β -Sheet is the bioactive conformation of the anti-angiogenic anginex peptide

Ruud P. M. DINGS^{*†}, Monica M. ARROYO^{*}, Nathan A. LOCKWOOD[‡], Loes I. VAN EIJK[†], Judy R. HASEMAN^{*}, Arjan W. GRIFFIOEN[†] and Kevin H. MAYO^{*1}

^{*}Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota Health Science Center, 6-155 Jackson Hall, 321 Church Street, Minneapolis, MN 55455, U.S.A.,

[†]Tumor Angiogenesis Laboratory, Department of Internal Medicine, University of Maastricht, University Hospital Maastricht, P.O. Box 5800, 6202 AZ Maastricht, The Netherlands, and

[‡]Department of Chemical Engineering and Materials Science, University of Minnesota Health Science Center, 6-155 Jackson Hall, 321 Church Street, Minneapolis, MN 55455, U.S.A.

Anginex is a designed peptide 33mer that functions as a cytokine-like agent to inhibit angiogenesis. Although this short linear peptide has been shown by NMR and CD to form a nascent β -sheet conformation in solution, the actual bioactive structure formed upon binding to its receptor on the surface of endothelial cells could be quite different. By using a series of double-cysteine disulphide-bridged analogues, we provide evidence in the present study that the β -sheet is in fact the bioactive conformation of anginex. CD and NMR spectral analysis of the analogues indicate formation of a β -sheet conformation. Three functional assays,

endothelial cell proliferation, apoptosis and *in vitro* angiogenesis, were performed on all analogues. As long as the placement of disulphide bonds preserved the β -strand alignment, as in the proposed bioactive conformation, bioactivities were preserved. Knowledge of the bioactive conformation of anginex will aid in the design of smaller molecule mimetics of this potent anti-angiogenic peptide.

Key words: apoptosis, disulphides, endothelial cell proliferation, NMR, peptide, structure.

INTRODUCTION

Angiogenesis, the formation of new blood vessels out of pre-existing capillaries, is pivotal to a broad array of biological functions, ranging from normal processes like embryogenesis and wound healing to abnormal processes such as tumour growth, arthritis, restenosis and diabetic retinopathy [1,2]. The use of agents that can inhibit angiogenesis *in vitro* and *in vivo*, particularly in anti-tumour research, has indicated that anti-angiogenic therapy can be a therapeutic modality in the future. Most anti-angiogenic agents have been discovered by identifying endogenous molecules, primarily proteins, which inhibit endothelial cell (EC) growth. This traditional approach has produced a number of anti-angiogenics, such as platelet factor-4 (PF4) [3], thrombospondin-1 [4], angiostatin [5], endostatin [6] and bactericidal-permeability increasing (BPI) protein [7].

Recently, we have reported [8,9] the anti-angiogenic activity of a novel peptide 33mer (β pep-25 or anginex). Anginex belongs to a family of homologous β pep peptides that were designed by using basic folding principles and incorporating short sequences from the β -sheet domains of α -chemokines and BPI protein [10]. All β pep peptides form β -sheets to varying degrees and can self-associate in solution as dimers and tetramers [9,10]. A high-resolution NMR structure of tetrameric β pep-4 demonstrated that its monomer subunits are folded into an amphipathic three-stranded anti-parallel β -sheet motif [11]. Two types of six-stranded anti-parallel β -sheet dimers can form and these dimers associate via their hydrophobic faces into tetramers. Although a high-resolution structure of anginex (β pep-25) could not be determined by using NMR because of aggregate exchange resonance broadening, CD and NMR studies [8,9] indicate that

anginex also folds primarily into tetrameric β -sheet sandwiches at millimolar concentrations. At concentrations in the micromolar range and lower, however, anginex exists in solution primarily in the monomer state and is composed of a relatively broad distribution of conformations with considerable random coil and some β -sheet character [12]. Therefore, at the micromolar concentrations used in *in vitro* bioassays and *in vivo* animal studies demonstrating the anti-angiogenic [8,9] and anti-tumour [13–15] potency of the peptide, anginex is mostly a random coil monomer. Therefore the bioactive structure of anginex, which is selected from this rather broad conformational distribution, remains unclear.

