Synthetic disulfide-bridged cyclic peptides mimic the anti-angiogenic actions of chondromodulin-I

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ChM-I mutants were then excised by NotI digestion and cloned into the pCAGGS expression vector."(14) Recombinant human ChM-I and its mutants were expressed using the Free-Style 293 Expression system (Invitrogen) and purified with the anti-FLAG M2 affinity gel (Sigma) as described previously."(12)

Synthesis of chondromodulin-I mimetic peptides. All of the ChM-I mimetic peptides used in this study were synthesized by standard Fmoc solid phase peptide synthesis, and analyzed by reverse-phase HPLC and mass spectrometry (Fig. 2). ChM-I cyclic peptide is a synthetic 19-amino acid product corresponding to residues 102-130 of hChM-I. It was cyclized through incubation at a concentration of 1 mg/mL in PBS for 30–60 min. Disulfide bond formation was confirmed by mass spectrometry. The C83 and C99 residues were replaced by serines in the ChM-I linear peptide. The ChM-I cyclic peptide with a tail is 40 amino acids in length and corresponds to the ChM-I linear peptide. The ChM-I cyclic peptide with the C83 and C99 residues were replaced by alanine. The Tnmd cyclic peptide with a tail is a chimeric peptide containing a cyclic domain of human Tnmd (R278-Y296) and the C-terminal tail is a glycosylation domain corresponding to residues I82-R100 of hChM-I. It was cyclized by standard Fmoc solid phase peptide synthesis, and analyzed by mass spectrometry. The C83 and C99 residues were replaced by alanine. The Tnmd cyclic peptide with the C83 and C99 residues were replaced by alanine.

Cell migration assay. HUVEC were grown in endothelial cell growth medium (EBM complete medium with supplements; Lonza, Walkersville, MD, USA) to subconfluence and used in the experiments at passages 4–7. The migration of HUVEC was assessed using a modified Boyden chamber assay as previously described."(13) Briefly, membrane filters (8-μm pore size, BD Biosciences) of cell culture inserts were coated with 1–2 μg/mL of vitronectin at 4°C overnight. HUVEC were serum-starved for 4 h in αMEM containing 0.5% FBS and were resuspended in αMEM containing 0.1% BSA (7 × 10^5 cells/200 μL) after trypsinization. The cells were preincubated with test samples for 30 min, and then seeded onto the vitronectin-coated cell culture inserts. Aqueous stock solutions of ChM-I mimetic peptides were prepared at a concentration of 300 μM, and a series of dilutions were added to the cultures. In some experiments, rhChM-I was treated with 10 mM DTT at 37°C for 1 h, and added to the culture at a 200-fold dilution. Cell migration was induced by the addition of VEGF-A (20 ng/mL) in the lower chamber. After 4 h, the number of cells that had migrated to the bottom surface of the insert was counted in five representative fields per insert. These assays were performed in triplicate and repeated three times.

Xenograft tumor model. Human chondrosarcoma OUMS-27 cells were cultured in DMEM containing 10% FBS at 37°C in 5% CO2."(15,16) Cell aliquots (5 × 10^6 cells) were suspended in 0.1 mL PBS and inoculated subcutaneously into the back of 4-week-old Balb/c nu/nu mice (Shimizu Laboratory Supplies, Kyoto, Japan). When tumors reached a size of 45 mm^3, the mice received 20 mg daily treatments of a ChM-I cyclic peptide with a tail, 5 μg rhChM-I or 50 μL PBS alone by
subcutaneous injection around the tumor daily for the initial 5 days. Tumor volumes were determined as width$^2 \times$ length $\times 0.52$.

