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Peptides derived from type I thrombospondin repeat-containing proteins of the CCN family inhibit proliferation and migration of endothelial cells

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Summary

Angiogenesis, or neovascularization, is tightly orchestrated by endogenous regulators that promote or inhibit the process. The fine-tuning of these pro- and anti- angiogenic elements (the angiogenic balance) helps establish the homeostasis in tissues, and any aberration leads to pathologic conditions. The type I thrombospondin repeats are a family of protein structural elements involved in the control of angiogenesis, and some proteins containing these repeats have been identified as negative regulators of angiogenesis. Here we identify a set of 11 novel, anti-angiogenic 18- to 20-amino acid peptides that are derived from proteins that belong to the CCN protein family and contain type I thrombospondin motifs. We have named these peptides spondinstatin-1, cyrostatin, connectostatin, nephroblastostatin, wispostatin-2, wispostatin-3, netrinstatin-5C, netrinstatin-5D, adamsostatin-like-4, fibulostatin-6.1, and complestatin-C6 to reflect their origin. We have shown that these peptides inhibit proliferation and migration of human umbilical vein endothelial cells in vitro. By conducting a clustering analysis of the amino acid sequences using sequence similarity criteria and of the experimental results using a hierarchical clustering algorithm, we have demonstrated that there is an underlying correlation between the sequence and activity of the identified peptides. This combination of experimental and computational approaches introduces a novel systematic framework for studying peptide activity, identifying novel peptides with anti-angiogenic activity, and designing mimetic peptides with tailored properties.

Keywords

Angiogenesis; inhibitors; endogenous; endothelial cell; proliferation; migration; spondinstatin-1; cyrostatin; connectostatin; nephroblastostatin; wispostatin-2; wispostatin-3; netrinstatin-5C; netrinstatin-5D; adamsostatin-like-4; fibulostatin-6.1; complestatin-C6

Introduction

Angiogenesis, the growth of new microvessels from the pre-existing vasculature, is tightly controlled by various endogenous regulators (Carmeliet, 2005; Folkman, 2004; Nyberg, Xie, & Kalluri, 2005). These regulatory elements include pro- and anti-angiogenic proteins or

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peptide fragments. Many of the angiogenesis regulators have been determined to be fragments of extracellular matrix proteins or of circulating factors (Folkman, 1996).

The thrombospondin family of angiogenesis regulators consists of a group of five prototypical proteins that are characterized by modular organization (Iruela-Arispe, Luque, & Lee, 2004). The thrombospondins contain a number of modules, among which are a globular amino terminal motif, a pro-collagen homology region, three type I thrombospondin repeats, three EGF or type 2 repeats, and a globular carboxy-terminal region. Two of the five members of this family, thrombospondin 1 (TSP-1) and thrombospondin 2 (TSP-2), have the highest degree of similarity, both in terms of amino acid identity and structural organization.

The thrombospondins are potent inhibitors of angiogenesis (Carpizo & Iruela-Arispe, 2000; N. Guo, Krutzsch, Inman, & Roberts, 1997; Ren, Yee, Lawler, & Khosravi-Far, 2006). Thrombospondin 1 was the first endogenous inhibitor of angiogenesis to be discovered. It has been shown to play a critical role in suppressing the formation of new vessels, and thereby inhibiting tumor growth and metastasis. Thrombospondin 1 inhibits both the proliferation and migration of endothelial cells both in vitro and in vivo (Lawler, 2002).

The ADAMTS (a disintegrin and metalloproteinase with thrombospondin motif) proteins are a separate group of 19 metalloendopeptidases that also regulate angiogenesis and show similarity in their metalloproteinase domain to that of reprotlysins, the snake venom metalloproteinases (Porter, Clark, Kevorkian, & Edwards, 2005). Like the thrombospondins, the ADAMTS proteins are also modular and contain a set of sequential domains (Apte, 2004). The most important of these domains are the metalloproteinase catalytic domain, with a reprotysin-like zinc binding motif; a disintegrin-like domain; and, most importantly, a type I thrombospondin repeat. This type I TSP repeat is similar to the type I repeats found in thrombospondin 1 and thrombospondin 2.

