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CLT1 Targets Angiogenic Endothelium through CLIC1 and Fibronectin

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Abstract

Angiogenesis is important for tumor growth and metastasis. CLT1 (CGLIIQKNEC), a peptide that binds to tumor interstitial spaces in the presence of fibrin-fibronectin, has structural similarity to the anti-angiogenic β -sheet peptides anastellin and anginex. This similarity is reflected in the ability of CLT1 to form co-aggregates with fibronectin that induce an unfolded protein response and cause autophagic cell death in proliferating endothelial cells. CLT1 cytotoxicity is mediated at least in parts by a novel CLT1 binding protein, Chloride Intracellular Channel 1 (CLIC1), which promotes internalization of CLT1-fibronectin co-aggregates in a mechanism that depends on the LIIQK amino acid sequence of CLT1. LIIQK encompasses amino acid residues relevant for CLT1 binding to CLIC1 and in addition, facilitates the formation of CLT1-fibronectin co-aggregates, which in turn promote translocation of CLIC1 to the endothelial cell surface through ligation of integrin $\alpha v \beta 3$. Paralleling the *in vitro* results, we found that CLT1 co-localizes with CLIC1 and fibronectin in angiogenic blood vessels *in vivo*, and that CLT1 treatment inhibited angiogenesis and tumor growth. Our findings show that CLT1 is a new anti-angiogenic compound, and its mechanism of action is to form co-aggregates with fibronectin, which bind to angiogenic endothelial cells through integrins, become internalized through CLIC1 and elicit a cytotoxic unfolded protein response. The simple structure and high potency of CLT1 make it a potentially useful compound for anti-angiogenic treatments.

Keywords

CLT1; fibronectin; chloride intracellular channel 1; angiogenesis; integrin

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ETHICAL STANDARDS

Experiments comply with current U.S. law.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

INTRODUCTION

Angiogenesis, the sprouting of new blood vessels from existing ones, is critical for expanding the blood supply in growing tumors [1]. A combination of hypoxia and genetic reprogramming leads tumor cells to secrete a plethora of pro-angiogenic factors that induce expression of receptor tyrosine kinases, integrins, proteases and extracellular matrix components in the angiogenic vasculature [2]. Pathways linked to these molecules promote endothelial proliferation, motility and invasion [3,4]. Conversely, inhibiting proteins that promote these functions with specific antibodies, peptides or small molecules has been shown to reduce angiogenesis and, consequently tumor growth [5–7]. On the forefront of anti-angiogenic treatments are tyrosine kinase inhibitors targeting VEGFR, PDGFR and c-KIT [7,8]. However, despite the potency of this class of angiogenesis inhibitors under preclinical conditions, their effectiveness under clinical settings has been rather transitory, as tumors rapidly develop resistance [9]. Additional problems resulted from side effects such as hypertension, thrombosis and bleeding [10,11]. These results underscore the continued need to develop efficient and well-tolerated anti-angiogenic treatments.

Endogenous angiogenesis inhibitors, such as endostatin, denatured antithrombin and anastellin represent an alternative to the compounds targeted to the tyrosine kinase receptors [12–15]. The common denominator of this heterogeneous group of peptides is their dependence on integrin-binding plasma adhesion proteins such as fibronectin or vitronectin for their inhibitory activity *in vivo* [14]. The interdependence of angiogenesis inhibitors and adhesion proteins has been extensively studied in the case of anastellin, a fragment of the first type III repeat of fibronectin, which initiates co-aggregation with plasma fibronectin as a means for RGD-dependent binding to integrins specifically expressed on angiogenic blood vessels [15,16]. The functional interrelationship of anastellin and plasma fibronectin is reproduced by anginex, a synthetic β -sheet peptide that shares common structural characteristics with a large number of angiogenesis inhibitors including anastellin, endostatin, denatured antithrombin and amyloid β [17–20]. β -sheet peptides such as anginex and anastellin contain exposed hydrophobic amino acid residues that are relevant for the co-aggregation of fibronectin [17,21]. In addition, the β -sheet conformation has direct effects on cell function, as in the case of amyloid β , which forms insoluble intracellular aggregates, or anginex, which has been shown to disrupt the endothelial cell membrane [22–26].

Phage library screening has led to the identification of a number of peptides that specifically home to blood vessels, lymphatics or tumor cells in tumors [24–26]. Combined with identification of the cognate receptors, these peptides have provided insight into the process of angiogenesis, while providing valuable reagents for the delivery of drugs to tumors. We previously screened a phage displayed peptide library on clotted plasma *in vitro* and identified two peptides, CLT1 and CLT2 that recognize various types of tumors, but not healthy organs [27]. Both peptides associate with fibrin and fibronectin in tumor interstitial spaces and cease to home to tumors grown in mice deficient for plasma fibronectin or fibrinogen. CLT1 (CGLIIQKNEC) contains a sequence of hydrophobic amino acids that is similar to the key functional residues in anastellin and anginex [17,21], whereas CLT2 (CNAGESSKNC) is largely hydrophilic. Here, we sought to determine if CLT1 has anti-angiogenic properties in addition to its tumor homing function.

MATERIALS AND METHODS

Peptides

Recombinant anastellin was purified as previously described [15]. CLT variants were purchased from Prim Biotech (Cambridge, MA). Anginex was synthesized as previously described [23]. Peptide quality was monitored by mass spectroscopy and HPLC. CLT

peptides were cyclized by exposure to air, which leads to the formation of intramolecular disulfide bonds between C- and N-terminal cysteines. A linear version of CLT1 was generated by replacing cysteines with alanine. A list of CLT1 variants is provided in Table 1. Peptides were conjugated to carboxyfluorescein (CF) or biotin via a 2-aminoethoxy-2-ethoxyacetic acid (AEEA)-linker for fluorescent studies or pull down assays, respectively. Peptides were free of endotoxin (< 0.01 EU/ml; ToxinSensor chromogenic LAL assay, GenScript).