**Immunohistochemical analysis.** Tumors were excised on day 21, fixed with 4% paraformaldehyde at 4°C overnight, embedded in the Tissue-Tek O.C.T. Compound (Sakura Fine-technical, Tokyo, Japan) and sectioned at a thickness of 8-10 μm. Frozen sections were washed with TBS containing 1% Tween 20 and incubated with 2% skim milk for 20 min in a humidity chamber. These sections were then incubated at 4°C overnight with a primary antibody (anti-CD31 antibody, 1:2000 dilution, or anti-type II collagen antibody, 1:800 dilution) and rinsed. For immunofluorescent analysis, the sections were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 594-conjugated goat anti-rat IgG secondary antibodies (1:300 dilution, Molecular Probes, Eugene, OR, USA). The CD-31-positive areas were measured from five random fields using ImageJ (ver. 1.39u). Sections were also stained with 0.05% toluidine blue (pH 2.5).

**Statistical analysis.** The significance of the differences between different groups was determined using Student’s t-test. The differences were considered statistically significant when the $P$-value was <0.01 or 0.05.

**Results**

Inactivation of recombinant human chondromodulin-I by the reducing agent. We first examined the effects of DTT on the anti-angiogenic activity of rhChM-I (Fig. 3). An optimum dose of VEGF-A (20 ng/mL) stimulated the migration of HUVEC approximately fourfold over the basal level. Recombinant hChM-I (1 μg/mL, 40 nM assuming an average molecular mass of 25-kDa) successfully inhibited this VEGF-A-stimulated migration (Fig. 3). However, following incubation with

![Fig. 2. Sequences of synthetic chondromodulin-I (ChM-I) mimetic peptides. The number beneath the amino acid symbol indicates the position of the amino acid residue from the N-terminus of ChM-I or Tnmd. The cysteine residues at the position of 103 and 109 were replaced by alanine in the peptides synthesized.](image)

| Peptide Type                          | Sequence                        | Position |
|--------------------------------------|---------------------------------|----------|
| ChM-I cyclic peptide (I$^{82}$–R$^{100}$) | ICEPLGGYYWPYNQYQGR              | 83       |
| ChM-I linear peptide (I$^{82}$–R$^{100}$) | ISEPLGGYYWPYNQYQSR              | 83       |
| ChM-I cyclic peptide (K$^{81}$–R$^{100}$) with a tail (S$^{101}$–V$^{120}$) | KICEPLGGYYWPYNQYQGCRSAARVIMPASWMVARILGMV | 99 103 109 |
| ChM-I reduced linear peptide (K$^{81}$–R$^{100}$) with a tail (S$^{101}$–V$^{120}$) | KICEPLGGYYWPYNQYQGCRSAARVIMPASWMVARILGMV | 99 103 109 |
| Tnmd cyclic peptide (R$^{278}$–R$^{297}$) with a ChM-I tail (S$^{101}$–V$^{120}$) | RVCPEPLLGYYPYCYQGGRSAARVIMPASWMVARILGMV | 280 292 |
| Tnmd reduced linear peptide (R$^{278}$–R$^{297}$) with a ChM-I tail (S$^{101}$–V$^{120}$) | RVCPEPLLGYYPYCYQGGRSAARVIMPASWMVARILGMV | 280 292 |

![Fig. 3. Effects of DTT on the anti-migratory activity of recombinant human chondromodulin-I (rhChM-I). Serum-starved HUVEC (7 × 10$^4$ cells) were preincubated in a modified Boyden chamber for 30 min with rhChM-I (1 μg/mL), which had been incubated with or without 10 mM DTT for 1 h at 37°C, and then seeded on vitronectin-coated cell culture inserts in serum-free medium. The cells were allowed to migrate for 4 h toward vascular endothelial growth factor (VEGF)-A (20 ng/mL) added in the lower chamber. The number of cells that had migrated to the bottom surface of the insert was counted. Values are the means ± SD of a triplicate assay. The data are representative of three independent experiments with similar results. *$P$ < 0.05; NS, not significant.](image)
DTT, rhChM-I completely lost its inhibitory activity, while DTT (50 μM) alone did not interfere with the migration of HUVEC.

