Development of a Novel Recombinant Disintegrin, Contortrostatin, as an Effective Anti-Tumor and Anti-Angiogenic Agent

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Key Words

Snake venom · Recombinant disintegrin ·
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Abstract

Contortrostatin (CN) (Mr 13,500 Da) is a novel homodimeric disintegrin isolated from the venom of *Agkistrodon contortrix contortrix* (Southern Copperhead) snake and displays two RGD motifs (one on each chain), which modulate its interaction with integrins on tumor cells and angiogenic vascular endothelial cells. In previous studies, we have shown that native CN administered in a liposomal formulation exhibits potent anti-angiogenic and tumor growth inhibitory activities. Current isolation of the protein from crude venom is difficult and prohibitively expensive for translation into the clinic. In this report, we describe a method amenable to large-scale production of a soluble monomeric form of recombinant CN with biologic activity; the protein is expressed directly in the cytoplasm of an engineered bacterial system with an expression yield of approximately 20 mg/l of culture. We

present here the in vitro assays as well as the anti-tumor and anti-angiogenic evaluation of liposomal recombinant CN in an orthotopic, xenograft model of human breast cancer.

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Introduction

Contortrostatin (CN), a disintegrin isolated from the venom of the southern copperhead snake, has been shown to possess potent anti-tumor and anti-angiogenic activity in a number of models of human cancer. CN exists as a very small fraction of the total protein in venom. For further development of CN as a therapeutic agent, a stable source of the protein must be identified. Structurally, CN is a cysteine-rich protein (10 cysteines per monomer) that displays no secondary structure and like other disintegrins has a complex folding pattern that relies on multiple disulfide bonds (four intrachain and two interchain disulfide bonds) to stabilize its tertiary structure [1]. By folding in a compact structure stabilized by multiple disulfide bonds, CN, like many other venom proteins, has a survival advantage, making it resistant to proteolysis and able to better survive in the harsher extracellular micro-environment. Its peculiar structure, the lack of data regarding specific chaperones involved in the folding pro-

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cess of disintegrins in the venom gland, and the fact that disintegrins interact with high affinity with integrins, a process that may interfere with their own production in mammalian recombinant systems, are formidable obstacles in designing a reliable recombinant system suitable for this class of polypeptides. For this reason, a number of attempts made in our laboratory to express CN in eukaryotic cells failed to generate a consistent recombinant molecule produced at a suitable expression yield. One possible explanation for such failure resides in the fact that the secreted recombinant product can interact at picomolar concentrations with the integrin receptors displayed by the mammalian expression system itself, and thus the secreted recombinant product may negatively regulate its own production by disrupting the integrin signaling pathways that are instrumental for the survival and proliferation of these mammalian recombinant factories.

More than a decade ago, it was discovered that disulfide bond formation in the prokaryotic periplasmic space is a catalyzed process [2]. As in the case of other organisms, in bacteria, proteins that are cysteine-rich and whose folding is dependent on correct disulfide-bridge formation are meant to be secreted outside the cell. These proteins are usually molecules that play important survival roles for the system: such as key components of the locomotor apparatus, the respiratory chain, or virulence factors like exotoxins or antibiotic-resistance enzymes. In order to fold correctly and acquire biological activity, all these proteins have to pass through the periplasmic space and be assisted in their folding by periplasmic oxidoreductases. Thus *E. coli* possess in the periplasmic space the complete enzymatic equipment required to assist the formation of disulfide bonds, which renders this compartment (reminiscent of the endoplasmic reticulum of eukaryotes) suitable for recombinant heterologous disulfide-rich protein production. The unique advantage of this complex bacterial compartment was explored and it has already been demonstrated that complex multiple disulfide-bond eukaryotic proteins can be expressed as biologically active native conformers through the periplasmic space. However, with the recent advent of a novel bacterial strain (FA113) that was engineered to carry a double mutation in the two key enzymes that control the main reductive pathways in *E. coli* rendering them inoperative, the expression of heterologous disulfide-rich proteins in bacterial cytoplasm also became a viable alternative.

