Anginex, a designed peptide that inhibits angiogenesis

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Novel β -sheet-forming peptide 33-mers, β pep peptides, have been designed by using a combination approach employing basic folding principles and incorporating short sequences from the β -sheet domains of anti-angiogenic proteins. One of these designed peptides (β pep-25), named anginex, was observed to be potently anti-angiogenic. Anginex specifically inhibits vascular endothelial cell proliferation and induces apoptosis in these cells, as shown by flow-cytometric detection of sub-diploid cells, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-nick-end labelling) analysis and cell morphology. Anginex also inhibits endothelial cell adhesion to and migration on different extracellular matrix components. Inhibition of angio-

genesis *in vitro* is demonstrated in the sprout-formation assay and *in vivo* in the chick embryo chorio-allantoic membrane angiogenesis assay. Comparison of active and inactive β pep sequences allows structure–function relationships to be deduced. Five hydrophobic residues and two lysines appear to be crucial to activity. This is the first report of a designed peptide having a well-defined biological function as a novel cytokine, which may be an effective anti-angiogenic agent for therapeutic use against various pathological disorders, such as neoplasia, rheumatoid arthritis, diabetic retinopathy and restenosis.

Key words: apoptosis, cytokine, design, endothelium, structure.

INTRODUCTION

Angiogenesis is crucial to numerous biological functions in the body, from normal processes like embryogenesis and wound healing to abnormal processes like tumour growth, arthritis, restenosis and diabetic retinopathy. The use of agents that can inhibit angiogenesis *in vitro* and *in vivo*, particularly in anti-tumour research (e.g. [1,2]), has indicated that anti-angiogenic therapy will be a promising therapeutic modality in the future. The search for angiogenic inhibitors has been focused on controlling two of the processes that promote angiogenesis: endothelial cell (EC) growth and adhesion [3,4], primarily because ECs are more accessible than other cells to pharmacological agents delivered via the blood and ECs are genetically stable and are not easily mutated into drug-resistant variants. Most anti-angiogenic agents have been discovered by identifying endogenous molecules, primarily proteins, that inhibit EC growth. This traditional approach has produced a number of anti-angiogenics, such as platelet factor-4 (PF4) [5], thrombospondin [6], tumour necrosis factor (TNF)- α (depending on its concentration) [7–9], interferon- γ -inducible protein-10 [10], angiostatin [11], endostatin and vasostatin [1,12] and bactericidal-permeability-increasing protein (BPI) [13]. In total, about 40 anti-angiogenic agents, identified using various approaches, are currently known.

A survey of amino acid sequences from anti-angiogenic proteins reveals that they are compositionally similar, containing numerous hydrophobic and cationic residues. Comparison of the three-dimensional structures of several anti-angiogenic proteins, e.g. endostatin [14], PF4 [15], TNF [16] and BPI [17], provides a higher level of structural commonality, in that they are comprised

primarily of anti-parallel β -sheet structure. Another biological function common to at least two of these proteins, PF4 [18] and BPI ([19] and references therein), is their bactericidal activity. In fact, BPI was first identified and is better known for its ability to kill bacteria and to neutralize the bacterial endotoxin lipopolysaccharide. These structural and compositional characteristics, therefore, appear to be functionally important to both anti-angiogenic and bactericidal activities, which may suggest an evolutionary link. The selection of novel anti-angiogenic proteins or peptides may be guided by considering all these points: composition high in hydrophobic and cationic residues, anti-parallel β -sheet structure and bactericidal activity. In this regard, β pep peptides, which are designed amphipathic β -sheet-forming peptide 33-mers [20,21] and are bactericidal [18], are obvious candidates.

β pep peptides were designed by using a combination approach employing basic folding principles and incorporating short sequences from the β -sheet domains of PF4, interleukin (IL)-8 and BPI [20]. Initially, the β -sheet domains from α -chemokines PF4 and IL-8 were used in part to create a presentation scaffold whose solubility in aqueous solution and structural stability were significantly enhanced using basic folding principles [20]. From the β -sheet domain of BPI (residues 82–108), a few residues thought to be key to bactericidal activity [19] were designed into β pep peptides, which were then shown to be, to varying degrees, bactericidal and capable of neutralizing lipopolysaccharide [18]. In BPI, this sequence of residues (82–108) is conformed in an amphipathic, anti-parallel β -sheet containing a large loop [17], and the solvent-exposed surface is cationic. NMR structural studies demonstrate that β pep peptides self-associate as dimers

Abbreviations used: EC, endothelial cell; HUVEC, human umbilical vein EC; bFGF, basic fibroblast growth factor; IL, interleukin; PF4, platelet factor-4; BPI, bactericidal-permeability-increasing protein; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-nick-end labelling; TNF, tumour necrosis factor; CAM, chorio-allantoic membrane.

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and tetramers whose monomer subunits are folded amphipathically in a three-stranded anti-parallel β -sheet motif [21]. Whereas the interior of the β pep β -sheet sandwich tetramer is composed primarily of aliphatic hydrophobic residues, the solvent-exposed surface displays a high net positive charge. The β pep peptide fold, although structurally similar, is different from those of the β -sheet domains of α -chemokines and BPI, and is also similar to those folds found in a number of other β -sheet-structured cytokines [22]: IL-1 [23], TNF [24], lymphotoxin (LT or TNF- β) [25] and transforming growth factor- β [26].

