the possibility that the CLT binding site is on fibronectin, and that the organization of plasma-derived fibronectin, and perhaps also its retention, in tumor stroma, requires simultaneous deposition of a fibrin meshwork.

The CLT peptides recognized every tumor we have tested, including five tumor types in mouse and some human tumors. Thus, the CLT peptides may be useful as a general imaging or drug delivery agent for tumors, as well as for tissue injuries. Fibrin-binding peptides and antibodies have been used for imaging, but only in cardiovascular applications (19, 20). Our peptides are likely to be different from these earlier reagents in that the probable target is fibronectin rather than fibrin. Also, we have demonstrated remarkably effective tumor targeting with our CLT peptides.

**Materials and Methods**

**Animals, Cell Lines, and Tissues.** B16F1 mouse melanoma, Lewis lung carcinoma, C8161 human melanoma, MDA-MB-435 human breast cancer, and PPC-1 human prostate cancer cells (American Type Culture Collection) were maintained in RPMI medium 1640 or DMEM supplemented with 10% FCS. Human tumor cells (1 × 10⁶) were injected into the mammary fat pad or the flank of nude BALB/c nu/nu mice to induce tumors. Mouse mammary tumor virus (MMTV) PyMT mice were provided by Robert Oshima (Burnham Institute for Medical Research). The MMTV PyMT mice develop breast cancer under the influence of a polyoma middle T antigen driven by the mouse tumor virus promoter (21). B16F1 tumors induced by injecting 1 × 10⁶ cells s.c. were grown in the flank of fibrinogen knockout mice (22), and of transgenic plasma fibronectin-deficient C57BL/6-Fn(f1/f1) Mx-Cre⁺ (15) and their wild-type littersmates. Deletion of the fibronectin gene in the liver was induced by poly(I):poly(C) (15, 23). Frozen human tissues from clinical breast cancers were provided by the Western Division of the National Cancer Institute Cooperative Human Tissue Network (Vanderbilt University Medical Center, Nashville, TN).

**Phage Library.** A cyclic peptide library with the general structure of CX8C (C = cysteine; X = any amino acid) was designed to express 5–15 peptide copies on a T7 10–3b phage vector (T7 Select kit; Novagen). CX8C-encoding oligonucleotides were synthesized with flanking EcoRI/HindIII adapters (n = any nucleotide, K = guanine or thymine, M = adenine or cytosine): 5′-AAATTCCCTGNNKNNKNNKNNKNNKNNKNNKNGTGA-3′ and 3′-GGAGCMNNMNMMNNNNMNNN-NNMNNMACGATTCA-5′.

The annealed oligonucleotide pairs were ligated to EcoRI/HindIII-digested phage vector according to the T7 Select protocol. The diversity of the peptide library was 5 × 10⁶ primary recombinants.

**Clot Formation.** Blood was anticoagulated with 0.4% sodium citrate and centrifuged at 2,500 × g. The plasma was collected, spun again to remove remaining blood cells, and frozen at −80°C. Clotting was initiated by adding CaCl₂ to 20 mM, and the clot was repeatedly washed with PBS.

**Phage Screening.** Human plasma clots were incubated with the CX8C peptide library at 22°C for 30 min and extensively washed with PBS. Plasma was then added to the clot to remove phage recognizing soluble plasma components. The phage pool that remained bound to the clot was quantified and amplified. The process was repeated until maximum clot binding was reached. The peptide-encoding DNA from 24 randomly picked phage clones in the selected pool was isolated and sequenced.

**Peptide Synthesis.** Peptides were synthesized and conjugated to fluorescein isothiocyanate as described (24) and cyclized by exposure to air.

**Imaging, Antibodies, and Immunohistochemistry.** Imaging under a blue light and examination of the distribution of fluorescein-conjugated peptides after tail vein injection have been described (24, 25).

The imaging methods are briefly described as follows. A Leica fluorescence stereomicroscope, model LZ12, equipped with a 50-W mercury lamp, was used for high-magnification imaging. Selective excitation of GFP was produced through a D425s60 band-pass filter and 470 DCXR dichroic mirror. Emitted fluorescence was collected through a long-pass filter GG475 (Chroma Technology, Brattleboro, VT) on a Hamamatsu C5810 three-chip cooled color charge-coupled device camera.
Images were processed for contrast and brightness and analyzed with the use of IMAGE PRO PLUS 3.1 software (Media Cybernetics, Silver Springs, MD). Images of 1,024 × 724 pixels were captured directly on an IBM PC or continuously through video output on a high-resolution Sony VCR model SLV-R1000 (Sony). Imaging at lower magnification that visualized the entire animal was carried out in a light box illuminated by blue light fiber optics (Lightools Research, Encinitas, CA) and imaged by using the thermoelectrically cooled color charge-coupled device camera as described above (26).

The mice were perfused through the heart 3 h after the peptide injection, and tissues were removed for examination. For fibrin and fibronectin immunostaining, sections were fixed with 4% paraformaldehyde and stained with biotinylated mouse fibrinogen antiserum (Nordic) or a polyclonal anti-mouse fibronecin antibody (Chemicon), followed by streptavidin Alexa Fluor 594 or anti-rabbit Alexa Fluor 594 (Molecular Probes), respectively. For peptide overlay, sections of frozen OCT-embedded tissue were incubated with 10 µg/ml fluorescein-conjugated peptide for 30 min at room temperature. Tissue fluorescence was quantified by using IMAGE PRO PLUS software. At least two images from representative microscopic fields were analyzed from each tissue sample, and data from individual mice that received the same treatment (n = 2–8) were pooled.

Wound Assays. The wire injury of the mouse femoral artery was induced as described (27). Thirty minutes after the wire injury, 250 µg of fluorescein-conjugated peptides were injected i.v. Muscle injuries were induced in mice by applying a crush injury to the quadriceps muscle, and 500 µg of fluorescein-conjugated peptides was injected i.v. 48 h later. Skin incisions were studied similarly. The mice were perfused through the heart 4 h after the peptide injection, and tissues were excised, fixed in 4% paraformaldehyde, and embedded in OCT.

We thank Drs. Reinhard Faessler (Max Planck Institute for Biochemistry, Martinsried, Germany) and Jay Degen (Cincinnati Children’s Hospital Medical Center, Cincinnati) for knockout mice, Drs. Lianglan Zhang (Burnham Institute for Medical Research) and Lionel Hebbard (Burnham Institute for Medical Research) for providing frozen tumor tissues, Dr. Fernando Ferrer for peptide synthesis, Robbin Newlin for help with histology, and Paul Kirsch for editing. This work was supported by Department of Defense Grant DAMD 17-02-1-0315 and National Cancer Institute Contract N01-CO-37007 from the National Cancer Institute. This work was supported at AntiCancer by National Institutes of Health Grants CA 699258 and CA 105563.