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Novel Anti-Angiogenic Peptides Derived From ELR-Containing CXC Chemokines

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Abstract Angiogenesis is tightly regulated by numerous endogenous pro- and anti-angiogenic proteins and peptides. Among these are the CXC chemokines, a set of multifunctional peptides. CXC chemokines containing the ELR motif act as pro-angiogenic agents by regulating both endothelial cell proliferation and migration. Here we show that a set of six 22–24-amino acid peptides derived from the pro-angiogenic ELR-containing CXC chemokines exhibit notable anti-proliferative and anti-migratory activity in vitro; we call these peptides chemokinostatins. The ability of the identified peptides to inhibit the basic components of angiogenesis even though they are derived from pro-angiogenic proteins contributes towards the understanding of the diverse role of the CXC chemokine family in angiogenesis. J. Cell. Biochem. 104: 1356–1363, 2008. © 2008 Wiley-Liss, Inc.

Key words: angiogenesis; inhibitor; endogenous; chemokinostatin; endothelial cell

Chemokines are multifunctional mediators that can promote immune responses, stem cell survival, development, and homeostasis, as well as trigger chemotaxis and angiogenesis. They are divided into four subfamilies (C, CC, CXC, and CX3C), based on their structural properties and primary amino acid sequence (Fig. 1A). The CXC chemokines are heparin-binding proteins. On a structural level, they have four highly conserved cysteine residues, with the first two cysteines separated by one non-conserved residue (Fig. 1A) [Romagnani et al., 2004]. The CXC subfamily includes several ligands, designated as CXCL. The CXC ligands along with their alternate names and abbreviations are summarized in Figure 1B [Belperio et al., 2000; Strieter et al., 2004].

Although the CXC motif distinguishes this family from other chemokine families, a second structural domain dictates their angiogenic activity. The NH_2 terminus of a subset of the CXC chemokines contains a three-amino acid motif, the ELR motif, or Glu-Leu-Arg. This motif precedes the first cysteine of the primary structure of the cytokines. The family members that contain the ELR motif (ELR+, or ELR-positive) are known to be potent promoters of angiogenesis [Belperio et al., 2000; Romagnani et al., 2004; Strieter et al., 2004]. Members of the CXC chemokine family that behave as pro-angiogenic factors include interleukin-8; epithelial neutrophil activating protein-78; growth-related genes GRO-α, -β and -γ; granulocyte chemotactic protein-2; and NH_2-terminal truncated forms of platelet basic protein. ELR+ CXC chemokines directly induce endothelial cell chemotactic and proliferative activity in vitro and also stimulate angiogenesis in vivo in the absence of prior inflammation. Interestingly, the ELR motif-containing chemokine GRO-β, has been shown to exhibit anti-angiogenic properties [Cao et al., 1995].

In contrast, members of the CXC subfamily that are induced by interferons and lack the ELR motif (ELR−, or ELR-negative) are potent inhibitors of angiogenesis. These angiostatic

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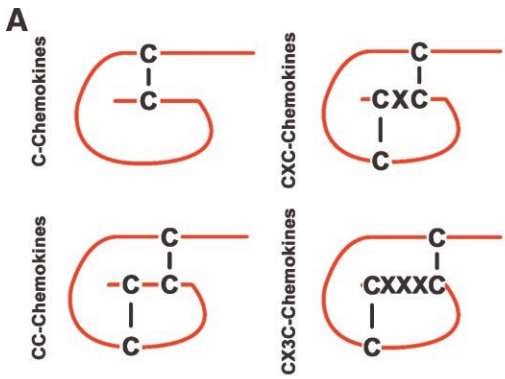