Peptides from the type I thrombospondin domains of ADAMTS-1 (METH-I) and ADAMTS-8 (METH-II) have been shown to be anti-angiogenic (Iruela-Arispe, Vazquez, & Ortega, 1999; Vazquez et al., 1999). Both can inhibit vascular endothelial growth factor (VEGF)-induced angiogenesis in the chick chorioallantoic membrane assay as well as fibroblast growth factor (FGF-2)-induced neo-vascularization in the corneal micro-pocket assay (Iruela-Arispe et al., 1999; Vazquez et al., 1999). Also two peptides, Mal II and Mal III, derived from the type I thrombospondin repeats of the thrombospondin 1 protein have been shown to exert anti-angiogenic activities both in vitro and in vivo (Dawson et al., 1999; Tolsma et al., 1993). There is also an increasing interest in using the thrombospondin-1 protein as a prototype for designing anti-angiogenic peptides with optimized activities (Haviv et al., 2005).

By using a bioinformatics algorithm, we have recently identified a set of 11 peptides of 18 to 20 amino acids that are similar to the type I thrombospondin domains of the aforementioned endogenous inhibitors of angiogenesis. These novel peptides are derived from the associated type I thrombospondin repeats of human endogenous proteins and share similarities to the known inhibitors. Several of the identified short peptides are derived from the Cyr61-Ctgf-Nov (CCN) protein family; these proteins are traditionally considered pro-angiogenic, since they promote the endothelial cell proliferation and migration (Brigstock, 2003). Here we demonstrate that peptides derived from the type I thrombospondin repeats of these proteins are anti-angiogenic, inhibiting the proliferation and migration of human umbilical vein endothelial cells (HUVECs) in vitro.

We have also performed two independent clustering analyses: one of the experimental results using a hierarchical clustering algorithm, and the other of the peptides based on sequence similarity criteria. The results of these analyses indicate that there is a correlation between the sequence of the tested peptides and both their function and relative potency. This correlation

can be used computationally to identify novel potent endogenous inhibitors of angiogenesis within proteins that contain type I thrombospondin repeats, as well as to design optimized synthetic anti-angiogenic peptides.

Material and Methods

Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) from a single donor were obtained from Cambrex (Walkersville, MD). The cells were propagated in EGM-2 medium, consisting of a basal cell medium with 2% FBS, growth factors (hbFGF and VEGF), and antibiotics (gentamicin/amphotericin B). All the cells used were from passage 3 to passage 6.

Peptide Synthesis and Handling

The peptides were produced using a solid-phase synthesis technique by the custom peptide synthesis facility in the Department of Oncology, Johns Hopkins University, and a commercial provider (Abgent, San Diego, CA). HPLC and mass spectroscopy analyses of each peptide were performed. The peptides were synthesized so that both the amino and carboxy terminals were free. For each of the peptides the synthetic procedure yielded 10 mg of >95% pure peptide. The peptides were provided in solid form and solubilized in water before use. The molecular weight of each peptide was confirmed by the mass spectral analysis. In the cases of highly hydrophobic peptides, dimethyl sulfoxide (DMSO) at a maximum concentration of 0.1% (v/v) was used as a solvent. We experimentally verified that at this concentration the solvent had no effect on the experimental results.

In Vitro Cell Viability Assay

We assessed the effects of our anti-angiogenic agents on the proliferation of endothelial by measuring the metabolic activity of the live cells using the colorimetric cell proliferation reagent WST-1 (Roche, Indianapolis, IN) (Ishiyama et al., 1996). Briefly, approximately 2×10^3 cells/well were seeded in a 96-well microplate without any extracellular matrix substrate and exposed for 3 days to different peptide concentrations: 0.01, 0.1, 1 and 10 $\mu\text{g/ml}$ and 20, 30 and 40 $\mu\text{g/ml}$. The molecular weight of each of the peptides is approximately 2 kDa thus the aforementioned concentrations are translated into 5 nM, 50 nM, 500 nM, 5 μM , 10 μM , 15 μM and 20 μM . Each of the concentrations was tested simultaneously in quadruplicate, and each of the experiments was repeated three times. As a positive control (i.e., decreasing viability) we applied 100 ng/ml (0.22 μM) TNP-470 (Farinelle et al., 2000). As a negative control (equivalent to normal viability) the cells were cultured without any agent in full medium, containing growth factors and serum. A more detailed description of the Materials and Methods is provided in (Karagiannis & Popel, 2007).

