Intravenous liposomal delivery of the snake venom disintegrin contortrostatin limits breast cancer progression


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Abstract
Despite significant research in this area, metastatic breast cancer remains a disease with a poor prognosis. Until an effective therapy is developed, it is imperative that new treatment modalities be investigated. In this report, we describe an effective method for delivery of a novel snake venom disintegrin, contortrostatin (CN), in an orthotopic, xenograft model of human mammary cancer in immunodeficient mice. CN (M, 13,500) is a homodimeric disintegrin isolated from venom of the Southern Copperhead snake. The homodimer possesses two Arg-Gly-Asp sites, which modulate its interaction with integrins on tumor cells and angiogenic vascular endothelial cells. Although our laboratory has previously described the antitumor activity of CN in a mouse model of human mammary cancer, the method of delivery, daily intratumor injection, was not translatable to clinical application. We now describe a clinically relevant method of administering CN, liposomal delivery (LCN). A unique liposomal system has been designed for i.v. administration of a biologically active protein with full retention of biological activity. Pharmacokinetics, biodistribution, platelet reactivity, and immunogenicity of LCN were determined and compared with similar characteristics of native, unencapsulated CN. There are several advantages to liposomal delivery of CN: (1) LCN has a significantly prolonged circulatory half-life compared with native CN; (2) LCN is passively accumulated in the tumor; (3) LCN has no platelet reactivity; and (4) LCN is not recognized by the immune system. Finally, antiangiogenic activity is an important component of CN’s mechanism of antitumor action. We have demonstrated that i.v. delivery of LCN leads to potent antiangiogenic activity in the orthotopic, xenograft human mammary tumor model.

Introduction
Despite significant improvements in the management of breast cancer, for women with distant metastases, the 5-year survival rate is only 21% (1). It is imperative, therefore, that new treatment modalities be developed for metastatic breast cancer. We are investigating the therapeutic potential and delivery options for a novel snake venom disintegrin, contortrostatin (CN), in an orthotopic, xenograft model of human breast cancer in nude mice.

CN (M, 13,500) is a homodimeric disintegrin isolated from venom of the Southern Copperhead snake; each of the monomeric chains contains 65 amino acids. Each chain of the disulfide rich homodimer possesses a Arg-Gly-Asp (RGD) motif at the tip of a flexible loop stabilized by disulfide bonds (2, 3). Interaction of CN with tumor cells is primarily modulated by the RGD motif (4). Although the potent antitumor activity of CN in a breast cancer model has been described previously (5, 6), daily intratumor (i.t.) injection was employed for delivery. This method of administration is not translatable to clinical use and an alternative, more clinically applicable delivery method was sought.

In the development of a more clinically relevant method of drug delivery, the increased vascular permeability associated with cancer-induced angiogenesis (7, 8) provides the foundation for the use of a liposomal delivery system. Liposomes are submicroscopic spheres composed of thin but durable membranes made primarily of phospholipids and cholesterol. The composition, number of lipid layers, size, charge, and permeability of the membrane can be altered to enable the delivery of a variety of therapeutic agents encapsulated inside the liposome (9, 10). Advantages of liposome-mediated drug delivery include the following: (1) ability to maintain therapeutically effective levels of drug for prolonged periods (sustained release); (2) possible decrease in the dosing frequency; and (3) facilitating the administration of drugs with short in vivo half-lives (11), including peptides like CN, by increasing their circulatory half-life. Liposomal encapsulation aids in the selective delivery of the entrapped contents to solid tumors in vivo by preferentially extravasating at the tumor sites. Liposome preparations containing doxorubicin (including Doxil and Evacet; Refs. 12–15) and one containing daunorubicin (DaunoXome; Refs. 16, 17) have been undergoing widespread clinical study. Liposomal encapsulation of these
Contortrostatin Limits Breast Cancer Progression

Contortrostatin (CN), a 50-kDa protein from the snake venom of Agkistrodon contortrix contortrix, has been shown to limit breast cancer progression (37). Most of the experimental failures can be blamed on a combination of issues. Included among these are the following: (1) patient selection, most patients receiving antiangiogenic therapy are very late-stage and therefore, the enhancement of antitumor activity by combining antiangiogenic therapy with chemotherapy may be a promising approach for treating metastatic cancer (35). Thus, the enhancement of the therapeutic activity by combining CN with the standard Saltz regime of irinotecan, 5-fluorouracil, and leucovorin (39, 40). These reports support the concept that antiangiogenic agents can be used successfully in the clinic to improve disease outcome. Avastin is an antibody directed against VEGF while CN is a protein that alters the signaling pathways associated with VEGF. The successes of this trial validate the theory that interference or alteration of the signaling pathways associated with VEGF can improve survival in cancer patients.

Materials and Methods

Materials

Venom of Agkistrodon contortrix contortrix was purchased from Miami Serpentarium (Punta Gorda, FL). CN was purified by multistep high-performance liquid chromatography according to an established protocol (42). Peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDS) was purchased from Life Technologies, Inc. (Gaithersburg, MD). Goat anti-mouse IgG conjugated with FITC was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). MDA-MB-435 cells, an estrogen receptor-negative cell line established from breast cancer cells isolated from the pleural effusion of a woman with metastatic ductal adenocarcinoma of the breast (43), were obtained from Dr. Janet Price (MD Anderson Cancer Center, University of Texas, Houston, TX). The monoclonal antibody against CD31 antigen was purchased from Sigma Chemical Co. (St. Louis, MO) and the immunohistochemistry detection kit was purchased from Zymed (South San Francisco, CA). Other reagents used in these studies were of the highest grade available from VWR (Pittsburgh, PA) and Sigma Chemical.

Liposomal Formulation of CN

Liposomes were prepared using different ratios of high-temperature transition lipids, cholesterol, and lipids derivatized with long-chain polymers (polyethylene glycol). The particles were formed using probe sonication according to previously described procedures (44). Briefly, drugs has been shown to maintain bioactivity of the drug following delivery to the tumor but with decreased systemic toxicity (18, 19). We have now developed a liposomal system that delivers CN to the tumor site and has significant translational potential as a clinically relevant method of administration.