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CXC Ligands	Alternative Name	Abbreviation
CXCL1	Growth-related oncogene-α	GRO-α
CXCL2	Growth-related oncogene-β	GRO-β
CXCL3	Growth-related oncogene-γ	GRO-γ
CXCL4	Platelet factor-4	PF-4
CXCL5	Epithelial neutrophil-activating peptide-78	ENA-78
CXCL6	Granulocyte chemotactic protein-2	GCP-2
CXCL7	Platelet basic protein	PBP
CXCL8	Interleukin-8	IL-8
CXCL9	Monokine induced by interferon-γ	MIG
CXCL10	Interferon-γ-inducible protein-10	IP-10
CXCL11	IFN-inducible T-cell α-chemoattractant	I-TAC
CXCL12	Stromal derived factor-1	SDF-1
CXCL13	B-cell chemoattractant-1	BCA-1
CXCL14	Breast and kidney-expressed chemokine	BRAK
CXCL16	Small inducible cytokine B16	SCY-B16

Fig. 1. The chemokines protein family. **A**: The four chemokine subfamilies. **B**: The CXC chemokine ligands and their alternative names along with commonly used abbreviations. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

members of the CXC chemokine family include PF-4, monokine induced by interferon-γ, and interferon-γ-inducible protein 10 [Belperio et al., 2000; Romagnani et al., 2004; Strieter et al., 2004].

Based on a bioinformatics analysis, we have identified a set of six short peptides (Table I) derived from pro-angiogenic CXC chemokines that contain the ELR motif. We call these peptides chemokinostatins and have assigned them numbers derived from the chemokine from which they originate: for example, chemokinostatin-1 is derived from CXCL1. Here we report the results of in vitro experiments testing the anti-angiogenic potency of these peptides. We show that, despite the fact that these chemokinostatins are derived from pro-angiogenic chemikines containing the ELR motif, they have anti-angiogenic properties: that is, they inhibit the proliferation and migration of human umbilical vein endothelial cells (HUVECs) in vitro.

Thus, even though the ELR motif-containing CXC chemokines have been shown to be pro-angiogenic, we hypothesized that within the sequences of these proteins there may be hidden (cryptic) short fragments with anti-angiogenic potency. Here we provide in vitro evidence that the short fragments we have identified possess anti-angiogenic properties, inhibiting endothelial cell proliferation and migration. These peptides have potential as anti-angiogenic agents with application to numerous diseases that are associated with excessive angiogenesis, including cancer, age-related macular degeneration, diabetic retinopathy, atherosclerosis, psoriasis, and rheumatoid arthritis.

MATERIALS AND METHODS

Cell Culture

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

Peptide Synthesis and Handling

The six test peptides used were identified by a computational bioinformatic analysis and synthesized by the custom peptide synthesis facility in the Department of Oncology, Johns Hopkins University, and a commercial provider

TABLE I. The Amino Acid Sequences of the Tested Peptides

Peptide name	Peptide origin	Accession #	Peptide sequence
Chemokinostatin-1	CXCL1/Gro-α	P09341(80–103)	NGRKACLNPASPIVKKIIEKMLNS
Chemokinostatin-3	CXCL3/Gro-γ	P19876(80–101)	NGKKACLNPASPMVQKIIEKIL
Chemokinostatin-5	CXCL5/ENA-78	P42830(86–108)	NGKEICLDPEAPFLKKVIQKILD
Chemokinostatin-6	CXCL6/GCP-2	P80162(86–109)	NGKQVCLDPEAPFLKKVIQKILDS
Chemokinostatin-7	CXCL7/PBP	P02775(100–121)	DGRKICLDPDAPRIKKIVQKKL
Chemokinostatin-8	CXCL8/IL-8	P10145(72–94)	DGRELCLDPKENWVQRVVEKFLK

(Abgent, San Diego, CA) using a solid-phase synthesis technique. HPLC and mass spectroscopy analyses of each peptide were performed. In each case, the synthetic procedure yielded 10 mg of >95% pure peptide. The peptides were solubilized in phosphate buffer solution (PBS) before use. 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 verified experimentally that at this concentration the solvent had no effect on the experimental results.

Mass Spectrometry

The peptides were solubilized in PBS or DMSO and diluted in final concentration of approximately 400 fmol/μl. The samples were also reduced with 50 mM of dithiothreitol (DTT) final concentration. The samples were analyzed by MALDI-TOF (Applied Biosystems Voyager) with DHB (50% EtOH/water-No TFA) matrix in reflector mode within 2–6 kDa mass range. The analysis was performed at the Johns Hopkins University Mass Spectrometry Facility.