The aim of the present study was to test the hypothesis that the bioactive conformation of anginex (β pep-25) is a β -sheet with the same strand alignment as in the NMR-derived structure of homologous β pep-4 (see Figure 1) [11]. To accomplish this, a series of disulphide-linked peptide analogues of anginex were made. By comparing activities from all 30 members of the β pep library, it has already been deduced [9] that functionally key residues in anginex are primarily hydrophobic residues, i.e. Leu⁵, Val⁷, Ile²⁰, Val²² and Leu²⁴. Because of this, cysteine residues replaced residues primarily at positions that would be on the hydrophilic face of the amphipathic anginex β -sheet. Moreover, to simplify peptide synthesis and purification, six functionally non-essential residues at the C-terminus of anginex [9] were removed, creating a peptide 27mer analogue of anginex that is shown in the present study to be equipotent with the parent peptide. Double-substituted cysteine pairs were made: Cys⁶–Cys²⁵, Cys⁸–Cys²³, Cys¹⁰–Cys²¹, Cys¹²–Cys¹⁹, Cys¹³–Cys¹⁸, Cys¹³–Cys¹⁹ and Cys⁶–Cys²⁶. These disulphide-bridged analogues were analysed in three functional assays: EC proliferation, apoptosis and *in vitro*

Abbreviations used: BPI protein, bactericidal-permeability increasing protein; bFGF, basic fibroblast growth factor; CP, cyclicized peptide; EC, endothelial cell; HUVEC, human umbilical-vein EC; NOE, nuclear Overhauser effect; PF4, platelet factor-4; PFG, pulsed-field gradient; PI, propidium iodide; RMS, root mean square.

¹ To whom correspondence should be addressed (e-mail mayox001@umn.edu).

angiogenesis. Peptide conformation was assessed with CD and ^1H NMR spectroscopies.

EXPERIMENTAL

Peptide preparation

Peptides were synthesized using fluorenylmethoxycarbonyl ('Fmoc') chemistry on a Milligen/Bioscience 9600 peptide solid-phase synthesizer, as described previously [8,9]. To form intramolecular disulphide bonds, crude peptides were solubilized at low concentrations (100 μM) and cysteine residues were oxidized by slowly bubbling wet oxygen gas into a stirred aqueous solution overnight at room temperature. Freeze-dried crude peptides (oxidized) were purified by preparative reverse-phase HPLC on a C18 column with an elution gradient of 0–60 % (v/v) acetonitrile with 0.1 % (v/v) trifluoroacetic acid in water. Purity and composition of the peptides were verified by HPLC (Beckman Model 6300), amino acid analysis and MS. The presence of the disulphide bond was checked in all peptides by using 5,5'-dithiobis-(2-nitrobenzoic acid (Ellman's reagent; 'DTNB') pre- and post-treatment with dithiothreitol.

CD

Aqueous solutions for CD were prepared by dissolving the freeze-dried peptide in the appropriate amount of water to give final peptide concentrations of 0.1 mM at pH 5.5. Concentrations were verified by tryptophan absorption at 280 nm. CD spectra were recorded on a Jasco J-710 spectrophotometer using either a 0.01 cm or 0.1 cm path-length thermally jacketed quartz cuvette maintained at 24 or 37 °C with a NesLab water bath. Acquisition was performed using a 50 nm/min scan rate, 1 nm bandwidth and 2 s response. The appropriate baseline was subtracted from each spectrum. Reported spectra are averages of six scans and are expressed as mean residue ellipticity. CD basis spectra (α -helix, β -sheet and random coil) were measured with poly(lysine) and poly(glutamic acid) (Sigma) using conditions and parameters reported previously [16,17]. Experimental CD spectra were fitted with a linear combination of α -helix, β -sheet and random coil basis spectra to estimate secondary structure contributions.

NMR measurements

For NMR measurements, the freeze-dried peptide was dissolved in water at a concentration of approx. 2 mM. The pH was adjusted to pH 5.5 by adding microlitre quantities of NaOH or HCl to the peptide sample. NMR spectra were acquired on a Varian UNITY Plus-600 NMR spectrometer using a homonuclear approach described previously [8]. Data were processed directly on the spectrometer or offline using VNMR (Varian, Inc., Palo Alto, CA, U.S.A.) or NMRPipe [18] on an SGI workstation and were analysed using Sparky (T. D. Goddard and D. G. Kneller, SPARKY 3, University of California, San Francisco, CA, U.S.A.).

Since cyclicized peptides (CPs) are relatively hydrophobic and amphipathic, pulsed-field gradient (PFG)-NMR self-diffusion measurements were performed as a check for peptide aggregation. PFG-NMR experiments were performed and analysed as described by Mayo et al. [10] using a Varian Unity-Plus 500 NMR spectrometer. The maximum magnitude of the gradient was 60 G/cm, and the PFG longitudinal eddy-current delay pulse sequence was used for all self-diffusion measurements which

were performed in $^2\text{H}_2\text{O}$ at temperatures of 5 °C and 40 °C. Peptide concentrations ranged from 0.1–2 mM.