Antitumor activity of CN is based on its high-affinity interaction with several integrins displayed on both cancer cells and newly growing vascular endothelial cells (20–22). CN blocks breast cancer cell adhesion and migration (22, 23); in addition, it inhibits tumor-induced angiogenesis by blocking important angiogenic pathways in endothelial cells mediated by integrins αvβ3, αvβ5, and αvβ5 (5, 22, 24). These diverse mechanisms of action provide CN with a distinct advantage over many other antitumor and antiangiogenic agents that only block a single angiogenic pathway and do not directly affect the growth of tumor cells (5, 6). The importance of the vitronectin receptor αvβ3 in angiogenesis is well known; αvβ3 undergoes up-regulation in tumor implantation sites in both animal (25) and chick embryo chorioallantoic membrane models (26). Integrin αvβ3 has also been found to play a role in angiogenesis and cancer metastasis (25, 27, 28). It was reported that an anti-αvβ3 antibody blocked angiogenesis induced by basic fibroblast growth factor whereas an anti-αvβ3 antibody blocked vascular endothelial growth factor (VEGF)-induced angiogenesis (29). This suggests that downstream signal transduction pathways of the two cytokines are distinct and indicate that there are two angiogenic pathways modulated by distinct α integrins. αvβ1 antagonists inhibit tumor angiogenesis (30) and cause regression of human tumor xenografts in animal models. Kim et al. (31) reported that αvβ3, and its ligand fibronectin, are coordinately up-regulated on blood vessels in human tumor biopsies. The central cell binding domain of fibronectin induces angiogenesis in a αvβ3-dependent manner. An antagonist of αvβ3 blocks angiogenesis induced by some growth factors but has little effect on angiogenesis induced by VEGF (32). αvβ1 and αvβ3 participate in the same pathways of angiogenesis (33) and inhibition of lumen formation can only be achieved by blocking both αvβ3 and αvβ1. Thus, integrin antagonism is an important approach for antiangiogenic and antimetastatic therapies (34). Targeting αvβ3 in combination with 5-fluorouracil infusion reduced liver metastases formation and improved survival in a colon cancer model (35). Thus, the enhancement of antitumor activity by combining antiangiogenic therapy with chemotherapy may be a promising approach for treating metastatic cancer (36).

Despite receiving several setbacks in recent clinical trials, antiangiogenic therapy remains a very promising form of therapy (37). Most of the experimental failures can be blamed on a combination of issues. Included among these are the following: (1) patient selection, most patients receiving antiangiogenic therapy are very late-stage and almost beyond cure; (2) dosage requirements for antiangiogenic agents; (3) traditional strategies that are used for evaluation in clinical trials for anticancer therapies are not appropriate for assessing the efficacy of antiangiogenic agents; and (4) most antiangiogenic agents are only targeted to a single angiogenic pathway, and because tumors produce different angiogenic factors at different times, antiangiogenic agents that target a single pathway are not likely to be very effective on their own (37). However, the recent success of Avastin (bevacizumab), a human monoclonal antibody to VEGF, in metastatic colorectal cancer trials is reassuring (38). In randomized phase III trials, Avastin increased survival time by >30% when given in combination with the standard Saltz regime of irinotecan, 5-fluorouracil, and leucovorin (39, 40). These reports support the concept that antiangiogenic agents can improve survival in cancer patients.

Antitumor activity of CN is based on its high-affinity interaction with several integrins displayed on both cancer cells and newly growing vascular endothelial cells (20–22). CN blocks breast cancer cell adhesion and migration (22, 23); in addition, it inhibits tumor-induced angiogenesis by blocking important angiogenic pathways in endothelial cells mediated by integrins αvβ3, αvβ5, and αvβ5 (5, 22, 24). These diverse mechanisms of action provide CN with a distinct advantage over many other antitumor and antiangiogenic agents that only block a single angiogenic pathway and do not directly affect the growth of tumor cells (5, 6). The importance of the vitronectin receptor αvβ3 in angiogenesis is well known; αvβ3 undergoes up-regulation in tumor implantation sites in both animal (25) and chick embryo chorioallantoic membrane models (26). Integrin αvβ3 has also been found to play a role in angiogenesis and cancer metastasis (25, 27, 28). It was reported that an anti-αvβ3 antibody blocked angiogenesis induced by basic fibroblast growth factor whereas an anti-αvβ3 antibody blocked vascular endothelial growth factor (VEGF)-induced angiogenesis (29). This suggests that downstream signal transduction pathways of the two cytokines are distinct and indicate that there are two angiogenic pathways modulated by distinct α integrins. αvβ1 antagonists inhibit tumor angiogenesis (30) and cause regression of human tumor xenografts in animal models. Kim et al. (31) reported that αvβ3, and its ligand fibronectin, are coordinately up-regulated on blood vessels in human tumor biopsies. The central cell binding domain of fibronectin induces angiogenesis in a αvβ3-dependent manner. An antagonist of αvβ3 blocks angiogenesis induced by some growth factors but has little effect on angiogenesis induced by VEGF (32). αvβ1 and αvβ3 participate in the same pathways of angiogenesis (33) and inhibition of lumen formation can only be achieved by blocking both αvβ3 and αvβ1. Thus, integrin antagonism is an important approach for antiangiogenic and antimetastatic therapies (34). Targeting αvβ3 in combination with 5-fluorouracil infusion reduced liver metastases formation and improved survival in a colon cancer model (35). Thus, the enhancement of antitumor activity by combining antiangiogenic therapy with chemotherapy may be a promising approach for treating metastatic cancer (36).

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Past studies have shown CN to be an effective agent in limiting tumor growth and spread (6, 41). The present report extends and enhances these studies by describing a clinically relevant delivery system for this highly effective antitumor agent. In the study reported here, we describe the antitumor and antiangiogenic efficacy of i.v. delivered liposomal CN (LCN). In addition to observations on tumor growth in an orthotopic, xenograft model of human breast cancer, we report on the pharmacokinetics and biodistribution of LCN and its lack of platelet reactivity and immunogenicity.

Materials and Methods

Liposomal Formulation of CN

Liposomes were prepared using different ratios of high-temperature transition lipids, cholesterol, and lipids derivatized with long-chain polymers (polyethylene glycol). The particles were formed using probe sonication according to previously described procedures (44). Briefly,
a proprietary mixture of lipids (including disteryocephosphatidylcholine, cholesterol, and polyethylene glycol derivatized lipid) is dissolved in a chloroform/methanol solution. Thin lipid films are created by pipetting aliquots of the lipid solutions into round-bottomed glass tubes and evaporating the solvent at 65°C under a stream of nitrogen gas. The films are placed under vacuum for at least 24 h to remove residual organic solvent. Liposomes are prepared by hydrating the lipid films with CN dissolved in 10 mM sodium phosphate and 9% sucrose (pH 7.2) and then incubating the suspension at 65°C for 5–10 min followed by probe sonication until the suspension is translucent. In the resultant suspension, unencapsulated CN is removed by ultrafiltration and the suspension is sterilized prior to use by passage through a 0.22 μm filter. The entrapped protein concentration is determined by disruption of the liposomes with chloroform/methanol/water (10:40:50) followed by centrifugation at 14,000 × g. The supernatant is analyzed for CN concentration using the BCA assay (Pierce Chemical Co., Rockford, IL) following an established protocol (45).