In Vitro Cell Viability Assay

The effects of our anti-angiogenic agents on the proliferation of HUVECs were assessed by measuring the metabolic activity of the live cells using the colorimetric cell proliferation reagent WST-1 (Roche, Indianapolis, IN). WST-1 is a substrate in a colorimetric assay that measures the metabolic activity of viable cells [Ishiyama et al., 1996]. Approximately 2×10^3 cells/well were seeded in a 96-well microplate in the absence of any extracellular matrix substrate. The viability of the cells was determined after a 3-day exposure to the peptide solution. During the course of the 3 days the medium containing the peptide solution as well as in the controls was not changed. Each peptide was tested at seven different concentrations: 0.01, 0.1, 1, and 10 μg/ml and 20, 30, and 40 μg/ml. The molecular size of each of the peptides is approximately 3 kDa; thus, these concentrations translate to 3.3, 33, 333, and 3333 nM and 6.67, 10, and 13.3 μM, respectively. 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 used 100 ng/ml (0.22 μM) TNP-470 (a synthetic analogue of fumagillin; provided by the National Cancer Institute) along with the full medium [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. The samples were read at 570 nm in a Victor3 V ELISA plate reader (Perkin Elmer). The amount of color produced was directly proportional to the number of viable cells. In order to estimate a quantity of the cells that are proliferating we have also determined a standard curve for the optical signal as a function of the cell population. Different concentrations of cells (500, 1000, 1500, 2000, 2500, 3000–10000 cells per well) were seeded on different wells and incubated with the WST-1 dye. A curve of the optical signal as a function of the cell population was fitted.

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. The lower compartment of the Boyden chamber was separated from the upper (containing the endothelial cells) by a laminin-coated polycarbonate filter with pores small enough to allow only the active passage of the cells (3-μm pore size).

The HUVECs were applied to the upper compartment of the chamber. Typically, 20×10^3 cells in a volume of 50 μl were added to each well. The growth factor VEGF (Invitrogen, Carlsbad, CA) was used as the activator. It was added to serum-free medium in the lower chamber to give a final concentration of 20 ng/ml in a total volume of 225 μl. VEGF in serum-free medium was also used alone as a positive control. The anti-angiogenic peptides to be tested were individually added to the lower chamber at different concentrations, together with 20 ng/ml VEGF in serum-free medium. The tested peptide concentrations in the migration experiments were 0.1, 1, 10, 20, and 30 μg/ml. The serum- and growth factor-free medium was used alone as a negative 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) 90 min prior to termination of the experiment. They were counted by measuring the fluorescence at 485 nm excitation and 510 nm emission in a fluorescence plate reader (Victor 3 V, Perkin Elmer).

Statistical Analysis

Statistical significance was assessed using Student's t-test, with $P < 0.001$ defined as significant.

RESULTS AND DISCUSSION

By using a computational bioinformatic analysis we have previously identified a set of six novel 22–24-amino acid peptides that are derived from CXC chemokines (Table I) and exhibit similarities to known anti-angiogenic peptides derived from this family. Specifically, the identified peptides bear similarities to short peptides derived from platelet factor 4, a well-established angiogenesis inhibitor [Bikfalvi, 2004; Bikfalvi and Gimenez-Gallego, 2004]. We initially observed that the anti-angiogenic active fragment of platelet factor 4 is localized within the CXC conserved protein domain. In Figure 2A, the amino acid sequence of PF-4 is shown. The CXC conserved domain is designated with blue letters whereas the active anti-angiogenic fragment is underlined. The localization of the active fragment within the conserved domain was indicative of the existence of similar fragments in the specific domain among all the CXC chemokines. We then constructed a bioinformatic algorithm to identify similar fragments in the whole human proteome. The most significant hits are shown in Figure 2B. We name the predicted fragments chemokinostatins followed by a number designating the CXC ligand that they are derived from. Here we have experimentally tested their anti-angiogenic potency using two in vitro assays that recapitulate the basic steps of the angiogenic process: endothelial cell proliferation and migration.