In-vitro Cell Migration Assay

A modified Boyden chamber migration assay (BD Biosciences, San Jose, CA) was used to examine endothelial cell migration in the presence of an activator and the peptide solution; in our case we used 20 ng/ml of VEGF (Invitrogen, Carlsbad, CA) and 1, 10, 20, and 30 $\mu\text{g/ml}$ of the tested peptide solution. A serum- and growth factor-free medium was used as a negative control and 20 ng/ml of VEGF were used as a positive control. The chambers were then incubated for 20 h at 37°C. The cells that had migrated into the lower chamber were stained with calcein (Invitrogen, Molecular Probes, Carlsbad, CA) 90 min prior to termination of the experiment and counted.

Computational Analysis

Statistical significance was assessed using Student's t-test, with p-values < 0.001 defined as significant. The dendrogram calculation was performed using the Clustalw algorithm (Thompson, Higgins, & Gibson, 1994) and visualized using Jalview (Clamp, Cuff, Searle, & Barton, 2004). The clustering and statistical analysis was performed using Matlab. We implemented a hierarchical clustering analysis using the Euclidean distances as a distance metric (Romesburg, 1984). Once the distance was calculated, the experimental observations were grouped into a binary, hierarchical cluster tree by linking the pairs of observations that were in closer proximity according to the metric used.

Results and Discussion

Using a bioinformatics analysis, we have identified a set of 11 peptides derived from type I thrombospondin repeats of different proteins that show similarity to known angiogenesis inhibitors derived from these repeats, such as METH-I (ADAMTS-1) and METH-II (ADAMTS-8) and the thrombospondin repeats of the thrombospondin 1 protein (Table 1). Here we first describe the parent proteins and then present the results of our proliferation and migration experiments.

The first peptide is derived from F-spondin (spondin 1), or vascular smooth muscle cell growth promoting factor (VSGP). F-spondin is an extracellular matrix protein that is expressed in the developing nervous system. Its expression patterns suggest potential roles for this protein in the guidance of neural cells and in growth cone migration (Adams & Tucker, 2000; Meiniel et al., 2003). We have named the newly identified anti-angiogenic peptide spondinstatin-1 because it is derived from F-spondin.

The second peptide is derived from CYR61, which is a member of the CCN (CCN1) protein family. Acting as an extracellular matrix-associated molecule, CYR61 promotes the adhesion of endothelial cells by interacting with integrins and augments growth factor-induced proliferation (Babic, Kireeva, Kolesnikova, & Lau, 1998). Here we show that a short peptide derived from a type I thrombospondin repeat of CYR61, and which we call cyrostatin, inhibits the proliferation and migration of human umbilical vein endothelial cells in vitro.

The third peptide is derived from connective tissue growth factor (CTGF). CTGF is another CCN protein (CCN2) and a growth factor secreted by vascular endothelial cells. It promotes proliferation and differentiation of chondrocytes and also mediates heparin- and divalent cation-dependent cell adhesion in many cell types, including fibroblasts, myofibroblasts, and endothelial and epithelial cells. As a total peptide it promotes the proliferation and migration of vascular endothelial cells in vitro (Brigstock, 2002; Shimo et al., 1999). Here we show that a short peptide derived from the type I thrombospondin domains of CTGF exhibits anti-angiogenic properties. This peptide derived from CTGF is designated connectostatin.

The fourth peptide is derived from nephroblastoma overexpressed gene (NOVH), the third member of the CCN protein family (CCN3). In endothelial cells, NOVH supports cell adhesion, induces directed cell migration, and promotes cell survival (Lin et al., 2003). The identified peptide derived from the type I thrombospondin motif of NOVH, which we call nephroblastostatin, reduces both the endothelial cell proliferation and migration in HUVECs.

The fifth identified peptide is derived from the type I thrombospondin repeat of WNT1 inducible signaling pathway protein 2 (WISP-2). WISP-2 is the fifth member of the CCN family (CCN-5). It is also considered an angiogenic factor (Brigstock, 2003) and has been shown to respond to estrogen in human breast cancer cells (Inadera et al., 2000). Wispstatin-2, the

peptide we have identified within the type I thrombospondin domain of WISP-2, has anti-angiogenic properties, inhibiting the proliferation and migration of HUVECs.