Effects of Cys to Ser mutations and a C-terminal deletion on the activity of recombinant human chondromodulin-I (rhChM-I). The importance of disulfide bonds to rhChM-I functions was further assessed by mutagenesis of this protein (Fig. 4A). The inhibitory action on the VEGF-A-stimulated migration was assessed by incubating these mutant proteins at a fixed dose (40 nM) in culture. The all-Ser rhChM-I mutant (1 μg/mL, 40 nM), in which all eight Cys were replaced by Ser, clearly failed to inhibit the VEGF-A-stimulated migration of HUVEC (Fig. 4B). The Ser(79,83,99,103) rhChM-I and the Ser(83,99) rhChM-I mutants were also non-inhibitory to this process. Interestingly, as shown by the Ser(83,99) rhChM-I mutant, the disruption of only one disulfide bond resulted in a significant reduction in its inhibitory activity (Fig. 4B). In agreement with this, the Cys(83,99) rhChM-I mutant, in which all of the Cys residues except for Cys83 and Cys99 were replaced by Ser, evidently inhibited the VEGF-A-stimulated migration of HUVEC (Fig. 4C). The Δ(Cys83-Cys99) rhChM-I mutant lacking the 17 amino acid residues from Cys83 to Cys99 exhibited only marginal effects, suggesting that the Cys83–Cys99 disulfide bond is important for the anti-angiogenic activity.

Naturally occurring bChM-I was purified from fetal bovine epiphyseal cartilage using a hCHM-5-conjugated affinity column followed by reversed-phase HPLC. The dose-response curve revealed a potent inhibitory effect of bChM-I on the VEGF-A-stimulated migration of HUVEC (ID50 = 1–2 nM, Fig. 5). The dose-response curve for rhChM-I was almost superimposable on that of bChM-I. The Cys(83,99) rhChM-I mutant gave a dose-response curve with a parallel slope to that of bChM-I (ID50/C25 = 6 nM). This parallel shift of the dose-response curve indicated that the Cys(83,99) rhChM-I mutant has an approximate fivefold weaker potency than bChM-I, but has a similar mode of action on HUVEC.

Although all eight Cys residues were maintained in the C-terminal deletion mutant, Δ(Trp111–Val120), of rhChM-I,

![Diagram](https://via.placeholder.com/150)

**Fig. 4.** Effects of the site-directed mutagenesis of Cys residues and truncations in recombinant human chondromodulin-I (rhChM-I). (A) Schematic representation of the rhChM-I mutants generated in this study. Specific pairs of Cys residues were substituted by Ser. Δ(Cys83-Cys99) and Δ(Trp111-Val120) rhChM-I are deletion mutants that lack the amino acids corresponding to Cys83–Cys99 and Trp111–Val120, respectively. (B,C) The anti-migratory activities of rhChM-I mutants on the vascular endothelial growth factor (VEGF)-A-induced migration of HUVEC were determined in a similar manner to Figure 2 at a fixed dose (40 nM) in culture. Values are the means ± SD of a triplicate assay and the data are representative of three independent experiments, which gave similar results. *P < 0.01 compared with control group (with VEGF-A alone); NS, not significant.
this truncated protein showed a significant loss of activity (Fig. 4C). However, this mutant was still clearly active, albeit at a significantly higher dose compared with the wild type protein (Fig. 5). The ID50 value of the Δ(Trp111-Val120) mutant was approximately 150 nM from the dose-response curve, which had a significantly shallow slope and was, therefore, clearly distinct from that of the full length rhChM-I or Cys(83,99) rhChM-I.