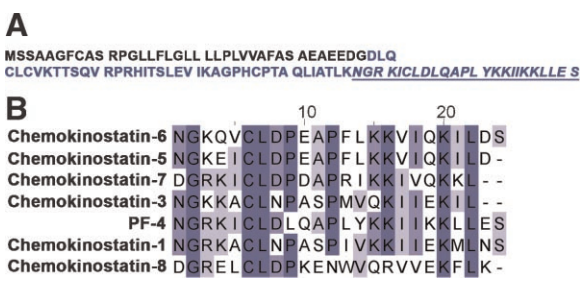

Fig. 2. The novel anti-angiogenic CXC chemokine derived peptides. **A**: The prototypical platelet factor 4 anti-angiogenic peptide (underlined) is located in the CXC chemokine/SCY conserved domain. **B**: The amino acid sequences of the novel peptides and similarities to the platelet factor 4 derived peptide. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Mass Spectrometry Analysis

The predicted peptides contain cysteines within their amino acid sequences. In order to investigate if the peptides dimerize in solution that could modulate their activity in our in vitro assays, we performed mass spectrometry analyses using the peptide solutions in normal and reducing conditions. Protein samples were analyzed with or without a reducing agent and their molecular weights were determined. As a reducing agent we used 50 mM of DTT. In both normal and reducing conditions, the peptides exhibited single peaks (Fig. 1S, Supplementary Material) and the molecular weight determined by the spectra was identical to the molecular weight calculated by using the amino acid sequence composition information. The only peptide that exhibited a dimer peak was chemokinostatin-3. Chemokinostatin-3 was the only peptide with which we used DMSO to solubilize it due to its hydrophobicity. The DMSO apparently oxidizes the cysteines inducing the formation of disulfide bridges among different monomers. In order to investigate the effect of DMSO on the dimerization of the peptides, we solubilized chemokinostatin-1 normally soluble in PBS and run a mass spectrometry analysis. In that case, we also observed the formation of dimers although those dimers are not present in the case that this peptide is solubilized in PBS. We thus conclude that the only situation that dimers may form among the peptides is in the case that we use DMSO as a solvent. This organic solvent is only required is in the case of chemokinostatin-3.

The Short Peptides Inhibit the Proliferation of HUVECs In Vitro

We first tested the ability of the six short peptides to inhibit the proliferation of HUVECS in vitro (Fig. 3) by culturing the cells with the individual peptide solutions for 3 days and then measuring the peptides' inhibitory potency relative to that of 100 ng/ml TNP-470. TNP-470 is a microtubule-stabilizing agent that has the ability to induce endothelial cell apoptosis [Farinelle et al., 2000]. It has been extensively used as a standard for quantifying angiogenesis inhibition [van der Schaft et al., 2004] thus adopted as a positive control for the in vitro proliferation assay. The optical signal from the proliferation assay was scaled so that 0% represented the signal from the negative control