The sixth peptide is derived from WISP-3 and is the sixth member of the CCN family (CCN6). Like WISP-2, the homologous WISP-3 is also considered to be angiogenic (Brigstock, 2003). We report here that wispostatin-3, the peptide we have identified within the type I thrombospondin repeat of WISP-3, has anti-angiogenic activity.

The next two peptides, which we call netrinstatin-5C and netrinstatin-5D, are derived from the netrin receptors UNC5C and UNC5D. Interestingly, the netrin receptors, like the semaphorins (Carmeliet, 2005), have been shown to regulate guidance events during the morphogenesis of the vascular system in zebrafish and mice (Lu *et al.*, 2004). Here we show that the two identified peptides derived from their type I thrombospondin motifs suppress endothelial cell proliferation and migration in vitro.

The ninth peptide is derived from thrombospondin repeat containing 1 (TSRC1) protein, or ADAMTS-like 4. This peptide, which we name adamtsostatin-like-4, also inhibits the proliferation and migration of HUVECs in vitro. The tenth peptide is derived from fibulin 6. Fibulins are extracellular matrix secreted glycoproteins that have been shown to modulate cell morphology, growth, adhesion and motility during tumor progression (Gallagher, Currid, & Whelan, 2005). Fibulins have been shown to abrogate the ability of endothelial cells to proliferate, invade Matrigel and form tubes in vitro by antagonizing VEGF signaling (Albig & Schiemann, 2004). Here we describe a peptide from the type I thrombospondin domain of fibulin 6, named fibulostatin-6.1, that also exhibits anti-angiogenic activity by inhibiting the proliferation and migration of endothelial cells in vitro.

The last identified peptide is derived from the type I thrombospondin repeat in complement component 6 (C6). We call it complestatin-C6. C6, a component of the complement cascade, is part of the membrane attack complex, which can insert into the plasma membrane and cause lysis of the cell. We now provide evidence that the complestatin-C6 peptide shows anti-angiogenic activity, inhibiting the proliferation and migration of HUVECs.

The tested peptides inhibit the proliferation of HUVECs in vitro

Evidence that an agent inhibits the proliferation of endothelial cells is critical for classifying it as anti-angiogenic. Therefore, we have tested the ability of the short peptides to inhibit the proliferation of a population of HUVECS in vitro, comparing the inhibitory activity of these peptides to the positive control inhibitor (TNP-470 at 100 ng/ml) and to full medium as a negative control.

Surprisingly, although some of the peptides are derived from parent proteins that had previously been shown to be pro-angiogenic, these peptides all demonstrated anti-proliferative effects, decreasing the viability of the human umbilical vein endothelial cells in vitro.

The results of these proliferation assays identified two distinct types of activity: One group of peptides, including connectostatin, nephroblastostatin, and wispostatin-2 and -3, showed a typical monotonic dose-response pattern of activity, with the anti-proliferative efficiency increasing as their concentration increased, until saturation was reached. The second group consisted of spondinstatin-1, netrinstatin-5C and -5D, adamtsostatin-like-4 and complestatin-C6. These peptides exhibited a biphasic (nonmonotonic) response: Their activity reached a maximum at an intermediate concentration and decreased as their concentration was increased. Some characteristic examples of such biphasic responses include the anti-proliferative effects of the full length endostatin in vitro and in vivo (Celik *et al.*, 2005; O'Reilly *et al.*, 1997) and its small fragment derivatives (Tjin Tham Sjin *et al.*, 2005), the anti-angiogenic fragments

derived from the thrombospondin-1 (Tolsma *et al.*, 1993) and thrombospondin-2 (Lopes *et al.*, 2003) proteins, anti-angiogenic fragments derived from SPARC (Chlenski *et al.*, 2004; Sage *et al.*, 2003) and urokinase (Y. Guo, Mazar, Lebrun, & Rabbani, 2002).

Connectostatin is an example of a typical monotonic dose-response peptide: It reached a maximal level of inhibitory activity (35% of that produced by the positive control, TNP-470) at 30 $\mu\text{g/ml}$; with increasing concentration of the peptide, its activity leveled off. Similar behavior was exhibited by nephroblastostatin, with a maximal inhibitory activity of 40% at 40 $\mu\text{g/ml}$. Wispostatins also showed a direct dose-response pattern of activity, with a maximal level of inhibitory activity of 40% at 40 $\mu\text{g/ml}$.