Structural modelling

Analysis of nuclear Overhauser effect (NOE) data and structural modelling (using X-PLOR [19]) were performed essentially as described previously [20]. Calculated structures were superimposed using the SwissPdbViewer [21] or Insight II (Accelrys, Inc., San Diego, CA, U.S.A.) and were analysed using X-PLOR analysis routines.

Human umbilical-vein EC (HUVEC) cultures

HUVECs were harvested from normal human umbilical cords by perfusion with 0.125 % (w/v) trypsin/EDTA. Harvested HUVECs were cultured in gelatin-coated tissue-culture flasks and subcultured 1:3 once a week in culture medium [RPMI-1640 with 20 % (v/v) human serum supplemented with 2 mM glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin].

Proliferation measurement

EC proliferation was measured using a [^3H]thymidine incorporation assay. HUVECs were seeded at 5000 cells/well in flat-bottomed tissue-culture plates and grown for 3 days in culture medium in the absence or presence of regulators. During the last 6 h of the assay, the culture was pulsed with 0.5 μCi of [^3H]thymidine/well. Results are expressed as the arithmetic mean c.p.m. of triplicate cultures.

Apoptosis measurement

HUVECs were cultured in fibronectin-coated tissue-culture flasks in culture medium [22]. Apoptosis was measured by determination of subdiploid cells after DNA extraction and subsequent staining with propidium iodide (PI) as described previously [23]. Briefly, HUVECs were cultured for 3 days in the presence of 10 ng/ml basic fibroblast growth factor (bFGF) and the conditions as mentioned above. Serum deprivation of HUVECs was used as a positive control for apoptosis. Cells were then harvested and subsequently fixed and permeabilized in 70 % (v/v) ethanol at -20 °C. After at least 2 h, the cells were centrifuged and resuspended in DNA extraction buffer [45 mM Na_2HPO_4 , 2.5 mM citric acid and 0.1 % Triton X-100 (pH 7.4)] for 20 min. PI was added to a final concentration of 20 mg/ml and red log-scale fluorescence was analysed on a FACS-calibur. Apoptosis was quantified as the percentage of cells with PI fluorescence below the fluorescence of cells in the G_0/G_1 (diploid) phase of the cell cycle. Cells with PI fluorescence less than 10 % of cells in G_0/G_1 phase were regarded as cells in the advanced stages of cell death.

In vitro angiogenesis assay

Sprouting and tube formation of bovine ECs were studied using Cytodex-3 beads overgrown with bovine ECs in a three-dimensional collagen gel (vitrogen-100; Collagen Corp., Fremont, CA, U.S.A.) as described by van der Schaft et al. [24]. Following gelation, culture medium containing 20 ng/ml bFGF, with or without anginex or CP analogues, was applied on top of the gel. Photographs were taken after 24 h of cell culture at 37 °C. Sprouts were traced on transparent paper and digitized by scanning using HPscan software. Analysis of the amount of sprouting was performed by binarization of the file and

Figure 1 Amino acid sequence and structure of anginex and CP analogues

The amino acid sequence of anginex is shown conformed as an anti-parallel β -sheet on the left-hand side of the Figure. The N-terminus is at the right bottom as labelled. CP analogues have only the first 27 residues and therefore are devoid of C-terminal residues Arg-Glu-Lys-Ser-Lys-Asp. The new C-terminal residue has its backbone carboxylate group amidated. The amino acid sequence for the control linear peptide 27mer (no cysteine residues) CP-1 is given on the right-hand side of the Figure. CP-2 to CP-6 [i.e. CP-2 (Cys⁶-Cys²⁵), CP-3 (Cys⁸-Cys²³), CP-4 (Cys¹⁰-Cys²¹), CP-5 (Cys¹²-Cys¹⁹), CP-6 (Cys¹³-Cys¹⁸)] have the same sequence as CP-1, with disulphide bridges positioned between β -strand 1 and β -strand 2 as indicated. CP-7 [Cys¹³-Cys¹⁹] and CP-8 [Cys⁶-Cys²⁶] are control peptides that alter the alignment of β -strands 1 and 2. Single-letter amino-acid notation is used.

quantification of sprout pixel count using Scion Image software, which yields a measure of sprout length [25].