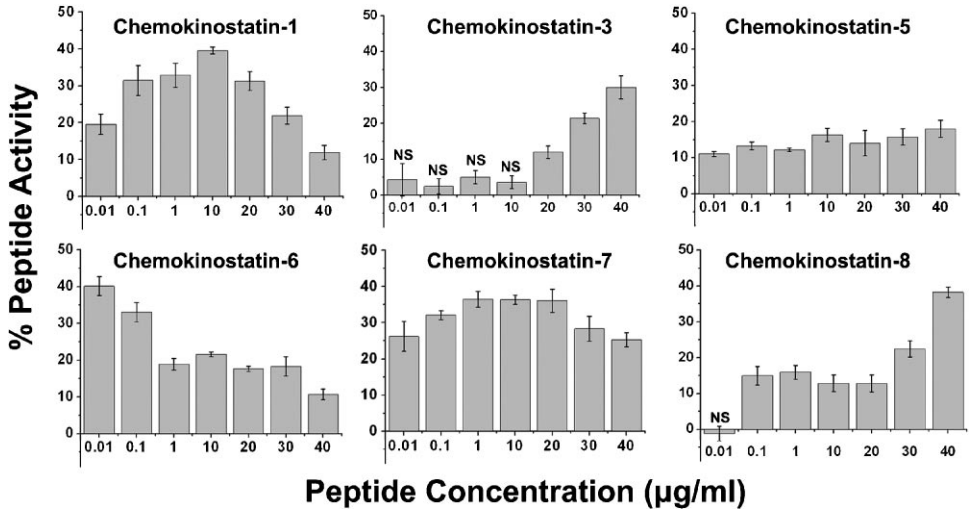

Fig. 3. Effect of the peptides derived from the CXC chemokines on the proliferation of HUVECs. After 3 days of incubation with the individual peptide solutions, the endothelial cells were stained and counted using the WST-1 colorimetric assay. The results are scaled so that 0% represents the amount of cells from the negative control (endothelial cells incubated with medium containing growth factor and serum, not shown) and 100% represents the amount of cells from the positive control (cells incubated with 100 ng/ml TNP-470, not shown). Vertical bars indicate the standard error. All values are significantly different from 0% at $P < 0.001$ except those marked by an NS sign (non-significant). In all cases, the standard error for the controls was $<3\%$ ($n = 8$).

and 100% represented the signal from the wells to which TNP-470 was added. We expressed this scaled result as peptide activity relative to the activity of TNP-470. The results from the proliferation experiments quantified solely from the optical signal and without any scaling using the TNP-470 control are presented in the supplementary material (Fig. 2S). In the case of chemokinostatin-3, a final volume of 0.1% DMSO was required for solubilization. In order to investigate the effect of DMSO on the proliferation of endothelial cells, we repeated the proliferation experiment with varying concentrations of solvent up to 0.15%. In this concentration range, we did not observe any effect of the solvent on the proliferation of the endothelial cells (Fig. 3S, Supplementary Material).

All of the six peptides derived from the CXC chemokines inhibited the proliferation of the endothelial cells, showing 20–45% of the inhibitory activity observed for the TNP-470 control. Chemokinostatin-1 reached approximately 40% of the positive control at 10 μg/ml and then declined at higher concentrations. A similar biphasic inhibitory response was seen for chemokinostatin-6 and -7. The chemokinostatin-6 activity peaked at ~45% at the lower peptide concentrations and declined as the concentration increased; chemokinostatin-7 activity peaked at 40%, then progressively decreased. This set of three peptides constitutes a class of peptides that were potent at lower concentrations (nanomolar range) and progressively lost their activity as their concentrations were increased. Chemokinostatin-3 and -8, in contrast, exhibited a typical monotonic dose-response pattern in which their inhibitory activity increased with increasing concentration. Chemokinostatin-3 reached its maximum activity (30% of positive control inhibition) at the highest tested concentration (40 μg/ml), whereas chemokinostatin-8 peaked at 40% of the control inhibition at the same concentration. Chemokinostatin-5 exhibited a consistent inhibitory activity of ~15% at all the concentrations tested; in this case the peptide was saturating at low concentrations.

We also quantified the activity of the predicted peptide fragments in the proliferation experiments relatively to a known short peptide derived from PF-4 [Jouan et al., 1999]. The short peptide from PF-4 (NGRKICLDLQAPLYKK-IIKKLLES) exhibited maximum activity of 80% relatively to the controls at concentrations greater than 30 μg/ml (Fig. 4S, Supplementary Material). Relative to this known peptide our novel peptides reach 60% efficiency, for chemokinostatin-1, 50% for chemokinostatin-3, 70% for chemokinostatin-6, 80% for chemokinostatin-7, and 60% for chemokinostatin-8. The scaled results using the PF-4 derived peptide as

a control are presented in the supplementary material (Fig. 5S).

In order to estimate a relative number of proliferating cells, we also used a standard curve to estimate the cell population after treating the cells with the chemokinostatins for 3 days. The curves in the proliferation experiment were replotted reporting now the number of viable cells (Fig. 6S, Supplementary Material).