Polymerization assay

Peptides (1 mg/ml) were mixed with 1 mg/ml albumin, fibronectin (EMD Chemicals), fibrinogen (Enzyme Research Laboratories) or PBS in a 96 well plate. Cross-aggregation was examined using an absorbance plate reader at 590 nm as previously described [15].

Cell lines and treatments

HUVEC were purchased from Lonza, human bladder epithelial cells were from Lifeline Cell Technology and neonatal human dermal fibroblasts (Hs58.Fs) were from ATCC. Cells were cultured at 37°C under a humidified, 5% CO₂ atmosphere according to manufacturers' specifications and as previously described [28]. Where indicated, cells were incubated at 4°C. Unless otherwise noted cells were treated at a plating density of ca. 50 % to ensure a linear growth rate. Peptides were diluted in H₂O to 2 mg/ml and added to cells at concentrations ranging from 2.5–150 µg/ml in the presence of 2% FBS. IAA94 (200 µM; Sigma Aldrich), bafilomycin A1 (EMD Chemicals), GRGDSP (RGD) or GRADSP (RAD) peptides (ea. 300 µM; EMD Chemicals) were added to cells at the time of peptide treatment. Fibronectin free media was generated by passing FBS through a gelatin agarose column to remove fibronectin. This media was then supplemented with fibronectin or fibrinogen (ea. 30 µg/ml) to generate fibronectin⁺ or fibrinogen⁺ media.

Cytotoxicity assay

Cell death was assessed at indicated times with commercially available reagents for the detection of LDH, DNA fragments and genomic DNA as previously described [23]. Results were normalized for background cell death observed in untreated cells. LDH release was maximal in response to 0.1% (v/v) Triton X-100, and DNA fragmentation was maximal in response to simultaneous treatment with camptothecin (1.4 µM; Sigma-Aldrich) and staurosporine (100 nM; Alexis Biochemicals).

RNA analysis

HUVEC were treated with 150 µg/ml CLT1 or CLT1-IK1 and monitored for changes in cell stress related gene expression after 7 hours. Total RNA was isolated from adherent cells using the Qiagen RNeasy kit (Qiagen) and hybridized to the Human Stress and Toxicity PathwayFinder PCR Array (SABiosciences) according to the manufacturer's instructions. RNA expression was measured using the iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories) and values were normalized to housekeeping genes present on the array. Genes that significantly changed in response to CLT1 treatment compared to CLT1-IK1 were identified.

Microscopy

To analyze internalization of CLT1, cultured cells grown on collagen-coated coverslips (BD Bioscience) were incubated with 10–25 µg/ml fluorescein-conjugated peptides for 1–24 hours, fixed in 4% paraformaldehyde (PFA) and stained with DAPI-containing mounting media (Vectashield; Vector Laboratories). Lysosomes were labeled with 100 nM LysoTracker Red DND-99 (Invitrogen) for 1 hour prior to fixation. For fibronectin, CLIC1

and integrin $\beta 3$ staining, PFA-fixed cells were permeabilized with 0.5% Triton x100 and incubated with anti-fibronectin (Millipore), anti-CLIC1 (Abcam), anti- $\beta 3$ (LM609; Millipore) or isotype control, followed by incubation with Alexa Fluor 488, 546 or 594-conjugated secondary antibody (Invitrogen) and analyzed using a fluorescence microscope (Zeiss Axioplan 2) or confocal microscope (Leica TCSSL) with image processing units. Non-fixed live cells were preincubated with Hoechst 33342 (5 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) and then labeled with anti-CLIC1 to identify CLIC1 cell surface expression. For confocal microscopy, nuclei were stained with Draq5 (eBioscience). Where indicated, coverslips were coated with fibronectin or vitronectin (ea. 10 $\mu\text{g}/\text{ml}$; BD Bioscience) at 37°C for 1 hour, washed and incubated with cells for 1 hour prior to staining with anti-CLIC1 antibody. CLT homing was analyzed in histological sections from matrigel plugs based on the unique green fluorescence of the peptides. For immunohistochemistry, tissues were fixed with acetone and incubated with anti-CD31 (BD Bioscience), anti-fibronectin, anti-CLIC1 or anti-LC3B (Cell Signaling Technology) and Alexa Fluor 546, 594 or 647-conjugated secondary antibody (Invitrogen). Endogenous IgG was blocked using the Mouse on Mouse Basic Kit (Vector Laboratories). Digitized images were processed with Adobe Photoshop.

Pull-down protein interaction assay and mass spectrometry

Biotinylated CLT peptides were conjugated to streptavidin beads using the ProFound™ Pull-Down Biotinylated Protein Protein Interaction Kit (125 μg biotinylated peptide/50 μl beads). Proliferating HUVEC cells were lysed in ice cold TBS containing 200 mM n-octyl- β -D-glucoside, 1 mM CaCl_2 and Halt protease inhibitor cocktail (Fisher Scientific). Protein aliquots (0.7–1 mg) were precleared with 50 μl streptavidin beads at 4°C for 2 hours, loaded onto the CLT biotinylated streptavidin columns and incubated overnight at 4°C on a rotor. Columns were washed according to manufacturer specifications and bound proteins were eluted using 50 μl of 4x XT sample buffer containing 20x reducing agent (BioRad). Eluted proteins were separated on 4–12% Criterion XT Bis-Tris polyacrylamide gels (BioRad) and visualized by coomassie stain. Protein bands were excised, digested in gel with sequencing grade trypsin (Promega) and peptides analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a linear ion trap (LTQ-XL, ThermoFisher Scientific) as described previously [29]. LC-MS/MS data were searched against the human proteome database (UniProt, Jan 2009 release) with SEQUEST (ThermoFisher Scientific) using high stringency filtering criteria [29]. Only proteins present in the CLT1-affinity purified material from two biological replicates were considered for further biological analysis.