RESULTS AND DISCUSSION

For the CP series, all CP analogues have the same sequence as anginex minus the six C-terminal residues Arg-Glu-Leu-Ser-Leu-Asp (Figure 1). CP-1 is the control linear peptide 27mer (no cysteine residues), and CP-2 to CP-8 are double-cysteine-substituted disulphide-bridged peptides: Cys⁶-Cys²⁵ (CP-2), Cys⁸-Cys²³ (CP-3), Cys¹⁰-Cys²¹ (CP-4), Cys¹²-Cys¹⁹ (CP-5), Cys¹³-Cys¹⁸ (CP-6), Cys¹³-Cys¹⁹ (CP-7) and Cys⁶-Cys²⁶ (CP-8). The amino acid sequences for parent anginex and CP-1 are given in Figure 1, and positions for disulphide bonds in CP-2 to CP-6 are indicated by curved lines at the right of the CP-1 sequence. Because functionally key residues in anginex are primarily hydrophobic, i.e. Leu⁵, Val⁷, Ile²⁰, Val²² and Leu²⁴, and would lie on one side of the amphipathic structure in a β -sheet conformation [9], residues on the hydrophilic face (and not the hydrophobic face) of the proposed β -sheet were substituted with cysteine residues. For assurance that cysteine-substituted positions did not affect activity of the parent peptide, single-substituted alanine variants (Ser⁶ $\rightarrow$ Ala, Gln⁸ $\rightarrow$ Ala, Lys¹⁰ $\rightarrow$ Ala, Phe¹² $\rightarrow$ Ala, Lys¹³ $\rightarrow$ Ala, Trp¹⁸ $\rightarrow$ Ala, Lys¹⁹ $\rightarrow$ Ala, Ile²¹ $\rightarrow$ Ala, Lys²³ $\rightarrow$ Ala, Asn²⁵ $\rightarrow$ Ala and Asp²⁶ $\rightarrow$ Ala) were also made and tested in the *in vitro* EC proliferation assay. Relative to the parent peptide, activities were unaffected solely by amino acid substitutions at these positions (results not shown). On forming an actual β -sheet in CP-2 to CP-6, positioning of disulphide bonds constrains the β -strand alignment to be the same as that proposed for the folding of anginex [8]. CP-7 [Lys¹³ $\rightarrow$ Cys and Lys¹⁹ $\rightarrow$ Cys] and CP-8 [Ser⁶ $\rightarrow$ Cys and Asp²⁶ $\rightarrow$ Cys] are control peptides that shift the β -strand alignment by one residue to be out of register with the other CP analogues.

Figure 2 CD spectra for CP analogues

Far-UV CD spectra for CP analogues are shown as mean residue ellipticity ($[\theta]$) versus wavelength. All spectra display a mixture of β -sheet and random coil conformations, as shown by linear fits of basis spectra (inset). Spectra shown were obtained at 37 °C; spectra at 24 °C were comparable. Peptide concentration was 0.1 mM in H₂O, pH 5.5. Other experimental conditions are given in the Experimental section.

CD

CD and NMR spectroscopies were used to assess the conformation of CP analogues. For all CP analogues, far-UV CD traces indicated a mixture of β -sheet and random coil conformations (Figure 2), with the degree of β -sheet character corresponding to the intensity of the characteristic 217 nm trough [17,26,27]. Linear combinations of secondary structure basis spectra (α -helix, β -sheet and random coil) fitted to the CP analogue spectra (Figure 2, inset) showed the largest β -sheet content (79%) in the linear CP-1 peptide and the smallest (40%) in CP-6. Fitted data indicate the absence of helical content for most of the CP analogues. Even though CP-4, CP-5 and CP-6 show 2–6% helix content, this contribution is small enough that it is within the error from the fitting process. In addition, because the CP analogues are relatively flexible, the secondary structure contributions are only meant to semi-quantify the trend in β -sheet content. Nevertheless, the CP analogue conformational distributions are consistent with that for anginex [11].

¹H NMR conformational analysis

Even though CD data demonstrate the presence of significant populations of β -sheets in all CP analogues, proton NMR spectra for all CP analogues, except CP-2, are characteristic of mostly random coil conformations (results not shown). CP-2 is the only CP analogue that shows well-dispersed resonances indicative of a well-folded structure [28,29]. Since the parent anginex peptide exists in solution in a monomer/dimer/tetramer equilibrium [29,30], PFG-NMR self-diffusion measurements were performed on CP analogues (results not shown). Analysis of the CP analogue diffusivities indicates that, at a concentration of 2 mM, CP-2 is mostly dimeric, whereas the other CP analogues are a mixture