Production of Recombinant CN

In the present study, we employed a commercially-available recombinant system for recombinant CN production. This system was designed to address the shortcomings of heterologous recombinant production in the natural periplasmic space, by shifting the production into a modified intracellular compartment: the cytoplasm of thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) dominant negative double-mutant *E. coli* strain. Although the ideal recombinant system would be the one designed to have key enzymes from both compartments (periplasmic and cytoplasmic) engineered to interact with each other, while assisting the proper folding of a heterologous protein in cytoplasm, we report here our experience with recombinant CN production in the cytoplasm of a commercially-available double-mutant strain [Origami B (DE3) pLysS].

The recombinant system consisted of Origami B(DE3)pLysS expression host in combination with the compatible pET32a vector with a strong T7lac promoter (EMD Biosciences, USA). Origami and Rosetta-gami host strains (EMD Biosciences) carry the same *trxB/gor* mutations as the original double-mutant strains (FA113), except that they are derived from a *lacZY* mutant of BL21. Moreover, the Origami strains combine the desirable characteristics of BL21 (deficient in *ompT* and *lon* proteases), Tuner and Origami hosts in one strain. As discussed above, the mutations in both the thioredoxin reductase *trxB* and *gor* genes greatly promote disulfide bond formation in the cytoplasm. Studies have shown that expression in Origami (DE3) yielded several-fold more active protein than in another host even though overall expression levels were similar [3].

Even in the absence of IPTG (isopropyl-beta-D-thiogalactopyranoside), there is some expression of T7 RNA polymerase from the *lacUV5* promoter in the DE3 lysogens and therefore basal expression of target protein. Any recombinant protein expressed at basal level in *E. coli* may interfere with the normal functioning of the cell and may, therefore, be 'toxic' to the bacteria. One approach to control basal expression is to use vectors that contain the tightly-controlled T7lac promoter [4, 5]. These plasmids contain a *lac* operator sequence just downstream of the T7 promoter. When this type of vector is used in DE3 lysogens, the *lac* repressor acts both at the *lacUV5* promoter level in the host chromosome to repress transcription of T7RNA polymerase gene by the host polymerase and at the T7lac promoter in the vector to block transcription of the target gene by any T7RNA polymerase that is

made in basal conditions. Another way of providing additional stability to target genes is to express them in host strains containing a compatible chloramphenicol-resistance plasmid that provides a small amount of T7 lysozyme, a natural inhibitor of T7RNA polymerase (the pLysS hosts) [6]. A pLysS host has little effect on growth rate and pLysS increases the tolerance of λ DE3 lysogens for plasmids with toxic inserts, thus improving stability of such plasmids. The presence of pLysS has the further advantage of facilitating the preparation of cell extracts because cells are less resistant to freezing and thawing cycles; by releasing small amount of T7 lysozyme they lyse easily.

The Tuner strain and derivatives (Origami B) are *lacY1* deletion mutants of BL21 and enable adjustable levels of protein expression by all cells in culture. The *lac* permease (*lacY1*) mutation allows uniform entry of IPTG (a lactose derivative) into all cells in the population, which produces a concentration-dependant, homogenous level of induction. By adjusting the concentration of IPTG, expression can be regulated to achieve the optimal level of target protein expression which may be attained at a significantly lower than the normally used IPTG concentration. This could be an advantage in that IPTG is an expensive substance and by using lower quantities per liter of culture, the manufacturing costs of recombinant proteins can be cut appreciably.

The pET32a vector (Novagen) is designed for cloning and high-level expression of peptides sequences fused with the 109-amino-acid TrxA, thioredoxin A wild-type protein [7]. Cloning sites are available for producing fusion proteins also containing cleavable His-tag sequence and S-tag sequence for detection and purification. By expressing various recombinant CN sequences as fusions with TrxA oxidase the result is an accelerated disulfide bond formation and an enhanced solubility of target protein.