With the hypothesis that β pep peptides might elicit angiostatic activity, the present study was aimed at assessing their ability to inhibit EC proliferation and angiogenesis. From a series of 30 β pep peptides, β pep-25 has been identified as a potent inhibitor of EC growth and angiogenesis. We call this peptide anginex. Anginex, which is more effective at inhibiting EC growth than PF4 and several other well-known angiogenesis inhibitors, such as angiostatin, endostatin, AGM-1470 and thrombospondin-1, acts by specifically blocking adhesion and migration of angiogenically activated ECs, leading to apoptosis and ultimately to inhibition of angiogenesis *in vitro* and *in vivo*. Sequence specificity is illustrated by results with a 91% similar and 67% identical peptide, β pep-28, which displays no anti-angiogenic activity at the concentrations investigated. Anginex has the potential to become a potent therapeutic agent against various pathological disorders like arthritis, restenosis, retinopathy and tumour growth.

EXPERIMENTAL

Peptide synthesis

Peptides were synthesized using a Milligen/Bioscience 9600 peptide solid-phase synthesizer using fluorenylmethoxycarbonyl chemistry. Lyophilized crude peptides were purified by preparative reversed-phase HPLC on a C_{18} column with an elution gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid in water. Pure peptides were subjected to gel filtration using a 1 cm $\times$ 9 cm Sephadex G-25 column and pyrogen-free saline buffered with 0.008 M citrate/phosphate buffer, pH 7.0, as elution buffer, and were used to confirm results obtained at every step of the study. The purity and composition of the peptides were verified by HPLC (Beckman Model 6300) analysis of amino acid composition of hydrolysates prepared by treating the peptides under argon in 6 M HCl for 24 h at 110 °C. The amino acid sequences of peptides were confirmed by N-terminal sequencing and MS.

NMR

For NMR measurements, freeze-dried peptide was dissolved in $H_2O/{}^2H_2O$ (9:1). Peptide concentration was 10 mg/ml. pH was adjusted by adding millilitre quantities of $NaO{}^2H$ or 2HCl to the peptide sample. NMR spectra were acquired on a Varian UNITY Plus-800 NMR spectrometer. The water resonance was suppressed by direct irradiation (0.8 s) at the water frequency during the relaxation delay between scans. Data were processed using VXRNMR software directly on the spectrometer console or remotely on an SGI workstation.

Two-dimensional homonuclear magnetization transfer [homonuclear Hartman–Hahn spectroscopy (HOHAHA)] spectra, obtained by spin-locking with a MLEV-17 sequence [27] and a mixing time of 60 ms, were used to identify spin systems. Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiments [28] were performed for conformational analysis. All two-dimensional NMR spectra were acquired in the TPPI

(time-proportional phase incrementation) [29] or States [30] phase-sensitive mode. The water resonance was suppressed by direct irradiation (0.8 s) at the water frequency during the relaxation delay between scans as well as during the mixing time in NOESY experiments. Two-dimensional NMR spectra were collected as 256–400 t1 experiments, each with 1024 or 2048 complex data points over a spectral width of 6 kHz in both dimensions, with the carrier placed on the water resonance. HOHAHA and NOESY spectra, normally 16 and 64 scans respectively, were time-averaged per t1 experiment. Data were processed directly on the spectrometer or offline using FELIX (Molecular Simulations) on an SGI workstation. Data sets were multiplied in both dimensions by a 30–60° shifted sine-bell function and zero-filled to 1024 in the t1 dimension prior to Fourier transformation.

Cells, cultures and reagents

Human umbilical vein ECs (HUVECs) were harvested from normal human umbilical cords by perfusion with 0.125% 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]. Human microvascular ECs were isolated from human foreskins and cultured in culture medium supplemented with ECGS (endothelial cell-growth supplement; 15 μ g/ml, Boehringer Mannheim, Mannheim, Germany). Before use in *in vitro* assays human microvascular ECs were starved for 48 h in culture medium without ECGS. Bovine capillary ECs were kindly provided by Dr M. Furie, State University of New York, Stony Brook, NY, U.S.A. Bovine capillary ECs were cultured in fibronectin-coated tissue-culture flasks in RPMI 1640 medium containing 10% fetal calf serum, glutamine and antibiotics. For isolation of recombinant PF4, the synthetic gene for human native PF4 was expressed as a non-fusion protein in *Escherichia coli* (BL21) cells (Repligen Corp., Cambridge, MA, U.S.A.). The protein was purified, cleaved and refolded essentially as described previously [31]. Purity was assessed by Coomassie Brilliant Blue staining of SDS/PAGE, analytical C_4 reversed-phase HPLC and amino acid analysis. Typically, several hundred milligrams of material (>95% pure) was isolated from 100 g of starting material.