Figure 3 TOCSY and NOESY spectra for CP-2

600 MHz ^1H TOCSY (A) and NOESY (B) spectra are shown for CP-2. Peptide concentration was 2.3 mM in $\text{H}_2\text{O}/^2\text{H}_2\text{O}$ (9:1, v/v), pH 5.5 at 40 °C. Spectra were accumulated with 8000 data points over 8000 Hz sweep width and were processed with 1 Hz line broadening. Only spectral regions downfield from the partially deuterated water resonance are shown and some key NOEs are identified. Several cross-peaks have been labelled to indicate intra- and inter-monomer $\alpha\text{H}-\alpha\text{H}$ NOEs, which have been used to indicate β -strand alignment. In (B), the cross-peaks are labelled with two numbers separated by a solidus to indicate which residues in the sequence are involved. Single-letter amino-acid notation has been used.

of dimers and monomers. None of the CP analogues apparently associates with the tetramer state like anginex.

NMR structure of CP-2

Because initial NMR experiments indicated that CP-2 is well-folded, a complete NMR structure analysis of this peptide was performed. The αH -NH region of a TOCSY plot of CP-2 shows high frequency dispersion of NH and αH resonances, indicating the presence of a well-folded β -sheet conformation (Figure 3A). This is also evident by the observation of cross-strand $\alpha\text{H}-\alpha\text{H}$ NOEs (Figure 3B and Figure 4A). Structurally, these NOESY data indicate the presence of the same anti-parallel β -sheet strand alignment as observed in $\beta\text{pep-4}$ and other βpep peptides [11].

For CP-2, conformational modelling was performed using NOE data acquired for the peptide. A total of 160 NOE distance constraints were derived from analysis of NOESY spectra. These include 94 intra-residue, 22 sequential, 16 medium-range ($|i-j| < 5$) and 28 long-range ($|i-j| > 5$) constraints. In addition, a total of eight hydrogen bonds were identified by inspection of initial CP-2 structures and from long-lived backbone NHs, giving rise to 16 hydrogen bond distance constraints. The total number of experimentally derived constraints was therefore 168, giving an average of six constraints/residue. Initially, 100 structures for CP-2 were calculated as described in the Experimental section. The best-fit superpositions of backbone $\text{C}\alpha$ atoms for the final 18 structures are shown in Figure 4(B). These structures showed no NOE violations greater than 0.5 Å (where 1 Å = 0.1 nm). Structural statistics (Table 1) show that the N-terminus is somewhat less structurally defined and that the

Table 1 Structural statistics for the calculated structures of CP-2 from NMR data

RMS deviations values are means $\pm$ S.D. for the 18 structures. Energy values represent means $\pm$ S.D.

Parameters	
RMS deviations from experimental distance restraints (Å)*	
NOE ($n = 176$)	0.047 ± 0.007
H-bond ($n = 16$)	
RMS deviations from idealized geometry	
Bonds (Å)	0.003
Angles (°)	0.5 ± 0.02
Impropers (°)	0.37 ± 0.02
Energies ($\text{kJ} \cdot \text{mol}^{-1}$)	
$E_{\text{NOE}}^\dagger$	21.3 ± 2.5
E_{BOND}	15.98 ± 2.5
E_{ANGLE}	164.8 ± 11.7
E_{IMPROPER}	80.3 ± 23.8
E_{TOTAL}	335.6 ± 43.1

*None of the 18 final structures exhibited distance restraint violations greater than 0.5 Å or dihedral angle violations greater than 5°.

†The final value of the NOE (E_{NOE}) was calculated with a force constant of $210 \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{Å}^{-2}$.

structures satisfy experimental constraints quite well. In addition, Φ and ψ angular order parameters are all > 0.8 . Taken together, the above data indicate that the structures used to represent the solution conformation of CP-2 are well converged. The root mean square (RMS) deviation for backbone atoms of β -sheet residues

Figure 4 NOE-derived structures of CP-2

(A) Key cross-strand NOEs and the overall fold for CP-2. Single-letter amino-acid notation is used. The best-fit superpositions of backbone $C\alpha$ atoms for the final 18 structures are shown in (B). The RMS deviation for backbone atoms of β -sheet residues 6–12 and 19–25 (excluding terminal and loop residues) is 0.61 Å, and for backbone atoms of residues 6–25 (excluding N- and C-terminal residues) it is 1.13 Å.

6–12 and 19–25 (excluding terminal and loop residues) is 0.61 Å and for backbone atoms of residues 6–25 (excluding N- and C-terminal residues) is 1.13 Å.

Even though only CP-2 is well-folded as an anti-parallel β -sheet, it is most probable that peptides CP-3, CP-4, CP-5 and CP-6 share the same overall β -sheet fold, because they only differ in the position of the disulphide bridge.