Bioactivity of synthetic chondromodulin-I mimetic peptides. We chemically synthesized a ChM-I cyclic peptide (I82–R100), which corresponded to the Cys83–Cys99 disulfide-bridged cyclic structure of ChM-I (Fig. 2). Owing to its hydrophobic nature, it was not possible to obtain a sufficient number of bioassay data points to prepare a complete dose-response curve, but the ChM-I cyclic peptide was clearly bioactive (Fig. 6A), with an estimated ID50 value of 2 μM. More importantly, the slope of the dose-response curve was particularly shallow and paralleled that of the C-terminal deletion mutant Δ(Trp111-Val120) rhChM-I. The ChM-I cyclic peptide, in which two Cys residues in the ChM-I cyclic peptide were replaced by Ser, did not show any appreciable activity.

We next prepared a ChM-I cyclic peptide with a C-terminal tail corresponding to the Lys87–Val120 region of the hChM-I protein (Fig. 2). The Cys103 and Cys109 residues were replaced by Ala to prevent inter-molecular and intra-molecular disulfide bonding. The addition of the C-terminal hydrophobic tail made the peptide further insoluble and, therefore, hampered the ability to perform the migration assay at higher doses (i.e. over several μM). However, this species still exhibited marked inhibitory activity in the assay (Fig. 6B). At higher doses, such as 2–3 μM, this 40-amino acid peptide inhibited cell migration almost completely. The slope of the dose-response curve became much steeper than that of the ChM-I cyclic peptide without the C-terminal tail and paralleled those of the parent rhChM-I or Cys(83,99) rhChM-I.

Tenomodulin (Tnmd), a ChM-I-related gene product, also contains a cysteine-rich domain.

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cells form a vascularized but translucent tumor mass with cartilage-like ECM in nude mice. Using this xenograft tumor model, we examined the effects of a ChM-I mimetic peptide on tumor angiogenesis in vivo. The OUMS-27 chondrosarcoma that developed in these animals was treated with PBS daily for the first 5 consecutive days, with the volume rapidly increased from 45 mm$^3$ to approximately 300 mm$^3$ by 3 weeks (Fig. 7A). In contrast, the tumors in this model that received rhChM-I (5 $\mu$g each for the initial 5 days) clearly grew slowly to reach a size of approximately 120 mm$^3$. ChM-I cyclic peptide with a tail (20 $\mu$g each for the initial 5 days) also clearly inhibited tumor growth (Fig. 7A). The invasion of blood vessels could be readily observed through the translucent tumor tissues (Fig. 7B) and the CD31-positive vasculature seen in the type II collagen-positive cartilage-like tumor ECM (Fig. 7C). As shown in Figure 7(D), tumor angiogenesis was clearly inhibited by the tailed ChM-I cyclic peptide.

**Discussion**

Chondromodulin-I and Tnmd form an anti-angiogenic protein family characterized by a unique disulfide-bridged hydrophobic domain at their C-termini. This structure is implicated in the heat-stable and reduction-sensitive nature of bChM-I. In fact, incubation of rhChM-I with DTT completely abolishes its activity against the VEGF-A-stimulated migration of HUVEC. The Cys(83,99) rhChM-I mutant in our current study successfully inhibited VEGF-A-stimulated migration of HUVEC. Deletion of the 17-amino-acid stretch from Cys83 to Cys99 resulted in the loss of this inhibitory activity. Synthetic ChM-I mimetic peptides revealed that even the disulfide bridge-closed cyclic structure alone could be inhibitory to this migratory event. Thus, this disulfide bridge-closed cyclic structure might represent a core structure that underlies the activity of the ChM-I/Tnmd family of proteins.