**Iodination of Native CN and LCN**

CN in solution can be directly iodinated, but CN in liposomes is iodinated by passive diffusion of radioiodine (125I or 131I) into the core of the liposome using a modification of the chloramine T method (46). Briefly, chloramine T is dissolved in PBS and added to buffered LCN or CN solution (100–600 μg at 0.3–2.0 mg/ml in PBS) containing Na 125I or Na 131I. Five successive aliquots of 15 μg chloramine T (in 5 μl) are added at 5-min intervals to the reaction mixture (final volume 400 μl). Following the final chloramine T addition, excess 125I or 131I is quenched through the addition of sodium metabisulfite (100 μg/400 μl reaction). Unreacted iodine is removed from the L-125I-CN solution and the buffer is exchanged by repeated filtrations through a 100-kDa cutoff filter in an Amicon pressure filtration device (Amicon, Inc., Beverly, MA). This allows iodine and any free CN to pass through the membrane and be separated from the LCN solution. With native CN, a membrane with 5-kDa cutoff is employed to retain the 13.5-kDa CN while changing buffer and removing unreacted iodine. The resultant retentate contains either intact L-125I-CN or 125I-CN. To establish that CN in LCN is iodinated, samples were included in which non-CN-bearing liposomes in an identical iodination reaction mixture were used or radioactivity of iodinated CN was determined following disruption of L-125I-CN, separation of the contents by SDS-PAGE (47), and counting bands of CN cut from the gel. 

**Assay of CN Activity following Iodination and Liposomal Encapsulation**

Liposome formulations used to deliver CN contain protein at concentrations greater than 1.5 mg/ml with encapsulation efficiency of greater than 80% as determined by BCA protein assay (45). Both radioiodinated and radioinert CN were analyzed for retention of biological activity on lytic release from liposomes. Activity was assessed by the ability of CN to inhibit ADP-induced platelet aggregation. Briefly, the inhibitory activity of native CN and CN released from liposomes (either iodinated or uniodinated) on ADP-induced platelet aggregation was determined using a platelet aggregometer (Platelet Aggregometer IV Plus; Helena Laboratories, Beaumont, TX) with human platelet-rich plasma (PRP). Whole human blood (36 ml) obtained from volunteers was drawn into 4 ml of 0.1 M sodium citrate. Donors had not received any aspirin-based medication for at least 2 weeks prior to blood donation. The blood was centrifuged (150 × g for 20 min) at 22°C. The supernatant, PRP, was carefully transferred to a new tube. PRP (1 ml) was placed in a microcentrifuge tube and centrifuged for 1 min at full speed. The supernatant was removed and retained as platelet-poor plasma (PPP). PPP was used as a blank to standardize the platelet aggregometer. Inhibition of ADP-induced platelet aggregation was monitored at 37°C by adding samples (iodinated CN and LCN) over a range of CN concentrations (0–250 nm) to PRP 1 min prior to the addition of ADP. The level of aggregation inhibition was plotted against the final concentration of CN added to each sample. A sample of LCN that had been lysed with a solution of methanol/chloroform/water (40:10:50) was included to demonstrate that encapsulated CN was active. Controls included PBS and native CN in lysis buffer. The rate of release of CN from intact LCN was determined following incubation of LCN in PPP. LCN (200 nM) was added to PPP and incubated for various times. At timed intervals, incubates were added to an equal volume of PRP that had been prepared no longer than 30 min prior to addition of samples. To verify platelet activity, ADP controls were run at each time point with the freshly prepared PRP to evaluate aggregatory response to ADP. After 1 min equilibration, ADP was added to the solution and the level of platelet aggregation inhibition was determined. The observed aggregation inhibition levels were plotted versus time of incubation in PPP.

**Binding of Native 125I-CN and L-125I-CN to Washed, Activated Platelets**

Using radioiodinated CN, binding activity of native CN and LCN to washed human platelets was evaluated. Radiolabeled CN and LCN were prepared as described above. To prepare washed platelets, whole human blood (36 ml) was drawn into 4 ml ACID anticoagulant [sodium citrate dihydrate (2.5 g), citric acid monohydrate (1.5 g), and dextrose (2.0 g) per 100 ml (pH 4.5)]. PRP was prepared by centrifugation and prostaglandin E1 (PGE1) was added to a final concentration of 2.5 mM. PRP was then centrifuged at 2500 × g for 15 min to pellet the platelets. The supernatant (PPP) was decanted and the inside wall of the centrifuge tube was wiped with a Kimwipe. Modified Tyrodes buffer [0.1% glucose, 0.8% NaCl, 0.1% NaHCO3, 0.02% KCl, 1% BSA in 10 mM HEPES (pH 7.2)] was added (1 ml/ml of original PRP) and PGE1 was again added to a concentration of 2.5 mM. A small amount (1–2 ml) of PPP was saved for a zero-platelet control in the binding experiments. After addition of Tyrodes buffer, platelets were resuspended by gently stirring the buffer with a disposable plastic pipette; platelets were gently drawn into and expelled from the pipette until small chunks of the
original pellet could no longer be seen. Pelleting and resuspension of the platelets was repeated thrice and final resuspension was in 6 ml modified Tyrodes buffer without PGE,

The platelet count was determined and the washed platelets were used to determine binding of different forms of CN (native and liposomal).

Determination of binding efficiency of iodinated CN and LCN to activated platelets required the following conditions: (1) a control containing no platelets, (2) platelets not activated with ADP, (3) platelets activated with ADP, and (4) platelets activated with ADP in the presence of a large excess of a peptide antagonist of GPIIb/IIIa. The peptide GRGDSP (at a final concentration of 2000 nm) was used as the fibrinogen receptor (GPIIb/IIIa) antagonist. The reaction mixtures (in triplicate) contained 200 μl of a solution containing 300,000 platelets/ml. These were placed in each test tube, except for the no platelet control set, which had an identical volume of Tyrodes buffer without platelets. Native 125I-CN was added to 12 tubes: 9 with washed platelets and 3 with buffer (control tubes); similarly, an additional 12 tubes had L-125I-LCN added. For all samples, both native CN and LCN, the final concentration of CN was 25 nm. GRGDSP was added to samples requiring competitive agent and ADP was added (final concentration 15 μm) to samples requiring platelet activation. The samples were then incubated for 15 min at 37°C along with the controls (no platelets added) and the unactivated platelet (no ADP) samples. Platelets were pelleted by centrifugation and the amount of radioactivity bound by platelets was determined by counting radioactivity in both the supernatant and the pellet.