CP analogues inhibit EC proliferation

CP analogues were tested for their ability to inhibit the proliferation of growth-factor-induced (bFGF, 10 ng/ml) HUVECs in the [3 H]thymidine incorporation assay [15]. Relative to the activity of the parent peptide anginex [8], CP analogues effectively inhibited the proliferation of HUVECs to various extents (Figure 5). Although the linear control, CP-1, and three of the disulphide-bridged variants, CP-4, CP-5 and CP-6, were essentially as effective as anginex, CP-2 and CP-3 appeared to

be almost as active, and control peptides CP-7 and CP-8 were only slightly active. The kinetics of these anti-proliferative effects on ECs were similar to those found for anginex, with half-maximal effects after 36–40 h and maximal responses after 3 days. As with anginex, the inhibition of proliferation by CP analogues was specific for ECs, since proliferation of fibroblasts obtained from human endometrium was unaffected (results not shown).

CP analogues promote apoptosis in ECs

To investigate whether CP-induced inhibition of EC growth is based on specific induction of apoptosis in these cells, as was demonstrated for anginex [8], HUVECs were exposed to 25 μ M peptide for 48 h, and the percentage of cells undergoing apoptosis was quantified by analysis of DNA fragmentation following DNA extraction, PI staining and flow cytometric analysis. Although all CP analogues demonstrated apoptotic activity, CP-4 and CP-5 were the most active and even slightly more effective, on average,

Figure 5 Inhibition of EC proliferation by CP analogues

Proliferation of bFGF-stimulated (10 ng/ml) HUVEC cultures was measured by quantification of [³H]thymidine incorporation. Proliferation is expressed as mean c.p.m. (± S.E.M.) of quadruplicate cultures of four independent experiments. Dose-response curves of CP analogues and parent anginex on bFGF-stimulated HUVEC proliferation are shown.

Figure 6 CP analogues induce apoptosis in ECS

HUVECs were cultured for 2 days in the absence or presence of 25 μM CP analogues or anginex (Ax). Upper panel, apoptosis induction was demonstrated by analysis of subdiploid cells after DNA extraction and staining with PI. Lower panel, total number of dead cells was determined by total number of diploid cells as quantified by FACS analysis. Quantification of the means (± S.E.M.) of four different experiments is shown.

Figure 7 Anginex inhibits *in vitro* angiogenesis in a collagen matrix

Bovine ECs were cultured on gelatin-coated Cytodex-3 beads in a collagen matrix. Sprouting was induced by addition of 20 ng/ml bFGF and 25 μM of the CP analogues or anginex (Ax) was added when tested. Tube formation of a representative experiment under control conditions (A; pixel count 1002) and in the presence of anginex (B; pixel count 211), CP-1 (C; pixel count 205) and CP-6 (D; pixel count 513) is shown. CP-7 had a pixel count of 990. Mean size of the beads is 170 ± 40 μm. (E) Quantification of the means (± S.E.M.) of four different experiments.

than anginex (Figure 6A). Since apoptosis in this assay is defined as those subdiploid cells with a minimum of 10% of the DNA content of the diploid cells, advanced or late stage apoptosis may result in less than 10% of that value. Consequently, the total number of dead cells was also quantified (Figure 6B). The loss of ECs in G₂ and M (4n DNA) phases of the cell cycle is approx. 10% or less (results not shown). The correlation between apoptosis induction and anti-proliferative effects suggests that CP analogues, like anginex, regulate EC growth by forcing ECs to undergo apoptosis.

***In vitro* angiogenesis assay**

Since angiogenesis is a complex process, which, aside from EC proliferation, depends on cell migration and differentiation, the

effects of CP analogues on angiogenesis were investigated in an *in vitro* collagen matrix-based sprout formation assay [13–15]. Figures 7(A–D) show raw data from this angiogenesis assay, and Figure 7(E) gives the percentage of sprouting relative to control, as detailed in the Figure legend and the Experimental section. Although all CP analogues were able to inhibit sprout formation to various extents, the best responses, comparable with those from anginex, were observed with CP-1 and CP-2. It was somewhat surprising that CP-4, CP-5 and CP-6, which were the most effective at inhibiting EC proliferation, were not as effective in this assay. One reason for this could be the complexity of the collagen gel sprouting assay itself. Because these peptides must first navigate through the gel before exerting their effect on ECs, it may be that CP-1 and CP-2 permeate the gel better than other CP analogues or interact somewhat differently with the gel matrix. Alternatively, it may be that the sprouting assay is indeed the more sensitive indicator of anti-angiogenic potential. In any event, bioactivity in this angiogenesis assay decreased most when the β -strand alignment was shifted, as in CP-7 and CP-8, indicating that that specific strand alignment is crucial to maintaining full bioactivity.