Interestingly, the disulfide bridge-closed cyclic moiety is rich in aromatic residues, such as Tyr and Trp. We speculate that this particular cyclic structure contributes to downstream signaling by presenting these residues to the putative cognate receptor(s), as exemplified by the single disulfide bridge-containing peptide mimetics that interfere with ErbB receptor signaling by presenting specific amino acid residues onto the ligand recognition surface of the receptor. Cryptic angiogenesis inhibitors derived from vascular basement membrane...
collagens, such as endostatin and tumstatin, also exhibit anti-inflammatory and anti-proliferative properties against cultured vascular endothelial cells.\(^{24,25}\) The disulfide-bridge closed cyclic peptide, which corresponds to the C-terminal region of endostatin (Fragment IV), was found to be an inhibitor of endothelial cell migration and proliferation.\(^{26}\) However, a non-cyclic N-hydrophilic peptide (Fragment I) that contains an α-helix also had an inhibitory effect on the migration of HUVEC and angiogenesis in vivo.\(^{27,28}\) In the case of tumstatin, the anti-angiogenic activity is not dependent on its disulfide-bridged structure.\(^{29,30}\) Unlike these cryptic inhibitors,\(^{31}\) function-blocking antibodies against β1 and αvβ integrins did not interfere with the inhibitory action of ChM-I,\(^{1,2}\) suggesting that ChM-I carries a unique anti-angiogenic sequence motif and acts on endothelial cells through a mechanism distinct from these cryptic angiogenesis inhibitors.

The conserved hydrophobic C-terminal tail is also characteristic of ChM-I and Tnm1.\(^{17}\) Previous measurements of far-UV circular dichroic spectra have suggested that the bioactive glycosylated rChM-I contains an α-helix.\(^{1,11}\) The primary sequence of ChM-I indicates that an α-helix is predicted to localize at its hydrophobic C-terminus. Deletion of these C-terminal residues renders rChM-I significantly less sensitive to the dosage. The dose-response curve of this truncated rChM-I mutant was found to be parallel to that of the ChM-I cyclic peptide. Addition of the ChM-I C-terminal tail to the ChM-I cyclic peptide resulted in a restoration of the normal dosage-sensitive response. Hence, we speculate that the hydrophobic tail (Trp\(^{101}–\)Val\(^{200}\)) of ChM-I may assist with its signaling by facilitating the presentation of its disulfide-bridged core structure (Cys\(^{83}–\)Cys\(^{99}\)) to the putative ChM-I receptor through the binding to a low-affinity site or co-receptor.

A ChM-I cyclic peptide conjugated directly to the C-terminal tail (Trp\(^{111}–\)Val\(^{200}\)) of ChM-I and a ChM-I cyclic peptide with a tail having the polyethylene glycol moiety (NH-CH\(_2\)-CH\(_2\)-O-\(\sim\)-CH\(_2\)-CO\(_3\)) in place of the intervening nine residues (Ala\(^{102}–\)Ser\(^{105}\)) were synthesized. Both of these peptides were substantially insoluble in culture medium where they formed aggregates and this prevented an examination of their bioactivity in vitro (data not shown). The results of MALDI-TOF-MS analysis suggests that a substantial portion (30–50%) of these peptides is spontaneously oxidized at the C-terminal Met\(^{119}\) residue. These observations led us to speculate that the disulfide-bridged cyclic structure and C-terminal tail have to be adequately separated from each other to function appropriately. Therefore, the intervening nine amino acids may contribute to the separation of these functional units, without which the C-terminal hydrophobic tail cannot be internally folded. In this regard, the parent ChM-I molecule has a disulfide-bridged cyclic structure (Cys\(^{83}–\)Cys\(^{99}\) that is effectively separated from the hydrophobic C-terminal tail by anti-parallel stretches (Cys\(^{88}–\)Cys\(^{97}\) and Cys\(^{99}–\)Cys\(^{105}\) interconnected with disulfide bonds.

In summary, we have in our present study dissected out the structural elements of ChM-I that are functionally important for its anti-angiogenic properties. The design and application of bioactive synthetic ChM-I mimetic peptides will also help with the identification of ChM-I/Tnm receptors and may contribute to the development of anti-angiogenic therapies.

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**Disclosure Statement**

Jun Kondo is an employee of the Science and Technology Research Center, Mitsubishi Chemical Group.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sets used in the mutagenesis experiments.

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