Western blot analysis

Cellular fractions, prepared using the Subcellular Protein Fractionation Kit (Thermo Fisher Scientific), and proteins eluted from the biotinylated CLT columns were separated by SDS-PAGE, transferred onto PVDF and stained with 0.05% Ponceau S (Sigma-Aldrich) to ensure equivalent protein loading. Immunoblots were blocked with 5% non-fat milk or bovine serum albumin and then probed overnight at 4°C with anti-Cathepsin C (R&D Systems), anti-Cathepsin D, anti-LC3B (Cell Signaling Technology), anti-CLIC1, anti-TGM2 or anti-EHD4 (Abcam). Immunoreactivity was detected using peroxidase-conjugated anti-mouse, goat or rabbit IgG antibodies and visualized by enhanced chemiluminescence.

Saturation binding assay

CLT1 binding affinity to CLIC1 was quantified by an ELISA-based assay. Copper coated microtiter wells (Pierce) were coated with 2 $\mu\text{g}/\text{ml}$ of purified his-tagged CLIC1 protein (Abcam) and incubated for 1 hour at room temperature with various concentrations of CF-CLT1 peptide in TBS containing 200 mM n-octyl- β -D-glucoside and 1 mM CaCl_2 . After washing with TBS, peptide binding to CLIC1 was monitored at 485 EX and 520 EM using a fluorescent plate reader (Molecular Devices). Non-specific binding of CF-CLT1 to wells

coated with control protein (his-tagged nucleolin; Abcam) was subtracted as background. Kd values were calculated using GraphPad Prism software (Version 4.03).

siRNA mediated gene silencing

All small interfering RNAs (siRNAs) were purchased from Dharmacon (On-TARGETplus SMARTpools). HUVEC cells were plated for 24 hours prior to transfection with 10 nM CLIC1 (L-009530-00), CLIC4 (L-013553-00), integrin $\beta 3$ (L-004124-00), integrin $\alpha 5$ (L-008003-00) or non-targeting control (D-001810-10) siRNA. Cells were transfected in Opti-MEM medium (Invitrogen) using LipofectAMINE 2000 reagent (Invitrogen) for 5 hours, then placed in normal culture medium and grown for an additional 43 hours prior to treatment with CLT1.

Flow cytometry assay

HUVEC were suspended in media and incubated on a rotor for 45 minutes at room temperature with fibronectin (30 $\mu\text{g/ml}$), CLT1 (100 $\mu\text{g/ml}$), CLT1-fibronectin aggregates in the presence or absence of RGD or RAD, fibrinogen (140 $\mu\text{g/ml}$), CLT1-fibrinogen aggregates or solubilized fibrin clot (140 $\mu\text{g/ml}$) prepared as previously described [30]. Cells were pelleted by centrifugation, incubated with CLIC1 antibody for 30 minutes at 4°C, washed with ice cold buffer, and incubated for 30 minutes on ice with Alexa Fluor 488 anti-mouse F(ab')₂ (Invitrogen). Cell viability was monitored by staining cells with 5 $\mu\text{g/ml}$ propidium iodide (Roche Applied Science). Fluorescence was examined on 15,000 viable cells per sample using a tabletop cytometer (Accuri C6).

Matrigel angiogenesis assay

Animal care was in strict compliance with the institutional guidelines established by the University of Pittsburgh. Female athymic nude mice were subcutaneously injected with 500 μl matrigel (BD Biosciences) containing 500 ng basic FGF (R&D Systems) in the abdomen at two locations. Mice were treated with 4 intravenous injections of CLT1, CLT2 (ea. 300 μg in 150 μl H₂O) or carrier over 6 days starting at day 1. After 7 days, plugs were harvested and frozen in OCT compound. For homing experiments, fluorescein-conjugated CLT peptides (500 μg) were injected intravenously. Matrigel plugs and control organs were harvested 1 or 24 hours after peptide injection following perfusion through the heart.

DU145 tumor treatment study

Male athymic nude mice bearing 2 week-old DU145 subcutaneous xenografts in the flank (average 40 mm³ in tumor volume) were injected intraperitoneally with carrier, CLT1 or CLT2 (300 μg) three times per week. Tumor volume (mm³) was monitored at each treatment time point using calipers. Mice were weighed on day 15, and tumors were harvested and frozen in OCT.

Statistical analysis

Data were analyzed using unpaired two-tailed Student's *t* test or one-way ANOVA followed by the posthoc Tukey's multiple comparisons test. Treatment differences with a two-sided *p* value < 0.05 were considered significantly different. Error bars show mean $\pm$ SEM.