Conclusions

In the present study, we demonstrate that the bioactive conformation of the anti-angiogenic anginex peptide is an anti-parallel β -sheet. This finding is consistent with earlier results [8,9] on anginex that in the β -sheet conformation all five hydrophobic residues (Leu⁵, Val¹⁷, Ile²⁰, Val²² and Leu²⁴), which were identified as being crucial to anti-proliferative activity, are proximal and are conformed on the hydrophobic face of the amphipathic β -sheet [9]. Comparison of the three-dimensional structures of several anti-angiogenic proteins, e.g. endostatin [31], PF4 [32], tumour necrosis factor [33] and BPI protein [34], provides a higher level of structural commonality among anti-angiogenic proteins in that they are comprised primarily of anti-parallel β -sheet structure. Furthermore, a survey of amino acid sequences from numerous anti-angiogenic proteins reveals that they are compositionally similar, containing numerous hydrophobic and cationic residues. These structural and compositional characteristics, which appear to be functionally important, are embodied in anginex [9]. As structure–function relationships in these and other anti-angiogenic proteins and peptides become known, it will be interesting to see if this is a common feature among anti-angiogenic proteins. Knowing that this β -sheet conformation is that which promotes anti-angiogenic activity will aid in the development of small molecule mimetics of the anginex peptide.

This work was supported by generous support from the National Institutes of Health (NIH CA-96090), the US Department of Defense (Army Grant #DA/DAMD17-99-1-9564), the Dutch Cancer Society, and ActiPep Biotechnology, Inc. N. A. L. was supported by a Whitaker Foundation Graduate Fellowship. NMR instrumentation was provided with funds from the NSF (BIR-961477) and the University of Minnesota Medical School. We are also grateful to Dinesha Walek of the University of Minnesota Microchemical Facility for peptide synthesis.