Immunogenicity of Native CN and LCN

The ability to elicit an antibody response by native CN and LCN has been tested on immunocompetent mice. Three groups each containing three BALB/c immunocompetent, tumor-free mice were injected i.v. daily with either 30 μg CN, 30 μg LCN, or sterile PBS for 5 consecutive days. On day 7, an initial blood sample was collected from each mouse. In the second week, the daily dosage cycle was repeated and a second blood sample was obtained from each animal on day 14. For long-term studies, animals were injected i.v. twice weekly with either CN or native CN for 8 weeks (50 mals were injected i.v. twice weekly with either LCN or CN). At the conclusion of the injection cycle, a final blood sample was collected. For the ELISA, native CN and LCN was obtained using gamma camera imaging. The mice previously had 5 105 tumor cells implanted in the mammary fat pads (MFPs) and the tumors were allowed to grow untreated for 6 weeks (average tumor volume 1130 ± 112 mm3). Twenty-four hours post-CN injection, the animals were sacrificed and native CN or LCN in 100 μl into MDA-MB-435 tumor-bearing mice. The mice previously had 5 × 105 tumor cells implanted in the MFP as described above. Mice were anesthetized and allowed to incubate for 1 h at room temperature. The samples were then washed again with PBS thrice. The bound antibody was detected using the chromogenic substrate p-nitrophenyl phosphate (Sigma Chemical) following the manufacturer’s directions. The samples were evaluated for color development using a Dynex MRX microplate reader (Dynex Technologies, Inc., Chantilly, VA) with wavelength setting at 405 nm.

A second study to quantitate immunogenic response was carried out in a similar manner, except that prior to the administration of CN or LCN, immunocompetent mice were injected s.c. with 200 μl of complete Freund’s adjuvant followed by a 1-week rest period. Animals were then given 50 μg of either CN, LCN, or unloaded liposomes i.v. daily for 1 week. Following treatment, a blood sample was obtained and analyzed for the presence of neutralizing anti-CN antibodies as described above. Additionally, at this time, the animals were injected s.c. with incomplete Freund’s adjuvant (200 μl) and allowed 1-week rest at which point the second blood sample was collected. Another week of CN, LCN, and empty liposome administration was then initiated; this cycle was repeated for 12 weeks. At the conclusion of the injection cycle, a final blood sample was taken. Blood samples obtained during the experiment were analyzed for anti-CN antibodies by ELISA as described above.

Pharmacokinetics of i.p. and i.v. Administered CN and LCN in Mice

Native 125I-CN and L-125I-CN (20 μg each) were injected i.v. into two separate groups of five tumor-free, immunocompetent BALB/c mice. Whole body counts of the mice were obtained at 1, 2, 6, 12, 18, 24, 48, and 72 h after injection using a Capintec CRC12 chamber radioactive dose calibrator (Capintec, Ramsey, NJ). Additionally, blood was collected (20 μl via the retroorbital sinus) at the above time points and counted in a Beckman gamma counter.

Once the baseline values for blood clearance had been established in tumor-free mice, we measured the differences in tumor uptake of native CN and LCN in groups of three tumor-bearing mice. For these studies, native 125I-CN or L-125I-CN (20 μCi) was injected through the tail vein of MDA-MB-435 tumor-bearing mice. The mice previously had 5 × 105 tumor cells implanted in the mammary fat pads (MFPs) and the tumors were allowed to grow untreated for 6 weeks (average tumor volume 1130 ± 112 mm3). Twenty-four hours post-CN injection, the animals were sacrificed and native CN or LCN in 100 μl into MDA-MB-435 tumor-bearing mice. The mice previously had 5 × 105 tumor cells implanted in the MFP as described above. Mice were anesthetized and
Tumor Xenografts and Dissemination of Orthotopically Implanted Breast Tumor Xenografts

LCN was evaluated for efficacy in an orthotopic, xenograft breast cancer model established by implantation of MDA-MB-435 cells (5 × 10⁵) in the MFP of nude mice as described previously (43). Two experimental trials were performed to determine effectiveness of liposomal delivery of CN. In the initial experiment, 5 mice/group were used and LCN was given (100 µl/injection) via three different routes: i.v. administration every other day (60 µg/dose), i.v. administration twice weekly (100 µg/dose), and daily i.t. administration (30 µg/dose). Controls for this experiment (100 µl/injection) included the following: i.v.-PBS (the buffer in which CN is diluted), empty liposomes both i.v. and i.t. injection, and native CN both i.v. (60 µg every other day) and i.t. (30 µg daily). During the experiment, the implanted tumors were measured by caliper weekly in a blind fashion. The tumors were measured for the maximum width (X) and length (Y) and the tumor volumes (V) were calculated using the formula: \( V = \frac{X^2Y}{2} \), as described previously (48). The average tumor volume for each study group was then plotted as a function of time and type of treatment.

A second experiment using 10 animals/group focused on the comparison between i.v.-LCN delivery and i.t.-CN. Three study groups were evaluated (100 µl/injection in all groups): LCN delivered i.v. twice weekly (100 µg/dose), daily i.t. administration of native CN (30 µg/dose), and PBS delivered i.v. twice weekly. As in the previous study, tumors were followed by weekly size measurement. Tumor volume was again plotted versus time during the 8-week course of the experiment and the effectiveness of treatment was assessed by inhibition of tumor growth.

Staining of the Tumor Microvasculature

Sections taken from MDA-MB-435 tumors following the efficacy study were analyzed for antiangiogenic activity of CN and LCN. Following surgical removal, tumors were immediately sliced into 2-mm-thick serial sections and fixed in 10% phosphate-buffered formalin overnight at 4°C. Tumor sections were then slowly dehydrated over a 5-h period in serial alcohol washes at room temperature and embedded in paraffin as described (49). For further analysis, the paraffin blocks were sectioned at 5-µm thickness, with the sections being placed on positively charged VWR Superfrost immunohistochemistry slides (VWR, West Chester, PA). These slides were then treated by two washes of 10 min each with 100% xylene and then slowly rehydrated through serial alcohol washes (95% ethanol to pure water in 5 steps, each step allowing 2 min for hydration). To enhance antigen retrieval, the method of Filieri et al. (50) was employed. Briefly, rehydrated sections were immersed in 1 mM EDTA-NaOH solution (pH 8.0) and processed in a microwave oven with a power setting of 750 W for three cycles (5 min each). After completion of the microwave procedure, sections were allowed to cool to room temperature for 20 min and rinsed in distilled water and then PBS. Following antigen retrieval, endogenous peroxidase activity was quenched by placement directly in 3% H₂O₂ for 10 min at room temperature in a humidity chamber. Antibody CD31 (Sigma Chemical) was then applied to the sample in a volume of 100 µl and allowed to react for 1 h at room temperature. The unbound primary antibody was then removed through multiple (thrice, 2 min each) washes with PBS. The secondary (detection) goat anti-rabbit antibody conjugated with peroxidase (Zymed) was then applied to the sample and incubated for 10 min at room temperature followed by removal of unbound antibody by multiple (thrice, 2 min each) washes with PBS. Detection of the secondary antibody using 3,3′-diaminobenzidine as the chromogen was performed following the manufacturer’s instructions (Zymed HistoMouse Max).