Spondinstatin-1 exhibited the biphasic pattern of behavior: It reached its maximal anti-proliferative activity of 30% at 1 $\mu\text{g/ml}$; at the maximum tested concentration of 40 $\mu\text{g/ml}$, its activity decreased to essentially background levels. Similarly, the anti-proliferative activity of cyrostatin reached a maximum of 15% relative to the control at 30 $\mu\text{g/ml}$ and decreased at 40 $\mu\text{g/ml}$. The netrinstatins were also biphasic: Netrinstatin-5C exhibited a maximal inhibitory activity of 30% at 1 $\mu\text{g/ml}$ and netrinstatin-5D a maximum of 25% at 10 $\mu\text{g/ml}$; in both cases, their inhibitory activity decreased with increasing peptide concentration. Adamtsostatin-like-4 was also biphasic, with a maximum activity of 30% relative to the control at 20 $\mu\text{g/ml}$. Fibulostatin's activity was relatively weaker, peaking at 15% at 10 $\mu\text{g/ml}$. Finally, complestatin-C6 also exhibited a biphasic response, with its maximum anti-proliferative potency of 25% at 0.1 $\mu\text{g/ml}$; above 30 $\mu\text{g/ml}$ its activity was minimal.

We also quantified the activity of the predicted peptide fragments in the proliferation experiments relative to known short peptides derived from the type I thrombospondin repeats of the thrombospondin 1 protein Mal II (SPWSSCSVTCGDGVITRIR) and Mal III (SPWDICSVTCGGGVQKRSK) (Dawson *et al.*, 1999; Tolsma *et al.*, 1993). Those short peptides exhibited maximum activity of 25% and 35%, respectively, at 10 $\mu\text{g/ml}$ (data not shown). Our peptides reach similar levels of potency relative to the activity of the known peptides. From the tested peptides spondinstatin-1, connectostatin, nephroblastostatin and the wispostatin-2 and -3 exhibit identical potency. The scaled results after using the maximum Mal II and III peptide activity (30%) as a positive control are presented in the supplementary material (Figure 1S).

In order to exclude false positives attributed to the nature of the used peptides, the proliferation experiments were repeated with two scrambled peptides, where the amino acid sequences of the active peptides were randomly permuted. We chose to create scrambled peptides based on the sequences of wispostatin-2 and netrinstatin-5D. Both of the peptides exhibited significant activity in the proliferation experiments and the former followed a monotonic dose response while the latter a biphasic. The amino acid sequences of the peptides were randomly permuted and the new random scrambled peptides that were created and tested were CGWTLPATGRMVGTTCSEA from wispostatin-2 and RAWRCVSCRQSKRTGWNGE from netrinstatin-5D. The results in the proliferation experiments using the scrambled peptides in seven different concentrations (0.01, 0.1, 1, 10, 20, 30 and 40 $\mu\text{g/ml}$) were not statistically different than the negative control (Supplementary Figure 3S).

The migration of HUVECs in the presence of VEGF is suppressed by the tested peptides

The second component of the angiogenic process is the migration of the proliferating population of endothelial cells in response to a chemoattractant or angiogenesis-promoting agent. Consequently, in order to validate the potency of the tested peptides as anti-angiogenic agents, we tested their ability to suppress the migration of the HUVECs in the presence of 20 ng/ml of VEGF in a modified Boyden chamber assay. In this assay, VEGF increases the motility of a population of endothelial cells, stimulating their migration through a porous laminin-

coated membrane into the opposite chamber. As a positive control (decreasing motility), we incubated the cells in serum- and growth factor (VEGF)-free cell medium. As a negative control, we incubated the cells with VEGF alone, and no peptide. The results are scaled so as to represent the percentage of migration inhibition relatively to the controls and are shown in Figure 2.