REFERENCES

- Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1**, 27–31
- Griffioen, A. W. and Molema, G. (2000) Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol. Rev.* **52**, 237–268
- Gupta, S. K. and Singh, J. P. (1994) Inhibition of endothelial cell proliferation by platelet factor-4 involves a unique action on S phase progression. *J. Cell Biol.* **127**, 1121–1127
- Rastinejad, F., Polverini, P. J. and Bouck, N. P. (1989) Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell (Cambridge, Mass.)* **56**, 345–355
- O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H. and Folkman, J. (1994) Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell (Cambridge, Mass.)* **79**, 315–328
- O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R. and Folkman, J. (1997) Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell (Cambridge, Mass.)* **88**, 277–285
- van der Schaft, D. W., Toebes, E. A., Haseman, J. R., Mayo, K. H. and Griffioen, A. W. (2000) Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. *Blood* **96**, 176–181
- Griffioen, A. W., van der Schaft, D. W., Barendsz-Janson, A. F., Cox, A., Struijker Boudier, H. A., Hillen, H. F. and Mayo, K. H. (2001) Anginex, a designed peptide that inhibits angiogenesis. *Biochem. J.* **354**, 233–242
- Mayo, K. H., van der Schaft, D. W. and Griffioen, A. W. (2001) Designed β -sheet peptides that inhibit proliferation and induce apoptosis in endothelial cells. *Angiogenesis* **4**, 45–51
- Mayo, K. H., Ilyina, E. and Park, H. (1996) A recipe for designing water-soluble, β -sheet-forming peptides. *Protein Sci.* **5**, 13001–11315
- Ilyina, E., Roongta, V. and Mayo, K. H. (1997) NMR structure of a *de novo* designed, peptide 33mer with two distinct, compact β -sheet folds. *Biochemistry* **36**, 5245–5250
- Mayo, K. H., Haseman, J. R., Ilyina, E. and Gray, B. (1998) Designed β -sheet-forming peptide 33mers with potent human bactericidal/permeability increasing protein-like bactericidal and endotoxin neutralizing activities. *Biochim. Biophys. Acta* **1425**, 81–92
- van der Schaft, D. W., Dings, R. P., de Lussanet, Q. G., van Eijk, L. I., Nap, A. W., Beets-Tan, R. G., Bouma-Ter Steege, J. C., Wagstaff, J., Mayo, K. H. and Griffioen, A. W. (2002) The designer anti-angiogenic peptide anginex targets tumor endothelial cells and inhibits tumor growth in animal models. *FASEB J.* **16**, 1991–1993
- Dings, R. P., Yokoyama, Y., Ramakrishnan, S., Griffioen, A. W. and Mayo, K. H. (2003) The designed angiostatic peptide anginex synergistically improves chemotherapy and antiangiogenesis therapy with angiostatin. *Cancer Res.* **63**, 382–385
- Dings, R. P. M., van der Schaft, D. W., Hargittai, B., Haseman, J. R., Griffioen, A. W. and Mayo, K. H. (2003) Anti-tumor activity of the novel angiogenesis inhibitor anginex. *Cancer Lett. (Shannon, Irel.)* **194**, 55–66
- Adler, A. J., Greenfield, N. J. and Fasman, G. D. (1973) Circular dichroism and optical rotary dispersion of proteins and polypeptides. *Methods Enzymol.* **27**, 675–735
- Greenfield, N. and Fasman, G. D. (1969) Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry* **8**, 4108–4116
- Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J. and Bax, A. (1995) NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR.* **6**, 277–293
- Brunker, A. T. (1992) X-plor Manual, Yale University Press, New Haven
- Mayo, K. H., Haseman, J., Young, H. C. and Mayo, J. W. (2000) Structure–function relationships in novel peptide dodecamers with broad-spectrum bactericidal and endotoxin-neutralizing activities. *Biochem. J.* **349**, 717–728
- Guex, N. and Peitsch, M. C. (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**, 2714–2723
- Relou, I. A., Damen, C. A., van der Schaft, D. W., Groenewegen, G. and Griffioen, A. W. (1998) Effect of culture conditions on endothelial cell growth and responsiveness. *Tissue Cell* **30**, 525–530
- Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P. and Traganos, F. (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* **13**, 795–808
- van der Schaft, D. W., Wagstaff, J., Mayo, K. H. and Griffioen, A. W. (2002) The antiangiogenic properties of bactericidal/permeability-increasing protein (BPI). *Ann. Med.* **34**, 19–27
- Wild, R., Ramakrishnan, S., Sedgewick, J. and Griffioen, A. W. (2000) Quantitative assessment of angiogenesis and tumor vessel architecture by computer-assisted digital image analysis: effects of VEGF-toxin conjugate on tumor microvessel density. *Microvasc. Res.* **59**, 368–376
- Johnson, W. C. J. (1990) Protein secondary structure and circular dichroism: a practical guide. *Proteins* **7**, 205–214
- Waterhouse, D. V. and Johnson, Jr, W. C. (1994) Importance of environment in determining secondary structure in proteins. *Biochemistry* **33**, 2121–2128
- Hanzawa, H., Shimada, I., Kuzuhara, T., Komano, H., Kohda, D., Inagaki, F., Natori, S. and Arata, Y. (1990) ¹H nuclear magnetic resonance study of the solution conformation of an antibacterial protein, sapecin. *FEBS Lett.* **269**, 413–420
- Selsted, M. E. and Harwig, S. S. (1989) Determination of the disulfide array in the human defensin HNP-2. A covalently cyclized peptide. *J. Biol. Chem.* **264**, 4003–4007

- 30 Bodenhausen, G., Vold, R. L. and Vold, R. R. (1980) Multiple quantum spin echo spectroscopy. *J. Magn. Reson.* **37**, 93–99
- 31 Hohenester, E., Sasaki, T., Olsen, B. R. and Timpl, R. (1998) Crystal structure of the angiogenesis inhibitor endostatin at 1.5 Å resolution. *EMBO J.* **17**, 1656–1664
- 32 Mayo, K. H., Roongta, V., Ilyina, E., Milius, R., Barker, S., Quinlan, C., La Rosa, G. and Daly, T. J. (1995) NMR solution structure of the 32-kDa platelet factor 4 ELR-motif N-terminal chimera: a symmetric tetramer. *Biochemistry* **34**, 11399–11409
- 33 Baeyens, K. J., De Bondt, H. L., Raeymaekers, A., Fiers, W. and De Ranter, C. J. (1999) The structure of mouse tumour-necrosis factor at 1.4 Å resolution: towards modulation of its selectivity and trimerization. *Acta Crystallogr., Sect.D: Biol. Crystallogr.* **55**, 772–778
- 34 Beamer, L. J., Carroll, S. F. and Eisenberg, D. (1997) Crystal structure of human BPI and two bound phospholipids at 2.4 Å resolution. *Science (Washington, D.C.)* **276**, 1861–1864

Received 21 February 2003/22 April 2003; accepted 23 April 2003

Published as BJ Immediate Publication 23 April 2003, DOI 10.1042/BJ20030295