Analysis of Stained Microvessels

Stained slides were defined as areas of high vascular density (41, 51). Hotspots are defined as areas of high vascular density (41, 51). Hotspots were selected in each sample slide under 100× magnification with an Olympus light microscope with digital images acquired with an Olympus E20N digital camera (5.0 MP acquisitions; Olympus America, Melville, NY). Following selection and capture of the hotspot images, the areas showing positive staining were quantitated in terms of pixels within a given hotspot using SimplePCI advanced imaging software (C-Imaging Systems, Cranberry Township, PA). To eliminate bias in this study, selection of the hotspots and subsequent analysis of the slides was carried out in a blind fashion. The significance of the data was determined using descriptive statistics (means, SD, and 95% confidence intervals) and ANOVA.

Results

Retention of CN Biological Activity following Iodination and Liposomal Encapsulation

CN retains platelet aggregation inhibitory activity following encapsulation in liposomes. Although intact CN shows no ability to inhibit platelet aggregation, when CN is released through lysis of liposomes, the entrapped CN has an IC₅₀ of 60 nM (data not shown). This value is very close to the IC₅₀ for inhibition of human platelet aggregation by native CN (49 nM).

Platelet aggregation studies following timed incubation in PPP revealed that the liposomes remain stable (CN is not released) during 1–2 h incubation. However, during a 20-h incubation in PPP, at 200 nM CN in liposomes, CN was slowly released from the liposomes, with essentially complete release by 20 h. Controls for this experiment were unencapsulated CN incubated in PPP and PBS both at 200 nM. After timed incubation, aliquots (250 µl) were added to fresh PRP (250 µl) and the level of ADP-induced platelet aggregation was measured (Fig. 1).
Unencapsulated CN in PPP or PBS inhibited platelet aggregation completely at all time points tested while LCN showed increasing levels of platelet aggregation inhibition with increasing time of incubation in PPP, with close to 100% inhibition of platelet aggregation following 20 h of incubation. It must be noted that an addition of fresh PRP was used at each time point to ensure that aggregation in response to ADP addition was the same for all time points. The results of this experiment reveal that CN encapsulated within liposomes is slowly released during prolonged incubation in plasma but retains almost complete biological activity. LCN incubated in PBS did not release CN during a 20-h incubation.

Radiiodinated CN was evaluated for its ability to inhibit platelet aggregation. Radiiodinated samples were incubated in PPP as described for radioinert CN. No difference was observed between CN and 125I-CN in the ability to inhibit platelet aggregation. Further, L125I-CN released CN as expected over the 20-h incubation period. Released LCN inhibited ADP-induced platelet aggregation identically to native CN. The IC50 values with respect to platelet aggregation inhibition for both radiiodinated and liposome-encapsulated forms of CN are in the range of 50–65 nM. Thus, neither encapsulation nor radiiodination had an effect on bioactivity of CN.

**Binding of CN to Washed Human Platelets**

CN has been shown previously to inhibit ADP-induced platelet aggregation through binding to GPIIb/IIIa on the platelet surface (4). The binding of LCN to washed human platelets under a variety of conditions was compared with that of native CN under identical conditions (Fig. 2). Neither CN nor LCN binds to unactivated washed human platelets. Following ADP activation of platelets, native CN efficiently binds to the platelets during the 10-min incubation while CN encapsulated in liposomes is unavailable for binding. Addition of a large excess of a competitive binding agent for GPIIb/IIIa, the peptide GRGDSP, inhibits binding of native CN to platelets. As shown in Fig. 1, LCN requires incubation in plasma to release active CN. Preincubation (20 h) of L125I-CN in PPP followed by addition of the preincubation mixture to washed platelets revealed that CN released from liposomes binds to platelets in a manner indistinguishable from native CN. Moreover, released CN requires ADP activation of platelets for binding, like native CN, and this binding is inhibited by preincubation of platelets with GRGDSP peptide.

**Immunological Response to CN**

The ability to elicit an antibody response to native CN and LCN has been tested in immunocompetent mice. Short-term exposure experiments consisted of two rounds of five daily i.v. injections of CN or LCN (30 μg CN in each injection) separated by 2 days with no injections. Blood samples were analyzed by ELISA on days 7 and 14 for the presence of specific neutralizing anti-CN antibodies. Seven dilutions of blood (1:30, 1:100, 1:500, 1:1,000, 1:5,000, 1:10,000, and 1:25,000) were used for analysis of the antibody response. The assay showed no significant immunogenicity in animals injected with CN or LCN. The results indicate that short-term exposure does not lead to the development of an immune response to CN or LCN. Similarly, in long-term exposure experiments (8 weeks) using immunocompetent tumor-free mice with twice weekly i.v. administration of CN or LCN (30 μg CN each injection), no neutralizing antibodies to CN were evident by ELISA.
We also examined the immunological response to CN and LCN administration following presensitizing of the animal (2 weeks prior to initiation of CN injections) with Freund’s adjuvant (Table 1). In this study, LCN was nonimmunogenic and elicited no antibody response following an adjuvant administration course over 12 weeks. However, using the same conditions, CN elicited an antibody response following 12 weeks of administration. Immune response in both cases was evaluated by ELISA with four dilutions of serum (1:30, 1:100, 1:500, 1:1,000). Anti-CN antibodies were only in adjuvant samples immunized with native CN, and in these cases, the response was minimal with antibodies observed in the 1:30, 1:100, and 1:500 dilutions of serum from the CN plus adjuvant-treated animals (Table 1).

**Pharmacokinetics of i.v. Injected CN and LCN in Mice**

Following i.v. administration of L-125I-CN and 125I-CN (30 μg) into groups of five tumor-free, immunocompetent BALB/c mice, whole body distribution was analyzed using a chamber dose calibrator. Whole body counts in 125I-CN-injected mice decreased with a linear decay curve: the half-life was 7 h. Mice injected with L-125I-CN exhibit a similar shape in the whole body elimination curve, but the half-life was 16.5 h. Whole body counts, however, do not provide information about the circulating form of CN. Therefore, we investigated the blood distribution of radioactivity following i.v. administration of 125I-CN or L-125I-CN. Gamma counting of blood samples collected from mice 1, 2, 6, 12, 18, 24, 48, and 72 h after injection revealed that there was <0.1% of the administered counts in the blood 2 h after i.v. injection of 125I-CN. However, in animals given i.v. L-125I-CN, the percentage of total injected counts in the blood reaches a maximum (1.6% of injected counts) 2 h postinjection and decreased gradually over the following 70 h. By plotting the decrease in radioactivity in blood over time following i.v. administration in tumor-free mice, we observed a circulatory half-life of 0.5 h for 125I-CN and 19 h for L-125I-CN (Fig. 3). Thus, encapsulation of CN in liposomes not only protects the protein but also maintains it in the circulation for a much longer period of time than native, unencapsulated CN. This increased circulatory half-life leads to an increased probability of LCN delivery to the tumor.