Of the 11 tested peptides, 9 substantially decreased the motility of the endothelial cells in the presence of VEGF to levels of migration inhibition ~70–90%. The nine inhibitory peptides were spondinstatin-1 ($IC_{50} = 4.8 \mu\text{g/ml}$ or $2.2 \mu\text{M}$), cyrostatin ($IC_{50} = 7 \mu\text{g/ml}$ or $3.7 \mu\text{M}$), connectostatin ($IC_{50} = 7.3 \mu\text{g/ml}$ or $3.8 \mu\text{M}$), nephroblastostatin ($IC_{50} = 16.7 \mu\text{g/ml}$ or $8.5 \mu\text{M}$), wispostatin-3 ($IC_{50} = 8.5 \mu\text{g/ml}$ or $4.2 \mu\text{M}$), netrinstatin-5C ($IC_{50} = 8.8 \mu\text{g/ml}$ or $3.8 \mu\text{M}$), adamtsostatin-like-4 ($IC_{50} = 2.6 \mu\text{g/ml}$ or $1.1 \mu\text{M}$), fibulostatin-6.1 ($IC_{50} = 12.5 \mu\text{g/ml}$ or $6 \mu\text{M}$) and complestatin-C6 ($IC_{50} = 7.6 \mu\text{g/ml}$ or $3.5 \mu\text{M}$). Netrinstatin-5D ($IC_{50} = 4.8 \mu\text{g/ml}$ or $2.1 \mu\text{M}$) and wispostatin-2 ($IC_{50} = 4.8 \mu\text{g/ml}$ or $2.7 \mu\text{M}$) were less effective, inhibiting the migration of the endothelial cells 40 to 60%.

We also quantified the ability of the tested peptides to suppress the migration of the endothelial cells in the modified Boyden chamber assay relative to the ability of the known peptides derived from the type I thrombospondin repeats of the thrombospondin protein (Mal II and Mal III). We tested the two control peptides using two concentrations of $10 \mu\text{g/ml}$ and $30 \mu\text{g/ml}$. The first control peptide, Mal II, inhibited the migration of the endothelial cells in the presence of VEGF by 60% at the lowest tested concentration of $10 \mu\text{g/ml}$ and maintained this activity at $30 \mu\text{g/ml}$ as well, whereas the second peptide, Mal III, reached maximum inhibition of 80% in the migration assay at $30 \mu\text{g/ml}$ (data not shown). The activity of our peptides is comparable to the activity of these known peptides. The scaled results in the migration assay, after using the maximum migration inhibition of 80% for Mal III as a control are presented in the supplementary material (Figure 2S).

The migration experiments were also repeated using the two scrambled peptides. The results in the migration experiment using three different peptide concentrations (1 , 10 and $30 \mu\text{g/ml}$) were also not statistically different than the negative control of normal motility (Supplementary Figure 4S).

Combinatorial identification of a sequence-function correlation for the short peptides

Given the differences in activity profiles that we observed for the various peptides, we asked whether there was any correlation between the observed experimental activities of these peptides and their corresponding amino acid sequences. In order to establish whether any such sequence-activity correlation exists, we performed a computational analysis in which we treated the sequence similarities of the peptides and their potencies in the proliferation assay as independent data sets. We then performed two independent clustering analyses to search for inter-relationships within each of the two independent data sets. These analyses had as an outcome two sets of peptide clusters: those that were similar at the amino acid sequence level, and those with similar activity profiles. We then matched the two sets of clusters that had initially been calculated independently.

For the clustering analysis of the peptides' amino acid sequences, we used Jalview to analyze the information from the multiple sequence alignments and build a tree of similar peptides based on their amino acid sequence similarities. The metric used to calculate the tree was their average distance, calculated on the basis of their percent sequence identity (Fig. 3A). This average distance algorithm is a bottom-up clustering method; it is a greedy algorithm that constructs the tree in a stepwise fashion. From the tree analysis we identified two major clades, a set of two clusters with peptides that share sequence similarities. The first cluster was composed of spondinstatin-1, fibulostatin-6.1, adamtsostatin-like-4, and netrinstatin-5D and

-5C, and the second was made up of connectostatin, cyrostatin, nephroblastostatin, wispostatin-2 and -3, and complestatin-C6.