A unique TEV protease cleavage site has been engineered just upstream of CN constructs in order to facilitate the subsequent cleavage of target protein from the thioredoxin fusion partner. The TEV protease recognizes with high specificity the canonical ENLYFQG amino acid sequence and cuts efficiently, leaving the recombinant disintegrin intact. We generated two constructs: one with a sequence identical to that of native CN (designated rCN), and one that carries a C-terminal graft (designated rCN+) as described below. One reverse primer was designed to introduce the HKGPAT amino acid sequence, which represents the C-terminal tail of the monomeric disintegrin echistatin grafted at the C-terminus

of the recombinant CN construct. This C-terminal graft was expected to improve recombinant disintegrin affinity toward integrin $\alpha 5 \beta 1$ [8], a key player in angiogenesis, and also to assist in the folding process in this region of the molecule.

By employing the recombinant system described above, we successfully expressed two Trx-CN fusion proteins (designed as Trx-CN and Trx-CN+, where the CN+ carried the C-terminal graft from echistatin) with high expression yields directly in the cytoplasm of Rosetta-gami B *E. coli* strain (fig. 1). The fusion proteins were efficiently proteolyzed by TEV protease (Invitrogen, USA) and the recombinant disintegrins (rCN and rCN+) further purified by HPLC by utilizing a protocol similar to the one previously employed to purify native CN from southern copperhead venom.

Evaluation of in vitro and in vivo Function of Recombinant CN

Functional in vitro Evaluation of rCN

In several in vitro integrin functional assays (inhibition of ADP-induced platelet aggregation and inhibition of MDA-MB-435 human breast carcinoma cell adhesion to fibronectin (FN), only the modified rCN (i.e. rCN+) construct showed biological activity, and exhibited an IC_{50} almost identical to native CN (fig. 2, 3). Surprisingly, mass spectrometry (MALDI-TOF and ESI) demonstrated that this form of recombinant CN is a monomer. We hypothesize that rCN+ folded correctly in the C-terminal part of the molecule, preserving the integrin-binding loop of the recombinant molecule and thus, accounting for the potent biological activity. However, it adopted a slightly different disulfide bridge combination at the N-terminus, possibly by being unable to form the long disulfide bridge between Cys-7 and Cys-30, which may have compromised the dimerization process. We further analyzed the rCN+ efficacy in vivo in a MDA-MB-435 animal model where the recombinant molecule was administered in a liposomal formulation as described in the following section.

Once the recombinant CN was produced, a pathway for delivery similar to that which has been used with the native protein was employed. Native CN is rapidly cleared from the circulation limiting its therapeutic potential. Therefore, in order to optimize the pharmacokinetic and potential therapeutic window the recombinant protein was encapsulated within small unilamellar liposomes.

Fig. 1. SDS-PAGE Coomassie staining of total protein fractions (TPF) collected from *E. coli* transformed with Trx-CN and Trx-CN+. Transformed and un-transformed Origami B *E. coli* in 1 liter culture were induced with 1 mM IPTG and grown at 37°C or 25°C in shaker flasks at 250 rpm for 3 h. TPF were collected in 4 ml of 20 mM Tris-HCl, pH 7.5, of which 10 µl were loaded and analyzed under non-reducing conditions. From left to right, lanes represent: TPF from Trx-CN+ cells induced at 37°C, TPF from un-transformed cells induced at 37°C, TPF from Trx-CN cells induced at 37°C, TPF from un-transformed cells induced at 25°C, broad-range prestained protein standards (Bio-Rad Laboratories, Hercules, Calif., USA), TPF from Trx-CN+ cells induced at 25°C, and TPF from Trx-CN cells induced at 25°C.