RESULTS

CLT1 is cytotoxic for proliferating endothelial cells

To determine if CLT1 has anti-angiogenic effects, we treated cultured HUVEC with CLT1 and analyzed for cell death. CLT1 treatment resulted in significant cytotoxicity in proliferating endothelial cells while there was little effect on fibroblasts, epithelial cells or

confluent endothelium (Fig. 1a–b). Endothelial cell death only occurred in the presence of CLT1 but not after treatment with the clot-binding peptide CLT2 or the anti-angiogenic peptide anastellin. Cytotoxicity was detected as early as 3.5 hours following CLT1 treatment with the majority occurring between 7 and 24 hours at CLT1 concentrations ranging from 7.5 to 150 $\mu\text{g/ml}$ (Fig. 1c). CLT1 at 150 $\mu\text{g/ml}$ was almost as effective as Triton X 100, which causes complete LDH release, whereas apoptosis-specific DNA fragments were not detected (Fig. 1b, d). The cell death activity of CLT1 required the LIIQK amino acid sequence, and linearizing the cyclic structure by replacing the N- and C-terminal cysteines eliminated the cytotoxic activity of CLT1 (Fig. 1e). Significantly, uptake of CLT1 into endothelial cells was mediated by the same amino acid residues, LIIQK, responsible for cell death (Fig. 1f). CLT1 internalization was inhibited at 4°C, which also protected endothelial cells from CLT1-induced cytotoxicity, suggesting that LIIQK-mediated internalization of CLT1 is a prerequisite for cell death (Fig. 1b, f).

CLT1 co-localized inside endothelial cells with lysotracker in enlarged and deformed lysosomes, which following CLT1 treatment leaked cathepsin C and D into the cytoplasm as a sign of lysosome stress (Fig. 2a–b, Supplementary Fig. 1). Lysosome dysfunction was preceded by an unfolded protein response with up-regulation of mRNA for CHOP, hemoxygenase 1 and heat shock protein 70B as well as by increased expression of the autophagy marker LC3-II (Fig. 2c–d). The inactive CLT1 variant IK1 did not induce LC3-II conversion or lysosome dysfunction (Fig. 2a–b, d). Conversion of LC3-I to LC3-II was further enhanced by bafilomycin A1 demonstrating that CLT1 causes autophagy and does not merely disrupt the maturation of autophagosomes (Fig. 2d). Together our results suggest that LIIQK-mediated internalization of CLT1 leads to ER stress, autophagy and cell death. Interestingly, pretreatment of HUVEC with bafilomycin A1, which prevents the fusion of autophagosomes with lysosomes also ameliorated cytotoxicity of CLT1, indicating that endothelial cell death is a direct consequence of CLT1-induced autophagy and subsequent lysosome dysfunction (Fig. 2e).

CLT1-fibronectin co-aggregation supports CLT1-mediated cell death

The anti-angiogenic peptides anastellin and anginex form large insoluble aggregates when mixed with fibronectin or fibrinogen [15,31]. We found that mixing CLT1 with fibronectin or fibrinogen also increased co-aggregation (Fig. 3a). Furthermore, CLT1 variants that failed to induce internalization or cell death were also significantly less sufficient in aggregating fibronectin (Fig. 3b). The interaction of CLT1 with fibronectin was also relevant in cultured endothelial cells (Fig. 3c–d). Confocal microscopy revealed that after 7 hours CLT1 co-localized completely with fibronectin in HUVEC cultured in the presence of fibronectin while CLT1 uptake was strongly reduced in absence of fibronectin (Fig. 3c). Paralleling these results we found that addition of fibronectin to the HUVEC culture media increased CLT1-induced cell death after 3.5, 7 and 24 hours whereas fibrinogen had the opposite effect (Fig. 3d–e). Overall, our results show that CLT1 forms co-aggregates with fibronectin and that this function accelerates CLT1 internalization and cell death.

CLT1 uptake and cytotoxicity is mediated by CLIC1

To further define the mechanism of CLT1 internalization, we analyzed endothelial cell extracts by CLT peptide affinity chromatography and LC-MS/MS. This approach resulted in the identification of 4 cell surface proteins out of a total of 86 that displayed a higher affinity for CLT1 than for CLT2 (Supplementary Table 1). The most promising candidates, TGM2, CLIC1 and EHD4, exhibited higher affinity for CLT1 over CLT2 in immunoblotting, however, only CLIC1 was selective for CLT1 compared to the inactive CLT1 variants IK1, QA and LCLT1 (Fig. 4a). Saturation binding of CLT1 to purified CLIC1 yielded an affinity of $K_d=10.2 \mu\text{M}$ (Fig. 4b). Using confocal microscopy, we found that CLT1 co-localized

with CLIC1 at the cell margins after 1 hour and in the perinuclear space after 7 hours (Fig. 4c). CLIC1 knockdown with siRNA significantly reduced internalization and cytotoxicity of CLT1 in HUVEC after 24 hours, while treatment with siRNA against CLIC4 or a saturating concentration of the CLIC1 channel blocker IAA94 had no effect on cell death induced by CLT1 (Fig. 4d–e; Supplementary Fig. 2). CLIC1 siRNA alone did not change endothelial cell survival indicating that CLT1 does not act as a CLIC1 inhibitor (Supplementary Fig. 2). Together, these results show that CLIC1 is critical for endothelial cell uptake and cytotoxicity of CLT1.