For our clustering analysis of the experimental data, we used the results of the proliferation experiments to identify peptides with similar potency profiles. The use of a range of concentrations in this assay provided a statistically significant population of data from which we could infer inter-relationships. For each of the peptides, we used the scaled potencies in the proliferation assay to perform hierarchical clustering, using the Euclidian distance of the results as a distance metric. The Euclidian distance is one of the most direct metrics, as it measures the absolute distance between two points in the results space, which in this case is defined by the eight vectors that include the scaled proliferation results. The average linkage was used to generate a hierarchical tree (McLachlan, 1992).

The results from the clustering analysis of the experimental results are shown in Figure 3B: The first cluster was composed of netrinstatin-5C and -5D, spondinostatin-1, complestatin-C6, and adamsostatin-like-4, and the second cluster fibulostatin-6.1, cyrostatin, connectostatin, nephroblastostatin, and wispostatin-2 and -3.

Our further comparative analysis showed that the two sets of clusters, calculated from completely independent data sets, coincided almost entirely, except in the case of fibulostatin-6.1 and complestatin-C6. Fibulostatin-6.1 was aligned at the interface of two clusters, and its assignment to a different cluster than in the sequence similarity-based tree can potentially be attributed to experimental variation. If that explanation is tenable, then that peptide clusters with adamsostatin-like-4 and netrinstatin-5D. The only peptide that was aligned significantly differently according to the hierarchical clustering algorithm was complestatin-C6. A notable difference between this peptide and the other studied peptides is the presence of an Asn residue in the eleventh position of its sequence, a position that is occupied by Gly in all the other peptides. This amino acid substitution may be responsible for its divergent clustering. Apart from this divergence, however, the overlapping of these two independently calculated sets of motifs is indicative of a strong sequence-activity correlation, suggesting that the activities of the peptides are inherent in their amino acid sequences.

In the present study we have identified a set of 11 novel peptides of 18 to 20 amino acids that are derived from proteins containing type I thrombospondin repeats, and we provide evidence that these 11 peptides have anti-angiogenic activity, inhibiting the proliferation and migration of HUVECs in vitro. Of particular note is the fact that some of these anti-angiogenic peptides are derived from larger proteins that belong to the CCN protein family and have previously been shown to be pro-angiogenic.

Using the information from the multiple sequence alignment, we have assigned each of the peptides to one of two clusters on the basis of their sequence similarities, and then independently assigned them to a second pair of clusters on the basis of their functional properties. The conjunction of these two independently calculated pairs of clusters was indicative of an underlying relationship between the sequence and anti-angiogenic potency of the individual peptides. Only in the case of one peptide did the structure and function clusters not match. In that case, the divergence may be attributable to the substitution of a polar Asn for the non-polar Gly that is present in all of the other peptides. This hypothesis, however, requires further experimental testing.

Extensive studies on the mechanistic details of the anti-angiogenic activity of thrombospondin 1, the prototype type I thrombospondin repeat containing protein, have implicated CD36 as the cell surface receptor that mediates its effects on endothelial cells (Dawson et al., 1997). CD36 is an 88 kDa transmembrane glycoprotein expressed on endothelial cells and a collagen binding molecule. Using affinity chromatography it has been demonstrated that various motifs

of the type I thrombospondin repeats of the thrombospondin 1 protein can bind to CD36 (Greenwalt et al., 1992). Recently $\beta 1$ integrins were identified as critical components of the thrombospondin repeats anti-migratory effects on endothelial cells (Calzada et al., 2004; Short et al., 2005). The epitope that is responsible for the $\alpha 3 \beta 1$ integrin binding is mapped within the amino-terminus of the thrombospondin-1 protein (Krutzsch, Choe, Sipes, Guo, & Roberts, 1999). Future studies including mutagenesis studies should be performed with the identified peptides in order to determine whether this receptor interaction can explain any or all of the present data. Furthermore the novel information inferred from the amino acid sequences of the identified endogenous peptides can help us design novel synthetic peptides or peptide mimetics derived from these thrombospondin repeats (Reiher et al., 2002).

Angiogenesis is regulated by the orchestrated expression of endogenous regulatory elements (Carmeliet, 2005; Folkman, 1996, 2002, 2004; Nyberg et al., 2005), which include an array of growth factors that stimulate and control the proliferation and migration of endothelial cells. There is also a growing population of endogenous regulators that work competitively by inhibiting both of these processes, thus suppressing angiogenesis. The fine control of these two opposing elements, referred to as the angiogenic balance (Folkman, 1995), is vital for the homeostasis of a physiologic tissue and is disrupted during pathologic conditions, such as cancer.