**Table 1. Antibody response in immunocompetent mice following sensitization with Freund’s adjuvant and immunization with CN, LCN, and PBS**

<table>
<thead>
<tr>
<th>Agent used as antigen</th>
<th>Serum dilution</th>
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<tr>
<td></td>
<td>1:30</td>
</tr>
<tr>
<td>CN</td>
<td>++</td>
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<td>LCN</td>
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*aTo ensure assay function, monoclonal antibody against CN was diluted in plasma at the following concentrations: 10 and 100 ng/ml and 1 and 10 μg/ml.

To measure differences in tumor uptake between LCN and native CN, MDA-MB-435 human breast cancer cells were implanted into the MFPs of nude mice and the tumors were allowed to grow untreated for 6 weeks. Following the growth period, either L-125I-CN or 125I-CN (20 μCi) was injected into the tail vein of tumor-bearing mice. Tissue distribution was assessed in the mice 24 h postinjection by surgical removal of tissues, weighing, and counting radioactivity. These studies revealed that there is a significant difference in tumor uptake between mice injected with LCN and those injected with native CN.

**Figure 4.** Tissue distribution of 125I-CN and L-125I-CN 24 post-i.v. injection. Two groups of three mice had MDA-MB-435 human breast tumor cells implanted into the MFP (20 μCi) and the tumors were allowed to grow for 6 weeks with no treatment. Each group was treated with a single i.v. administration of either 125I-CN or L-125I-CN (20 μCi) to each animal. Animals were sacrificed 24 h postinjection and the organs were surgically removed. All organs were counted in a gamma counter and the numbers of counts obtained were normalized for the weight of the tissue. There is more accumulation of LCN and CN in the liver, spleen, and kidney than in the other organs examined. Importantly, there is significantly increased uptake of radiolabeled LCN in the tumors following i.v. injection than is observed with native CN. Columns, averages; bars, SD.
Due to the rapid elimination of native CN, the percentages of the injected protein retained in the individual organs of the mice 24 h post-i.v. injection were significantly less than observed in mice treated with LCN. Mice injected with LCN showed significantly increased tumor uptake (Fig. 4) as compared with animals given native CN. Analysis of the internal organs of tumor-bearing animals reveals retention of radioactivity in liver, kidney, and spleen for both L-125I-CN and 125I-CN groups. The radioactivity observed in the kidneys suggests that CN is excreted through the kidney (radioactivity was also observed in urine).

Following the observation that there is significantly increased tumor uptake by LCN compared with native CN, we compared tumor and tissue uptake of L-131I-CN and 131I-CN using real-time observation. L. 131I-CN and 131I-CN were injected i.v. (30 µg native CN or LCN in 100 µl) into MDA-MB-435 tumor-bearing mice. Mice were anesthetized and imaged by a Siemens gamma camera at 3-h intervals following injection. As can be seen (Fig. 5), L-131I-CN is distributed to two distinct areas, the tumor and the abdomen, with significant radioactivity accumulating in the tumor. By contrast, unencapsulated CN is found exclusively in the abdominal region. Results of this experiment correlate nicely with results of the efficacy studies, which indicate effective inhibition of tumor growth by LCN following i.v. administration but no inhibition following i.v. administration of native CN (see below).

Intravenous Administration of LCN Is Effective in Inhibiting Tumor Growth

LCN was given to nude mice, which had MDA-MB-435 human mammary cancer cells (5 x 10^5) implanted into MFP 2 weeks previously. Two experimental studies were performed to examine the effectiveness of liposomal delivery of CN (Fig. 6). In the initial experiment, five mice were used per group and LCN was given via three different modes: i.v. administration every other day (100 µl, 60 µg LCN/injection), i.v. administration twice weekly (100 µl, 100 µg LCN/injection), and daily i.t. administration (100 µl, 30 µg LCN/injection). Controls for this experiment included the following: i.v.-PBS, empty liposomes both i.v. and i.t., and native CN both i.v. (60 µg CN every other day) and i.t. (30 µg CN/day). Interestingly, as observed in Fig. 6B, i.v.-LCN has nearly identical efficacy to direct i.t. administration of native CN regardless of whether LCN is delivered i.v. every other day or twice weekly. However, LCN has no effect on tumor growth when delivered i.t. (Fig. 6A). The reason for this is under study, but it may be related to the method of injection. I.t. injection of LCN is to the necrotic core of the tumor where i.t. pressure may inhibit release of CN. It is postulated that with i.v.-LCN, CN is presumably released slowly from the deposits of liposomes that accumulate as they leak from the vasculature throughout the tumor. This experiment showed that there is a statistically significant difference (P < 0.0001) between both of the i.v.-LCN groups and the untreated PBS control group.

The second experiment supported the results from the first and expanded the number of study animals to 10 per group. Three groups were evaluated: LCN delivered i.v. twice weekly (100 µg LCN/injection), daily i.t. introduction of native CN (30 µg CN/injection), and PBS control (Fig. 6C). The results of this study again reveal statistical significance (P < 0.0001) between both treatment groups and control and the data correlate well with previous observations about tumor growth rates and the effect of CN on tumor size. Daily i.t. injection of CN is effective, but twice weekly i.v. delivery of LCN is equally effective and represents a much more clinically relevant method of drug delivery.

Limitation of Angiogenesis following Therapy with LCN and CN

Tumors from the control or treated animals were removed surgically and fixed by immersion in 4% formalin.
The samples were then embedded in paraffin and sectioned at 5 μm. After antigen retrieval, these sections were immunostained for the presence of CD31, an endothelial cell marker. Evaluation of the tumor sections revealed a significant decrease (>82%) in vessel count in the i.v.-LCN and i.t.-CN samples, indicating inhibition of angiogenesis as observed on stained slides (Fig. 7A) and when the stained areas were quantitated in terms of pixel count (Fig. 7B). Of the vessels that stain positive in the LCN and CN samples, the vessels were larger and showed weaker CD31 staining, which is consistent with weaker staining of older vessels. This is compared with the PBS control, which reveals small, strong staining vessels. Both i.t.-CN and i.v.-LCN effectively inhibited angiogenesis as compared with PBS control and this may help to explain the effective limitation of tumor growth observed following CN administration.

**Discussion**

At diagnosis, over 60% of breast cancer patients have metastatic disease. The most common cause of death in breast cancer patients is due to the metastatic spread of the cancer cells from the primary tumor site to remote sites and growth of the breast cancer cells at the distant location. Metastasis is a complex process and requires several distinct actions including the following: (1) migration of the tumor cells through the extracellular matrix surrounding the tumor; (2) invasion of tumor cells into angiogenic blood vessels growing into the tumor; (3) adhesion of the metastatic cell at a distant site where the microenvironment is receptive to tumor growth; and (4) newly attached cells must proliferate and induce angiogenesis at the metastatic site. Due to the complex multistep nature of the metastatic process, a combination of inhibitory actions is thought to be necessary to effectively limit this process (52).