CLT1-fibronectin co-aggregation supports CLIC1 cell surface expression

CLIC1 has been shown to shuttle between the cytosol and the cell membrane [32]. Using fluorescence microscopy, we found that CLIC1 was highly concentrated in the cell membrane at the leading edge of substrate-attached proliferating HUVEC, whereas confluent HUVEC exhibited a more diffuse distribution of CLIC1 (Fig. 5a). The localization of CLIC1 to lamellipodia was seen in endothelial cells cultured on fibronectin and vitronectin but not on collagen. Using live, non-permeabilized cells for immunohistochemistry, we confirmed that CLIC1 was expressed on the cell surface but failed to detect cell surface CLIC1 in detached endothelial cells by flow cytometry (Fig. 5a–b). These results suggest that adhesive interactions with RGD-containing extracellular matrix proteins promote CLIC1 cell surface expression. Significantly, adding CLT1-fibronectin co-aggregates or solubilized fibrin complexes to suspended endothelial cells compensated for the loss of substrate contacts and led to robust induction of cell surface CLIC1 (Fig. 5b–c). The cell surface localization induced by the CLT1-fibronectin co-aggregates was partially inhibited by a peptide containing the integrin-binding RGD motif, but not by an inactive peptide with an RAD sequence. Fibronectin, fibrinogen and CLT1 alone or co-aggregated with fibrinogen had only minor effects. Moreover, blocking integrin function with the RGD peptide or knocking down integrin $\alpha v \beta 3$, which co-localizes with CLT1-fibronectin-coaggregates in endothelial cells, inhibited the cytotoxic function of CLT1 after 24 hours to a level similar to that achieved by knocking down CLIC1 (Fig. 5d–e, Supplementary Fig. 3). Together, the results indicate that CLT1-fibronectin co-aggregates contribute to CLT1-mediated cytotoxicity by up-regulating CLIC1 in an integrin-dependent manner.

CLT1 has anti-angiogenic and anti-tumor activity in vivo

Fibronectin co-aggregation has been shown to support the homing of anti-angiogenic peptides to angiogenic vasculature [15]. Fluorescein-conjugated CLT1 when intravenously injected into mice with subcutaneous bFGF-impregnated matrigel plugs, homed to the angiogenic blood vessels within the plugs (Fig. 6a). Using fluorescence microscopy, we found that CLT1 co-localized with both CLIC1 and the vascular marker CD31, and that an intact LIQK motif was needed for the angiogenesis homing (Fig. 6a–b). The homing was specific, as no CLT1 fluorescence was seen in the mature blood vessels of other tissues, which also expressed significantly less CLIC1 than angiogenic blood vessels in matrigel or tumor xenografts (Fig. 6c). Confocal microscopy confirmed that CLT1 associates with CLIC1 in tumor blood vessels and revealed co-localization of CLT1 with fibronectin in large aggregates (Fig. 6c, 7a–b). CLT1 treatment significantly inhibited the formation of new blood vessels in matrigel plugs and DU145 prostate tumor xenografts, which correlated with overall inhibition of tumor growth, while the body weight of the mice remained unchanged (Fig. 8a–d). CLT2 did not home to angiogenic blood vessels and, therefore, had no anti-angiogenic or anti-tumor effects (Fig. 6b; Fig. 8a–b, d). Notably, we detected co-localization of LC3 with CLT1 co-aggregates in matrigel blood vessels, indicating that CLT1 has similar effects *in vivo* as *in vitro* (Fig. 8e). Overall our results show that CLT1

possesses anti-angiogenic and anti-tumor activity that correlates with the ability of this peptide to form fibronectin aggregates and to interact with CLIC1 through the LIIQK motif.

DISCUSSION

Screening a phage display library for binding to clotted plasma, we previously identified a peptide, CLT1 that strongly homes to tumor interstitial spaces in the presence of fibrin and fibronectin [27]. Here we demonstrate that CLT1 is anti-angiogenic *in vivo* and attribute this activity to the LIIQK sequence motif in CLT1, which mediates homing of CLT1-fibronectin co-aggregates to angiogenic blood vessels, CLIC1-mediated internalization into endothelial cells and autophagic cell death.

CLT1 consists of a hydrophilic C-terminal portion that shares homologies with CLT2 and a unique hydrophobic peptide sequence, LIIQK, towards the N-terminus that is critical for the anti-angiogenic effects of CLT1. This specific structure activity relationship is reminiscent of amyloidogenic β -sheets, which, as a result of exposed hydrophobic amino acids, have a high propensity to generate insoluble, cytotoxic aggregates [33]. Inside the cell, these protein aggregates typically induce ER stress, autophagy, and, ultimately, cell death as a result of lysosome dysfunction [34]. The β -sheet structure also lends itself to interactions with plasma adhesion proteins such as fibronectin, which is essential for the anti-angiogenic activity of a number of angiogenesis inhibitors including endostatin, anastellin and anginex [15,14]. The requirement for fibronectin as an anti-angiogenic co-factor *in vivo* correlates in the case of anastellin and anginex with the capacity to aggregate fibronectin *in vitro* [15]. With CLT1, we identified a third fibronectin-aggregating peptide with strong anti-angiogenic and anti-tumor activity *in vivo*. CLT1 was highly cytotoxic in actively proliferating HUVEC, which mimic key aspects of angiogenic cells [35]. At the same time, CLT1 lacked activity in primary epithelial cells, fibroblasts or confluent HUVEC that represent the mature endothelium [36]. CLT1 cytotoxicity was promoted by fibronectin and mechanistically linked to two cell surface proteins, CLIC1 and integrin $\alpha\text{v}\beta\text{3}$, which both have been shown to be specifically upregulated in angiogenic blood vessels, suggesting that $\alpha\text{v}\beta\text{3}$ and CLIC1 together contribute to the selectivity of CLT1-fibronectin co-aggregates for angiogenic cells [24,37].