The six novel peptides proved to be anti-proliferative, despite the fact that they were derived from proteins that had previously been shown to be pro-angiogenic. This surprising finding supports our hypothesis that within the sequences of the pro-angiogenic CXC proteins reside shorter (cryptic) fragments with anti-angiogenic potency.

The Short Peptides Inhibit the Migration of HUVECs in the Presence of VEGF

An important aspect of the angiogenic process is the migration of endothelial cells in the presence of an activator or a chemoattractant. In order to assess the ability of the short peptides to inhibit the migration of endothelial cells, we used a modified Boyden chamber assay to measure the suppression of VEGF-stimulated migration by HUVECs through a porous membrane covered by laminin (Fig. 4). In the migration experiments, we performed a dose response analysis to determine ability of the novel peptides to inhibit the migration of endothelial cells in vitro. We tested five different concentrations of 0.1, 1, 10, 20, and 30 μg/ml of peptide. The results were scaled so that 100% represented the positive control, and 0% represented the number of migrating cells in the negative control (Fig. 3). The results from the migration experiments by direct measurement of the optical signal and without any scaling using the controls are presented in the supplementary material (Fig. 7S).

Of the tested peptides, chemokinostatin-8 was the most potent inhibitor of migration as it inhibited the migration of endothelial cells by 80% at 1 μg/ml. For the other tested peptides, chemokinostatin-1 reached 80% of inhibition at the largest tested concentration of 30 μg/ml, and chemokinostatin-3 and -5 exhibited linear dose response with maximum inhibitory activity of 40% for the former and 60% for the latter. In the case of those two peptides, the smallest tested concentration was 1 μg/ml as at this level their inhibitory activity reached the minimal, non-statistically significant, activity. Chemokinostatin-6 and -7 also exhibited linear dose response with maximum activity of 60% for chemokinostatin-6 and 70% for chemokinostatin-7,

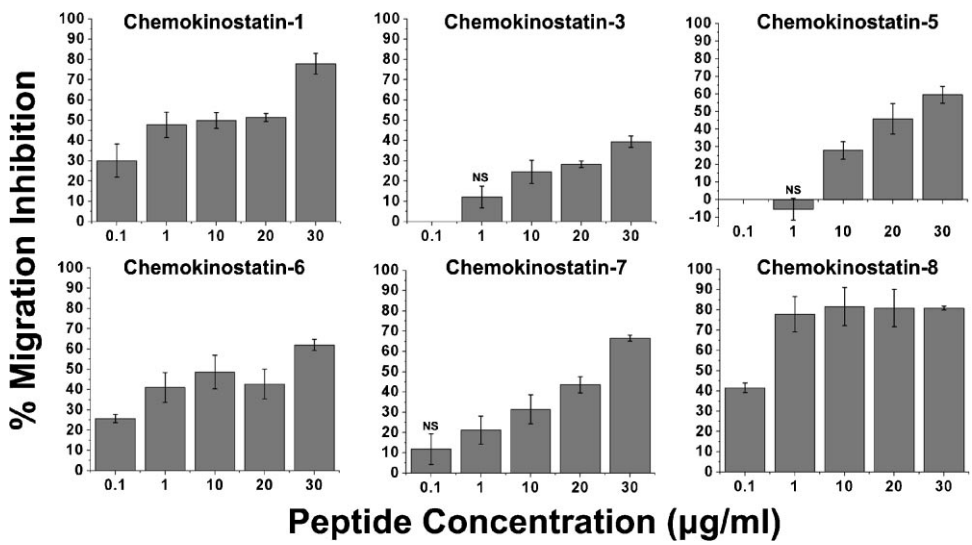

Fig. 4. Effect of the peptides on the migration of HUVECs in a modified Boyden chamber migration assay. Cells were allowed to migrate for 20 h in the presence of 20 ng/ml VEGF and 0.1, 1, 10, 20, and 30 μg/ml peptide, then stained with calcein and counted. The fluorescent signal was scaled so that 0% represents the negative control (endothelial cells in serum- and growth factor-free medium, not shown) and 100% the positive control (migration in the presence of 20 ng/ml VEGF, not shown). Vertical bars indicate the standard error. All values are significantly different from 0% at $P < 0.001$. In all cases, the standard error for the controls was <5% (n = 8).

present at the maximum tested concentration of 30 μg/ml.