Integrins have been shown to be vital to the processes involved in tumor metastasis (53). Cell migration, invasion, matrix degradation, proliferation, and angiogenesis are all mediated by integrins and integrin signaling (54). Previous studies from our laboratory have shown that CN, a unique homodimeric disintegrin, binds to and affects the function of several integrins expressed on cancer, platelets, and endothelial cell surfaces, including α5β3, α3β1, α4β1, and αIIbβ3 (4–6, 24, 55–57). Interactions between CN and integrins disrupt several steps critical to tumor growth, angiogenesis, and metastasis (4–6).

In the present communication, we show that CN can be effectively encapsulated within liposomes while retaining full biological activity. Liposomal encapsulation of CN allows for its i.v. administration, a method of drug delivery that is clinically relevant. Further, liposomal encapsulation eliminates two potential side effects: (1) CN has been shown to effectively inhibit ADP-induced platelet aggregation by binding to GPⅡb/Ⅲa on the surface of activated platelets. We were concerned that administration of native CN i.v. could lead to binding to circulating platelets. However, we found that this is not the case. In order for CN to bind to platelets, integrin GPⅡb/Ⅲa (the fibrinogen receptor) on the platelet surface must be in the activated

**Figure 6.** Inhibition of MDA-MB-435 tumor growth by LCN. Human mammary tumors (MDA-MB-435) were established by implantation of $5 \times 10^5$ cells into the MFPs of 5-week-old female nude mice. Eight groups, each containing five mice, were used. Tumor sizes were measured by caliper and the tumor volumes were calculated by standard methods (48). The tumors were grown until just palpable (day 14) and CN treatment was initiated at that time. A, CN, LCN, and control solutions were delivered by daily i.t. injection. Four groups were employed: i.t.-CN (100 μl, 30 μg CN/day), i.t.-LCN (100 μl, 30 μg CN/day), i.t.-PBS (100 μl), and (i.t. unloaded liposomes (IT-UL); formulation identical to CN loaded liposomes, 100 μl). B, four i.v. delivery groups were evaluated: i.v.-CN (100 μl, 60 μg every other day), i.v.-LCN (100 μl, 60 μg every other day), i.v.-LCN twice weekly (IV2x-LCN; 100 μl, 100 μg twice weekly), and i.v.-PBS. Both i.v. and i.t. control samples with unloaded liposomes and PBS showed no effect on tumor growth. In the i.v.-LCN and i.t.-CN groups, there was a significant ($P < 0.0001$) decrease in tumor growth, which was shared by the IV2x-LCN while the i.t.-LCN and i.v.-CN groups showed no effect on tumor growth. C, in a second experiment, nude mice were divided into three groups of 10 mice each and treated either daily with i.t.-CN (100 μl, 30 μg/day), i.v.-LCN (100 μl twice weekly, 100 μg/injection), or i.v.-PBS (100 μl). In this experiment with a larger number of animals/group, the results are statistically significant and identical to those seen in the earlier experiment (A and B). LCN was statistically as effective as daily i.t.-CN delivery when both were compared with control ($P < 0.0001$). Thus, i.v. administration of LCN is an effective means of limiting tumor growth in the orthotopic, xenograft model of human breast cancer. Points, geometric mean of the tumor sizes; bars, 95% confidence intervals.

**Points,** geometric mean of the tumor sizes; **bars,** 95% confidence intervals.
state; in the absence of integrin activation, there was no measurable interaction of CN with platelets in vitro. These studies also confirmed that binding of CN to activated platelets is primarily mediated by GPIIb/IIIa because the interaction could be effectively competed with a large excess of a RGD-containing peptide. Our findings show conclusively that CN and LCN do not bind to unactivated platelets. (2) There were concerns about possible immunogenicity of CN. However, in both short-term (1 week) and long-term (8 weeks) studies, there was no observable immune response to CN or LCN under the conditions used in these studies. CN was found to be weakly immunogenic in the presence of Freund’s adjuvant, but under identical conditions, LCN was not immunogenic. If results in mice translate to humans, these findings would suggest that LCN should be suitable for long-term use in the clinical setting.

We also investigated the pharmacokinetics and biodistribution of LCN and CN in tumor-free and tumor-bearing animals. In these experiments, there were distinct advantages to the liposomal formulation of CN. First, the circulatory half-life (t1/2) in mice of native CN is ~0.5 h whereas that of LCN is 19 h. The increased time in the circulation enhances the opportunity for delivery to the tumor as supported by the real-time imaging and the results of the efficacy studies. Further, examination of the tumor and internal organs of tumor-bearing mice following administration of L-131I-CN or 125I-CN showed that uptake of LCN in the tumor far exceeds that of native CN. Distribution to the internal organs of mice was comparable between LCN and CN when taking into consideration the actual amount of radioactive material remaining in the animals. These data correspond well with real-time gamma camera images of 131I-CN and L-131I-CN distribution following i.v. administration in tumor-bearing mice. L-131I-CN is delivered both to the tumor and an organ(s) in the abdominal area, but native 131I-CN is distributed solely to the abdominal area of the mouse. These experiments indicate the significant advantages of i.v.-LCN delivery over i.v. delivery of native CN: LCN is available in the circulation for a substantially longer period of time and accumulates at the site of the tumor. Passive accumulation of LCN is presumably due to the “leakiness” of the angiogenic vasculature of the tumor (58). Previous reports have shown that newly growing vasculature at the site of tumors is not intact and the tight junctions of the mature vasculature are disrupted (59–62). The size of liposomes with encapsulated CN is ~100–150 µm. Thus, liposomes leak out of the newly growing angiogenic vessels and deliver CN into the tumor. Once the liposomes exit the “leaky” vessels and form depots in the tumor, presumably lipases and other extracellular enzymes derived from tumor or stromal cells aid in the slow degradation of the liposome, releasing the entrapped protein (63). On release from the liposome, CN binds to integrins on both tumor and endothelial cells thereby blocking both tumor growth

Figure 7. Inhibition of angiogenesis by CN. A, visualization of CD31 staining. MDA-MB-435 tumors were dissected and immediately fixed in formalin at 40°C for 5 h. Following fixation, tumors were dehydrated, infiltrated with paraffin, and sectioned at 5 µm thickness (see Materials and Methods). The slides were then stained for endothelial cell marker, CD31 (brown areas). CN delivered via i.t. injection (daily injection 30 µg; CN-IT) and LCN delivered i.v. twice weekly (100 µg LCN per tail vein injection; LCN-IV) showed potent inhibition of angiogenesis in the tumor sections as compared with PBS-treated tumors. B, quantitation of microvessels. Slides stained for CD31 were subjected to “hotspot analysis.” Hotspots are defined as areas of high vascular density. The positively stained regions were then quantitated in terms of pixels with Simple PCI advanced imaging software. The CN-IT group shows 82% inhibition while LCN-IV shows 94% inhibition of angiogenesis as compared with PBS-treated animals.
and angiogenesis. The development of the liposomal delivery system eliminates the problems of how to overcome the short circulatory half-life of CN and repress or eliminate the potential immunogenicity of the snake venom protein. Inhibition of both tumor growth and blood vessel growth are hallmarks of CN action and both activities have been demonstrated in the animal model studies described here using i.v. delivery of LCN. Thus, once CN is delivered to the tumor, it functions as a potent antiangiogenic and antitumor agent.