We identified CLIC1 on proliferating endothelial cells as a novel CLT1-binding protein by using an unbiased proteomics approach. CLIC1, a member of six highly homologous CLIC family proteins, was originally identified as a chloride channel and while chloride conductance has been implicated in CLIC1-mediated amyloid β cytotoxicity, we found that CLT1-mediated cytotoxicity was unaffected by saturating concentrations of the CLIC1 channel blocker IAA94 [38]. More recently, CLIC1 has been shown to promote pro-angiogenic functions such as endothelial cell migration and branching morphogenesis by regulating integrin turn-over [39]. Consistent with this study, our results provide evidence that CLIC1 functions on angiogenic endothelial cells as a receptor for CLT1 that mediates internalization of CLT1-fibronectin aggregates and cell death in cooperation with integrin $\alpha\text{v}\beta\text{3}$. Notably, CLT1 internalization and cell death was uniquely mediated by CLIC1 whereas the only other CLIC family member expressed by endothelial cells, CLIC4, had no effect on CLT1 function [40]. Several lines of evidence show that CLIC1 binds to CLT1 and, thus, mediates CLT1 function. First, pull down assays using CLT1 as bait resulted in binding of CLIC1, whereas inactive CLT1 variants with mutations within the LIIQK motif were unable to bind CLIC1. Using the inverse procedure, we show saturating binding of CLT1 to immobilized CLIC1. Second, CLIC1, which completely co-localized with CLT1 in endothelial cells, is required for CLT1 internalization as well as CLT1-induced cell death. Third, intravenously injected CLT1 homed to areas of increased CLIC1 expression in matrigel and tumor blood vessels.

The same structural motif of CLT1 that takes part in mediating CLIC1 binding, LIIQK, is also relevant for complex formation with fibronectin, an abundant plasma protein, which is readily available to form complexes with circulating anti-angiogenic peptides [41]. An important function of the fibronectin complexes is to deliver anginex and anastellin to endothelial cells in angiogenic blood vessels that express fibronectin-binding integrins on their luminal surface as a part of their angiogenic signature [24,42]. Paralleling this finding, we demonstrate that fibronectin acts as a co-factor for CLT1 internalization into angiogenic cells and as such significantly promotes CLT1 cytotoxicity. The concept that CLT1 and fibronectin co-operate is further supported by our data showing that the adhesion-dependent translocation of CLIC1 to the cell membrane can be induced by RGD-dependent engagement of endothelial integrins with the fibronectin component of the CLT1-fibronectin co-aggregates. Interestingly, while CLT1 was able to transform plasma fibronectin from an inactive dimer into an adhesive multimer that promotes CLIC1 cell surface expression, CLT1 co-aggregation of fibrinogen had no effect on CLIC1, thus explaining why fibronectin promotes CLT1-induced cell death whereas fibrinogen does not. Moreover, the specific composition of CLT1 aggregates could explain the dichotomy of CLT1 homing, with fibronectin directing CLT1 to angiogenic blood vessels in a manner similar to anastellin and with fibrinogen sequestering CLT1 in the tumor extracellular matrix [15,27].

Based on our results, we propose a model where CLT1-fibronectin complexes bind to endothelial integrins through the RGD motif in fibronectin and then trigger their internalization through LIIQK-dependent interaction of CLT1 with CLIC1. This mechanism appears to be important for the cytotoxic activity of CLT1 because inhibition of CLT1 uptake at low temperatures or treatment with the autophagy inhibitor bafilomycin A1, which blocks the clearance of autophagosomes through the lysosome pathway [43], significantly reduced CLT1 cytotoxicity. The concept that CLT1 promotes autophagic cell death is also supported by our finding that CLT1 aggregates co-localize with the autophagy marker LC3 in angiogenic blood vessels *in vivo*. Autophagy and subsequent lysosome dysfunction are most likely initiated by the unfolded protein response that follows the internalization of aggregate-prone CLT1 complexes. It is also possible that the CLT1-fibronectin-mediated internalization of integrin $\alpha v \beta 3$ contributes to cell death through destabilization of endothelial lipid rafts and subsequent suppression of Erk, PI-3 kinase and mTOR [44,45]. Interestingly, displacement of activated H-ras from lipid rafts has been attributed to the anti-angiogenic function of anginex, which conveys strong cytotoxic activity towards endothelial cells [46]. Anginex has been shown to bind to galectin-1 in addition to fibronectin and this interaction correlates with anginex internalization into endothelial cells [47]. While this function is reminiscent of the interaction of CLT1 with CLIC1, it remains to be seen if anginex-treated cells undergo autophagic cell death and if this mechanism can be promoted by fibronectin.

The function of autophagy for endothelial cell fate has been shown to be highly context dependent as it can be protective as well as death promoting [48,49]. This could explain why we found no cytotoxic effects of anastellin for proliferating endothelial cells despite its similarity to CLT1. Interestingly, the LIIQK motif of CLT1 shares homologies with the LISIQ sequence of anastellin (residues 55–59), which encompasses residues critical for binding and co-aggregating fibronectin [17]. This leads us to speculate that anastellin and by extension fibronectin, from which anastellin is derived, are physiological binding partners of CLIC1 and that CLIC1, consistent with its pro-migratory and pro-invasive function, could be involved in the turnover of matrix fibronectin [39]. While CLIC1 appears to have overall pro-angiogenic function, its interaction with CLT1-fibronectin co-aggregates leads to strong cytotoxicity, thus converting CLIC1 into an anti-angiogenic receptor. Together, our study suggests that utilizing the CLIC1 pathway for the internalization of amyloidogenic peptides such as CLT1 offers great promise as an anti-angiogenic therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. CLT1 cytotoxicity for proliferating endothelial cells depends on LIIQK-mediated internalization of CLT1