Fig. 2. rCN+ and native CN show a similar inhibitory profile for MDA-MB-435 cell adhesion to immobilized fibronectin. Pretreatment of MDA-MB-435 cells with various concentrations (0–1,000 nM) of either native CN or rCN+ for 30 min inhibited adhesion of MDA-MB-435 cells (100 µl of cells, 10⁵ cells/ml) to immobilized fibronectin. Pretreated cells were allowed to adhere for 1 h at 25°C, and after non-adherent cells were washed away, the number of adherent cells for each condition was estimated using the MTS cell viability assay.

Fig. 3. rCN+ and native CN exhibit identical effect on platelet aggregation. Different concentrations of rCN, rCN+, or native CN, were incubated with platelet-rich plasma. Addition of ADP induces platelet aggregation; either CN, rCN or rCN+ were added 1 min prior to ADP. Both CN and rCN+ inhibit aggregation with an IC₅₀ of ~60 nM. Thus, rCN+ interacts with platelets identically to native CN; rCN had no activity.

Fig. 4. A LrCN inhibits the growth of MDA-MB-435 tumors in a mouse xenograft model. In two independent studies, three groups of five nude mice had MDA-MB-435 human mammary carcinoma cells implanted in the mammary fat pad. Two weeks following implantation small tumors were palpable and treatment was commenced. Animals were treated with LCN or LrCN (105 µg, twice weekly, i.v. administration), a PBS-treated control was included. A significant reduction ($p < 0.005$) of tumor growth is observed in both treatment groups. **B** Inhibition of angiogenesis by LCN and LrCN in the MDA-MB-435 breast cancer model. Quantitation of microvessels by CD31-immunohistochemistry. Positive staining areas on CD31-stained slides were quantitated in terms of pixels with SimplePCI advanced imaging software. The LCN group shows 90% inhibition, while LrCN shows 92% inhibition of tumor induced angiogenesis as compared to PBS-treated animals. Five animals were used per group and the data represents 10 areas from three sections from each mouse, the average area in pixels of CD31-stained vessels in each treatment group is presented ($\pm$ SD).

Liposomal Encapsulation of Recombinant CN

The rCN+ construct has been formulated into liposomes using the same procedure as developed for encapsulation of native CN. For details of liposome preparation the reader is referred to the chapter in this volume by Swenson et al. Since the rCN+ construct represents the only form of recombinant CN to be encapsulated in liposomes and further studied in vivo, we will refer to this liposomal formulation as LrCN. The encapsulation efficiency has been determined through BCA protein determination following disruption of LrCN with a solution of H₂O:methanol:chloroform. We found that 72% of the recombinant protein in the encapsulation solution is entrapped within the liposomes, as compared to 80% with native CN. LrCN shows identical stability and size distribution (average particle size 140 nm) as encapsulated native CN.

Treatment of Breast Cancer with LrCN

LrCN has been administered to MDA-MB-435 human mammary cancer bearing mice with no visible toxicity. Following administration of LrCN (twice weekly IV administration for 7 weeks, 105 µg per dose, 10 mice per group) there is a significant inhibitory effect on tumor growth (~80% inhibition) as compared to the PBS-treated control; efficacy was equal to or better than that observed with LCN (fig. 4). The results of this in vivo experiment indicate that rCN+ functions as effectively as native CN.

Anti-Angiogenic Activity of LCN and LrCN in Breast Cancer

Since previous in vivo studies with CN indicated that there was a dramatic inhibitory effect on angiogenesis in growing tumors [9–11], we examined the effect of LCN

on tumor angiogenesis in the MDA-MB-435 model (methodology described in the chapter by Swenson et al.). Anti-CD31 (PECAM-1) monoclonal antibody was used for immunohistochemistry because it has been reported to be highly expressed on angiogenic vasculature (approximately one million copies of PECAM-1 reported on the surface of an endothelial cell) [12]. This property of CD31 makes it an optimal reporter molecule for determination of angiogenic growth. Figure 5 shows photomicrographs of sections stained for CD31 in the MDA-MB-435 studies and a graph of the quantitation of CD31 staining in the different treatment groups: PBS, IV-LrCN (105 μ g twice weekly) and IV-LCN (105 μ g twice weekly). There is a statistically significant ($p < 0.0005$) reduction of microvascular density in the LrCN and LCN groups as compared to the control, which corresponds to a 92% reduction of angiogenesis in the IV-LrCN group, and 90% reduction in the IV-LCN group.