CONCLUSIONS

We have identified a set of six short anti-angiogenic peptides derived from the CXC chemokines as potent angiogenesis inhibitors, and we have demonstrated their ability to inhibit the proliferation and migration of HUVECs in vitro, in assays that mimic key processes that occur during angiogenesis.

The six novel peptides inhibited the proliferation of these cells in vitro, with inhibitory potencies that were 20–45% of that exhibited by 100 ng/ml TNP-470, a potent anti-proliferative agent that is frequently used as a standard for testing cell viability. In some cases, the peptides exhibited biphasic (non-monotonic) response. Their activity reached a maximum at an intermediate concentration and decreased as their concentration was increased. This biphasic response is typical of the endogenous peptide inhibitors of angiogenesis. Some characteristic examples of such responses include the anti-proliferative effects of the full-length endostatin in vitro and in vivo [O'Reilly et al., 1997; Celik et al., 2005] and its small fragment derivatives [Tjin Tham Sjin et al., 2005] or the anti-angiogenic fragments derived from thrombospondin-1 [Tolsma et al., 1993]. The CXC-derived peptides also significantly suppressed the ability of HUVECs to migrate in response to VEGF. These two pieces of evidence strongly indicate that the six tested peptides possess anti-angiogenic properties and comprise a set of potent angiogenesis inhibitors.

The major receptor that has been implicated with the anti-angiogenic activity of the CXC chemokines is CXCR3. CXCR3 exists in three alternative splice isoforms, CXCR3A, CXCR3B, and CXCR3-alt [Strieter et al., 2005; Strieter et al., 2006]. The CXC chemokines that interact with CXCR3 inhibit the proliferation and migration of human microvascular endothelial cells. The second isoform of CXCR3, CXCR3B, has been shown to mediate the anti-angiogenic activity of CXCL4, CXCL9, CXCL10, and CXCL11 on human microvascular endothelial cells [Lasagni et al., 2003]. The third splice isoform, CXCR3-alt, has also been involved in the anti-angiogenic activity of CXC chemokines. This activity is primarily modulated by CXCL11 binding as well as CXCL9 and CXCL10 [Ehlert et al., 2004]. Future studies should be performed with the identified peptides in order to determine whether the CXCR3 receptor interaction can explain their anti-angiogenic properties.

We do not yet have evidence that these fragments are actually present in tissues under physiological or pathological conditions. The bioavailability of these fragments as circulating active agents remains to be determined by analytical techniques such as mass spectrometry of biological fluids. Evidence that these peptides may be biologically available can be provided by the existence of cleavage sites at locations that can yield the identified peptides within the total sequences of the proteins. A tool that can be used in order to determine the existence of such sites is the PeptideCutter tool at the Expasy database [Wilkins et al., 1999]. Enzymes that can cleave the whole proteins that the peptides are derived from include Arg-C proteinase, pepsin, typsin, thrombin, and thermolysin.

The unexpected origin of these peptides is of particular significance: All six of the anti-angiogenic peptides we identified were derived from ELR motif-containing chemokines, which are considered pro-angiogenic. The amino-terminal ELR motif is thought to be responsible for these pro-angiogenic properties. We have now shown that peptides from ELR motif-containing chemokines, derived from the carboxy-terminal of these proteins and similar to the well-established anti-angiogenic chemokine PF-4, possess anti-angiogenic properties. Our discovery can be supported by the evidence that Gro-β an ELR containing chemokine was shown to be anti-angiogenic and suppressed the growth of Lewis lung carcinoma in mice [Cao et al., 1995]. This demonstration of the ability of some proteins to exhibit two opposing activities contributes to the elucidation of the angiogenic balance [Folkman, 1996; Folkman, 2002; Folkman, 2004].

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