Our original studies showed that native CN effectively inhibited tumor growth in an orthotopic, xenograft model of breast cancer (5, 6). However, these studies employed daily i.t. injection, which is not clinically relevant. This prompted our investigation of the efficacy of i.v. delivery of LCN, a method readily translatable to the clinic. Two studies were performed using an orthotopic, xenograft model of breast cancer to evaluate the antitumor efficacy of LCN following i.v. administration. The first trial included several negative controls including PBS and unloaded liposomes. Native CN produced good antitumor efficacy following daily i.t. injection with results identical to those reported previously (6), but it had no effect on tumor growth when given i.v. Conversely, for animals treated with LCN, there was no effect on tumor growth following daily i.t. injection, but with i.v. injection (every other day), the tumor growth was inhibited just as effectively as observed following daily i.t.-CN administration. An additional animal group received twice weekly i.v.-LCN and it was observed that this dose schedule was as effective as administering i.v.-LCN every other day.

In a follow-up study, we compared daily i.t.-CN with twice weekly i.v.-LCN. The results of this study were identical to those of the initial study; the clinically relevant twice weekly i.v.-LCN administration shows equal efficacy to daily i.t. CN injection, both showing significant inhibition of tumor growth (P < 0.0001). The inhibitory effect shown by i.v.-LCN in the animal tumor model is supported by the biodistribution, pharmacokinetics, and biological activity studies of LCN. This mode of administration is one that can be directly translated to clinical use in breast cancer patients.

Although CN does not inhibit proliferation of cancer cells in vitro (6), it significantly suppresses growth of the MDA-MB-435 cancer in vivo following daily i.t. administration (5, 6). In view of the inhibition of tumor growth observed after exposure to i.v.-LCN, we examined the antiangiogenic activity of CN delivered by this route and by direct i.t. delivery. For these studies, we used immunohistochemistry with an antibody to CD31 (PECAM-1). CD31 has been reported to be highly expressed in the angiogenic vasculature with ~1 million copies reported on the surface of endothelial cells (64). CD31 (PECAM-1) has been reported to be involved with the initial formation and stabilization of cell-cell contacts at lateral junctions of endothelial cells, the maintenance of the vascular permeability barrier, the regulation of cell migration, and the formation of new blood vessels during angiogenesis (65–70). Overexpression of CD31 (PECAM-1) leads to increased cellular migration rates and increased formation of angiogenic vessels. Thus, using CD31 immunohistochemistry allows for the observation and quantitation of newly growing angiogenic microvessels as opposed to using factor VIII immunohistochemistry, which detects all vasculature (71). In both i.t.-CN-treated and i.v.-LCN-treated tumors, there is a statistically significant reduction of microvascular density, 82% reduction in the number of stained pixels in the i.t.-CN group and 94% reduction in the i.v.-LCN group. This phenomenon can be explained by the observed antiangiogenic activity of disintegrins (4, 42, 72, 73). Angiogenesis plays a central role in cancer growth and dissemination (74, 75). In animal models, antiangiogenic therapies have been shown to inhibit growth and metastasis of xenograft tumors and keep the tumors in a long-term dormant state (76–78). Several integrins have been shown to be closely associated with the antiangiogenic process and blocking or modulation of these integrins produces significant inhibition of angiogenesis and tumor growth (26, 27). CN is unique in that we have shown that the disintegrin interacts with several integrins increasing its versatility and offering the opportunity to block several integrin-mediated processes involved in tumor growth, dissemination, and angiogenesis. Despite several recent failures of antiangiogenic agents in clinical trials, the target is still valid and it is reassuring to learn of the positive results with the antiangiogenic agent Avastin (bevacizumab), a recombinant human monoclonal antibody to VEGF, in the recent colorectal cancer phase II clinical trial (39).

The studies reported here show that CN limits tumor growth and angiogenesis and that the protein can be efficiently packaged within liposomes. Importantly, the efficacy studies reveal that it is possible to deliver a biologically active protein to the site of a tumor using liposomal encapsulation. Previous studies using liposomal entrapment of chemotherapeutic agents have demonstrated the ability to deliver small molecules and nucleic acids to tumors (44, 79, 80). Recently, it has been reported that a cationic liposome preparation consisting of the gene for salmosin (a disintegrin found in the venom of Gloydius saxatilis) was successful in both delivery of the gene and in vivo production of an active protein (81). While this was a successful display of the ability of disintegrins to limit tumor progression, it differs from our system in that a gene was delivered rather than an intact biologically active protein. Demonstration of the ability to successfully deliver a biologically active protein opens new frontiers in designing drug delivery systems and expands the limits of what payloads can be employed in liposomal delivery systems for therapeutic options. However, it should be noted that CN is a unique molecule that is stabilized by several intrachain and interchain disulfide bonds. Further, CN is stable to extremes of pH and to organic solvents (like most disintegrins) as evidenced by its purification by reverse-phase high-performance liquid chromatography (82–84). Thus, CN is a small, very stable protein that is uniquely suited for liposomal encapsulation. Nonetheless, future development of liposomal delivery systems and the encapsulation of other biologically active proteins can address facets of tumor biology and treatment not previously available.
In summary, our data demonstrate that ~100 µg twice weekly i.v. administration of LCN not only limited tumor growth but also significantly reduced microvascular density in the MDA-MB-435 xenograft model. Blockage of integrin αvβ3 on breast cancer cells with peptides or antibodies has been shown previously to limit angiogenesis (25); CN administration not only limits tumor growth and angiogenesis but also severely curtails tumor metastasis (6). CN can be effectively given i.v. by a clinically acceptable delivery method. On introduction into the systemic circulation, LCN passively accumulates at the tumor site where it exerts an effect on both tumor growth and angiogenesis. Our findings further demonstrate that in an animal model, CN in a liposomal formulation does not interact with components of the circulatory system and does not elicit an immune response.

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References