(a), the effects of CLT1, CLT2 (ea. 150 µg/ml) and anastellin (1 mg/ml) on proliferating (CLT1-p, CLT2, ANA) or confluent (CLT1-c) HUVEC were analyzed by treating the cells with the peptides for 24 hours. Cell death was assessed by measuring propidium iodine (PI) uptake with flow cytometry. *, $P < 0.05$ versus CLT1-c and CLT2. (b), LDH release in HUVEC cultured at 4°C versus 37°C and neonatal human dermal fibroblasts (NHDF) or human bladder epithelial cells (HBEC) cultured at 37 °C after addition of 150 µg/ml CLT1 or Triton x100 (Tx) for 24 hours. ***, $P < 0.001$ versus CLT1 at 37°C. (c), LDH release was assessed in HUVEC treated with increasing concentrations of CLT1 over time. (d), HUVEC were treated with 150 µg/ml CLT1 or a mixture of camptothecin/staurosporin (C/S) and analyzed for apoptosis-specific DNA fragments. ***, $P < 0.001$ versus C/S. (e), a lysine/alanine scan of CLT1. LDH release was assessed after treating HUVEC for 24 hours with CLT1 and the variant CLT1 peptides (ea. 150 µg/ml). CLT2 (150 µg/ml), which lacks the LIIQK sequence, was used as a negative control. ***, $P < 0.001$ versus CLT1. (f), HUVEC were treated for 24 hours with fluorescein-conjugated CLT1 at 37°C or 4°C, and with fluorescein-conjugated CLT1 variants at 37°C (10 µg/ml of peptide). Peptide internalization (green) and nuclear staining (DAPI, blue) was analyzed by fluorescence microscopy. Representative images from two independent experiments are shown (scale bar, 50 µm).

Fig. 2. CLT1 internalization induces lysosome dysfunction

(a), HUVEC were treated with 10 $\mu\text{g/ml}$ fluorescein-conjugated CLT1 (upper panel, green) or CLT1 IK1 (lower panel, green) for 24 hours, stained with lysotracker (Lyso; red) and analyzed by fluorescence microscopy. Nuclei were stained with DAPI (blue). Scale bar, 10 μm . (b), cytosolic fractions from HUVEC treated with CLT1, IK1 (75 $\mu\text{g/ml}$; 24 hours) were immunoblotted for cathepsins C (CTSC) and D (CTSD). (c), expression of mRNA for CHOP, hemoxygenase 1 (HO1) and heat shock protein 70B (HSP70B) after 7 hours of treatment with CLT1 (fold change over HUVEC treated with the inactive IK1 variant). (d), cytosolic fractions from HUVEC were immunoblotted for LC3 after 7 hour treatment with CLT1 compared to IK1 (7.5–75 $\mu\text{g/ml}$; upper panel) or CLT1 (75 $\mu\text{g/ml}$) in the presence of bafilomycin A1 (B40–400, 40–400 nM; lower panel). (b, d) Ponceau S (PS) staining shows equal protein loading. (e), LDH release in HUVEC treated with 20 nM bafilomycin A1 for 1 hour prior to incubation with CLT1 (25 $\mu\text{g/ml}$) for 24 hours. ***, $P < 0.001$ versus CLT1.

Fig. 3. CLT1 induces fibronectin and fibrinogen aggregates

(a), the ability of CLT1, CLT2 and annexin to form aggregates when incubated with 1 mg/ml fibronectin, fibrinogen or BSA was analyzed by measuring the optical density at 590 nm. ***, $P<0.001$ versus BSA. (b), the fibronectin aggregating activity correlates with the presence of the LIQK motif in CLT1. **, $P<0.01$ versus CLT1. (c), HUVEC cultured with or without fibronectin (upper panel, FN+, 30 $\mu\text{g/ml}$; middle panel, FN-) were treated with 25 $\mu\text{g/ml}$ fluorescein-conjugated CLT1 (green) for 7 hours, stained with an anti-fibronectin antibody (FN, red) and analyzed by confocal microscopy. (c, lower panel) Control IgG of CLT1-treated HUVEC in FN+ media (scale bar, 10 μm). Nuclei were stained with Draq5 (blue). (d-e), HUVEC cultured in presence or absence of fibronectin (d; FN+, 30 $\mu\text{g/ml}$, solid line; FN-, dotted line) or fibrinogen (e; FG+, 30 $\mu\text{g/ml}$, solid line; FG-, dotted line) were treated with increasing concentrations of CLT1 and probed for LDH release after 3.5, 7 and 24 hours. *, $P<0.05$, *** $P<0.001$ for 150 $\mu\text{g/ml}$ CLT1 in FN+ media versus FN- media at a given time point.

Fig. 4. CLIC1 is a CLT1-binding protein

(a), HUVEC cell extracts were fractionated by chromatography on affinity matrices conjugated with the indicated peptides. The eluates were analyzed by mass spectrometry and immunoblotted for 3 promising candidate CLT1-binding proteins, TGM2, EHD4 and CLIC1. (b), the binding of increasing amounts of fluorescein-conjugated CLT1 to immobilized CLIC1 protein. Non-specific binding to a control protein was subtracted. (c), HUVEC were treated with 25 μg/ml fluorescein-conjugated CLT1 (green) for 1 hour (upper panel) or 7 hours (middle panel), stained with an anti-CLIC1 antibody (CLIC1, red) and analyzed by confocal microscopy. (c, lower panel) Control IgG after 7 hours of CLT1 treatment (scale bar, 10 μm). Nuclei were stained with Draq5 (blue). (d), CLT1-induced cell death was measured by LDH release in HUVEC after treatment with IAA94 or siRNAs targeting CLIC1 or CLIC4 compared to non-targeting control siRNA. ***, $P < 0.001$ versus control. (e), uptake of fluorescein-CLT1 was analyzed in HUVEC treated with control siRNA, siRNA targeting CLIC1 or CLIC4 siRNA by fluorescence microscopy. Data are shown as percent CLT1-positive endothelial cells (EC) per optical field (40x). ***, $P < 0.001$ versus control.