We have now identified an additional set of anti-angiogenic peptides that have potential application as agents to help restore the angiogenic balance when it has been disrupted. The ability of these 11 short peptides to inhibit the proliferation and migration of endothelial cells is strong evidence that within the sequences of some pro-angiogenic proteins can reside short (cryptic) fragments with anti-angiogenic potency. Furthermore the aforementioned properties of the identified peptides make them suitable candidates for use as anti-angiogenic pharmaceutical agents in numerous therapeutic applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effect of four peptides derived from the type I thrombospondin repeat-containing proteins on the proliferation of HUVECs that exhibit maximum anti-proliferative activity. After 3 days of incubation with the test peptides, the endothelial cells were detected using a WST-1 colorimetric assay and counted. The results are scaled so that 0% represents the optical signal from the negative control (endothelial cells incubated with medium containing growth factor and serum, data not shown) and 100% represents the signal from the positive control (cells incubated with 100 ng/ml TNP-470, data not shown). Vertical bars indicate the standard error. All values are significantly different from 0% at $p < 0.001$ except those marked by NS (non-significant). In all cases, the standard error for the controls was $< 3\%$ ($n=8$).

Figure 2.

Effect of the peptides on the migration of HUVECs in a modified Boyden chamber migration assay. Endothelial cells were allowed to migrate for 20 h in the presence of 20 ng/ml VEGF and 1, 10, 20 and 30 µg/ml peptide solution, then stained with calcein and counted. The fluorescent signal was initially scaled so that 0% represents the negative control (endothelial cells in serum- and growth-factor-free medium) and 100% the positive control (migration in the presence of 20 ng/ml VEGF) and based on the scaled results the percentage migration inhibition as shown in the figure was calculated. Vertical bars indicate the standard error. All values are significantly different from 0% at $p < 0.001$ except those marked by NS (non-significant). In all cases, the standard error for the controls was $< 5\%$ ($n=8$). Also the IC₅₀ concentration for each of the peptides is calculated.

Figure 3.

Cluster analysis of the activities of the individual peptides using as independent data sets the sequence similarities and the peptide activity in the proliferation assays. **A.** The multiple sequence similarity dendrogram calculated using as a metric the average length of the peptides, based on the percentage sequence identity. The peptides were clustered on the basis of their sequence identity. **B.** The heatmap represents the scaled activities of the peptides in the in vitro proliferation assay. In the associated colormap, the black color represents the cases in which the scaled activity is $\leq 0\%$. The peptides were aligned according to their similarities in terms of activity profile, and the associated hierarchical tree was calculated. The peptide clusters, calculated using the amino acid sequence similarities and shown as a tree in A, overlap with the clusters derived from the experimental results in B.

Table 1

Amino acid sequences of the short anti-angiogenic peptides derived from the type I thrombospondin repeat-containing proteins.

Peptide Name	Peptide Origin	Accession Number	Peptide Sequence
Spondin-1	F-spondin	Q9HCB6(621–639)	SEWSDCSVTCGKGMRTQR
Cyrostatin	CYR61	O00622(234–251)	TSWSQCSKTCGTGISTRV
Connectostatin	CTGF	P29279(204–221)	TEWSACSKTCGMGISTRV
Nephroblastostatin	NOVH	P48745(211–228)	TEWTACSKSCGMGFSTRV
Wispostatin-2	WISP-2	O76076(199–216)	TAWGPCSTTCGLMATRV
Wispostatin-3	WISP-3	Q8TE59(232–249)	TKWTPCSRTC GMISNRV
Netrin-5C	UNC-5C	O95185(267–285)	TEWSVCNSRCGRGYQKRTR
Netrin-5D	UNC-5D	NP_543148.1(254–272)	TEWSACNVRCGRGWQKRSR
Adams-1-like-4	TSRC1	Q6UY14(790–809)	SPWSQCSVRCGRGQRSRQVR
Fibulin-6.1	Fibulin-6	Q96RW7(4536–4554)	SAWRACSVTCGKGIQKRSR
Complement component C6	Complement component C6	P13671(30–48)	TQWTSCSKTCNSGTQSRHR