Thus our findings from in vitro analysis of inhibition of ADP-induced platelet adhesion and inhibition of cancer cell adhesion to components of the extracellular matrix have been extended to in vivo observations in the mouse model of human cancer. These findings conclusively demonstrate that the recombinant disintegrin performs as expected and can be utilized for further clinical development.

Discussion

In the present study, we show for the first time that recombinant CN can be efficiently produced in a soluble active form and at high expression yields in a novel recombinant bacterial system engineered to better assist the proper folding of difficult eukaryotic proteins devoid of a secondary structure and displaying multiple disulfide bridges like CN.

CN cDNA has been previously cloned in our laboratory and we found that the CN gene is part of a precursor composed of pro-protein, metalloproteinase, and disintegrin domains [1]. The disintegrin domain encodes 65 amino acids with a molecular weight equal to that of the CN subunit. CN is truncated at its amino-terminus compared to other disintegrins; the missing sequence contains two cysteine residues. All cysteines in other disintegrins are involved in disulfide bonds; since there are two unpartnered cysteine residues in the residual CN sequence, they must be involved in its dimerization [1]. However, until recently the production of CN in a recombinant system suitable for development and commercialization had

proven to be a difficult challenge for this otherwise promising agent.

By employing the novel recombinant system described above, we have been able to generate a soluble monomeric form of recombinant CN that retains the full activity of native CN and was expressed at a yield of approximately 20 mg/l. CN wild-type disintegrin domain or the disintegrin domain with a C-terminal graft imported from a different disintegrin, echistatin, have been directionally cloned by PCR into the pET32a vector, downstream of TrxA sequence. The vector was transformed into Origami-gami B (DE3) pLysS expression host.

A unique TEV protease cleavage site has been engineered just upstream of CN constructs in order to facilitate the subsequent cleavage of target protein from the thioredoxin fusion partner. The TEV protease recognizes a specific amino acid sequence and cleaves it efficiently, leaving the recombinant disintegrin intact. The HKGPAT amino acid sequence, which represents the C-terminal tail of the monomeric disintegrin echistatin, has been grafted at the C-terminus of rCN+. This C-terminal graft was expected to improve recombinant disintegrin affinity toward integrin $\alpha 5 \beta 1$ [8], a key player in angiogenesis, and also to assist the folding process in this part of the molecule. It is well known that disintegrins are structurally designed in a way that enables them to establish deep and extensive contacts with the integrin-binding pocket that is created between the α -subunit β -propeller and β -subunit I-like domain displaying the crucial metal ion-dependent adhesion site that locks the negatively charged residue in the RGD motif. It has been long believed that the C-terminal tail of disintegrins played an important role in assisting and stabilizing the integrin-binding loop of disintegrins to lock it into the binding pocket of the integrins. In this way, disintegrins make better ligands than cyclic RGD peptides and are expected to outperform the latter as therapeutic agents. New structural studies suggest that the disintegrin-integrin-binding loop comes in close contact with its C-terminal tail, folding together as a functional unit, and that the latter assists the integrin-binding loop in the binding pocket by making additional contacts [13]. Moreover, the C-terminal tail on some disintegrins displays one or more strategic negatively charged residues that may interact with one additional metal ion-dependent adhesion site found at the β -subunit integrin level.

In summary, we have generated a functional recombinant monomeric form of CN (designated rCN+), successfully encapsulated it in unilamellar liposomes (LrCN),

and further demonstrated the potent anti-tumor and anti-angiogenic activity of this liposomal formulation in the MDA-MB-435 human breast cancer xenograft animal model.

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