Fig. 5. CLIC1 expression on the endothelial cell surface depends on ECM contacts (a, upper panel), immunohistochemical staining with anti-CLIC1 (green) or IgG control in fixed, permeabilized endothelial compared to live, non-permeabilized (non-fixed) endothelial cells. (a, middle panel), staining with anti-CLIC1 (green) in endothelial cells attached to fibronectin (FN), vitronectin (VN) or collagen for 1 hour, followed by fixation and permeabilization. (a, lower panel), CLIC1 staining (green) of proliferating (Pro) and confluent (Con) HUVEC. Nuclei are stained with DAPI (blue). Scale bar, 10 μ m. (b), endothelial cells suspended in media (control) with or without CLT1, fibronectin (FN), CLT1-fibronectin aggregates (CLT1-FN), and CLT1-fibronectin with either an integrin-blocking RGD peptide or control RAD peptide were analyzed for CLIC1 cell surface expression by flow cytometry. ***, $P < 0.001$ versus CLT1-FN. (c), percentage of endothelial cells expressing cell surface CLIC1 following treatment of suspended cells with fibrinogen (FG), CLT1-fibrinogen aggregates (CLT1-FG), or solubilized fibrin complexes (Fib). (d), LDH release after treatment with CLT1 in the presence of the RGD and RAD peptides or in HUVEC transfected with CLIC1, integrin $\alpha 5$, integrin $\beta 3$ or control siRNA. *, $P < 0.05$; ***, $P < 0.001$ versus vehicle and control siRNA. (e), HUVEC cultured with FN (30 μ g/ml) were treated with 25 μ g/ml fluorescein-conjugated CLT1 (green) for 2 hours, stained with an anti- $\beta 3$ antibody ($\beta 3$, red, upper panel) and analyzed by confocal microscopy. (e, lower panel) Control IgG after 2 hours of CLT1 treatment (scale bar, 10 μ m). Nuclei were stained with Draq5 (blue).

Fig. 6. CLT1 co-localizes with CLIC1 in angiogenic blood vessels in vivo

(a), mice carrying subcutaneously placed, angiogenic matrigel plugs were intravenously injected with fluorescein-conjugated CLT1 (500 μ g) as indicated. Histological sections of matrigel plugs isolated 1 hour after the injection of fluorescein-CLT1 (green) were stained for the blood vessel marker CD31 (upper panel; red) or for CLIC1 (lower panel) and analyzed by fluorescence microscopy. Scale bar, 20 μ m. (b), fluorescence microscopy of matrigels after injection of fluorescein-labeled LCLT1, CLT2 or CLT1 variants. Nuclei were stained with DAPI (blue). Scale bar, 20 μ m. (c), DU145 tumor xenografts (upper panel) and control organs (middle panel, heart; lower panel, skin) isolated 1 hour after the injection of fluorescein-CLT1 (green) were stained with anti-CLIC1 (red)/anti-CD31 (blue) and analyzed by confocal microscopy. Scale bar, 10 μ m.

Fig. 7. CLT1 co-localizes with fibronectin in angiogenic blood vessels in vivo

(a), matrigel plugs isolated 24 hours after the injection of fluorescein-CLT1 (green) were stained with anti-CD31 (red)/anti-FN (blue) and analyzed by confocal microscopy. Scale bar, 10 μ m. Magnified images of the inset show co-localization of CLT1 aggregates with FN and CD31 (right panel). (b), DU145 xenografts isolated 1 hour after the injection of fluorescein-CLT1 (green) and stained with anti-CD31 (red)/anti-FN (blue). Scale bar, 10 μ m.

Fig. 8. CLT1 is anti-angiogenic in vivo

(a), number of CD31⁺ blood vessels per microscopic field (40x) in histological sections from matrigel plugs after 1 week of treatment with vehicle, CLT1 or CLT2 (n=7–10; ea. 300 µg i.v.). (b), tumor volume was assessed in mice bearing DU145 tumor xenografts (n=12/treatment group) after treatment with vehicle, CLT1 or CLT2 (300 µg, 3x/week i.p.). *, $P < 0.05$ versus control and CLT2. (c), body weight was assessed at day 15 of the treatment study. (d), number of CD31⁺ blood vessels per microscopic field (20x) in histological sections from DU145 xenografts after 15 days of treatment. (e), matrigel plugs isolated 24 hours after the injection of fluorescein-CLT1 (green) were stained with anti-CD31 (blue)/anti-LC3 (red) and analyzed by confocal microscopy. Scale bar, 10 µm.

Table 1

Amino acid sequence of the CLT1 variants

CLT1	CGLIIQKNEC
CLT1-GA	CALIIQKNEC
CLT1-LK	CGKIIQKNEC
CLT1-IK1	CGLKIQKNEC
CLT1-IK2	CGLIKQKNEC
CLT1-QA	CGLIIAKNEC
CLT1-KA	CGLIIQANEC
CLT1-NA	CGLIIQKAEC
CLT1-EA	CGLIIQKNAC
LCLT1	AGLIIQKNEA