Proliferation measurement

EC proliferation was measured using a [3H]thymidine incorporation assay. ECs were seeded at 5000 cells/well in flat-bottomed, 96-well tissue-culture plates and grown for 3 days, in the absence or presence of regulators, in culture medium. During the last 6 h of the assay, the culture was pulsed with 0.5 μ Ci of [$Me-{}^3H$]thymidine/well. Results are expressed as the mean c.p.m. values ($\pm$ S.E.M.) from triplicate cultures.

Apoptosis measurement

HUVECs were cultured in fibronectin-coated tissue-culture flasks in culture medium [32]. Apoptosis was measured by determination of sub-diploid cells after DNA extraction and subsequent staining with propidium iodide as described before [33]. In short, HUVECs were cultured for 3 days in the presence of 10 ng/ml basic fibroblast growth factor (bFGF) under the conditions mentioned above. Serum deprivation of ECs was used as a positive control for apoptosis. Cells were then harvested and subsequently fixed and permeabilized in 70% ethanol at –20 °C. After at least 2 h the cells were spun down and resuspended in

DNA extraction buffer (45 mM Na_2HPO_4 /2.5 mM citric acid/0.1% Triton X-100) for 20 min. Propidium iodide was added to a final concentration of 20 $\mu\text{g}/\text{ml}$ and red log-scale fluorescence was analysed on the FACScalibur flow cytometer. Apoptosis was quantified as the percentage of cells with propidium iodide fluorescence below the fluorescence of cells in the G_0/G_1 (diploid) phase of the cell cycle. Cells with propidium iodide fluorescence less than 10% of the cells in the G_0/G_1 phase were regarded as cell fragments and were excluded from apoptosis measurements. TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-nick-end labelling) staining of cells was performed by immunocytochemistry on cytospin preparations of cultured cells, according to the manufacturer's instructions (Mebstain Apoptosis kit, Immunotech, Marseille, France).

Cell adhesion and migration

HUVEC cultures were exposed to 100 $\mu\text{g}/\text{ml}$ $\beta\text{pep-25}$ (anginex) or culture medium (see above) alone. Following 24 h in culture, cells were harvested by trypsinization and washed twice in culture medium. Cells were allowed to adhere for time periods from 10 to 120 min in fresh 96-well culture plates coated with matrix components, vitronectin, fibronectin, collagen or hyaluronic acid, as described previously [32]. At the indicated time points, non-adherent cells were washed away and adherent cells were enumerated under an inverted microscope. EC migration was measured in the wound assay. EC cultures were grown to confluence and wounds were made in the monolayer by removing cells with a sterile glass bar. Cultures were washed and the medium was replaced by fresh medium containing 10 ng/ml bFGF and incubated with or without inhibitors. Wound width was measured in triplicate cultures at four predefined locations at the start and at 2, 4, 6, 8 and 24 h after addition of the inhibitors. Student's *t* test was used to determine statistical significance of observed differences.

FACS analysis of endothelial adhesion molecule expression

Immunofluorescence using indirect phycoerythrin-conjugated reagents required three separate incubations. HUVECs (1×10^5 cells; cultured for 24 h with 10 $\mu\text{g}/\text{ml}$ $\beta\text{pep-25}$ or culture medium alone and harvested following incubation with 0.125% trypsin in PBS) were washed, fixed for 1 h in 1% paraformaldehyde, resuspended in 20 μl of appropriately diluted monoclonal antibody and then incubated for 1 h on ice. Subsequently, cells were washed twice in PBS/0.1% BSA and incubated for another 30 min with biotinylated rabbit anti-mouse IgG (Dako, Glostrup, Denmark). After another two washings, cells were incubated with streptavidin-phycoerythrin conjugate (Dako). Stained cells were analysed on a FACScalibur flow cytometer. For each sample, forward scatter, side scatter and fluorescence signals from 5000 cells were recorded. Data analysis was performed using CellQuest software (Becton Dickinson, Mountain View, CA, U.S.A.).

In vitro angiogenesis assay

A semi-natural matrix of collagen type I was prepared by mixing 8 vol. of vitrogen 100 (Collagen Corporation, Fermtom, CA, U.S.A.) with 1 vol. of $10\times$ concentrated minimal essential medium (Life Technologies) and 1 vol. of sodium bicarbonate (11.76 mg/ml). The matrix was dispensed into culture plates and allowed to gel at 37 °C. Confluent bovine capillary ECs were trypsinized and seeded on top of this collagen matrix. When cells were grown to a confluent monolayer, the medium was replaced

by fresh medium, containing 25 ng/ml bFGF or medium containing 25 ng/ml bFGF and the peptides. Aside from experiments that added the angiogenesis inducer on top of the matrix, other experiments had colon cell line (LS174T) spheroids embedded in the collagen matrix prior to gelation in order to use a more realistic tumour-release model. Peptides were added to the culture simultaneously with the ECs. In both experiments, after 48 h of incubation the (sprouting) endothelial monolayers were photographed with a Zeiss inverted phase-contrast photo microscope. The amount of sprouting in each well (i.e. the total length of the sprouts) was quantified by the computer program NIH Image [34]. Student's *t* test was used to determine statistical significance of observed differences.

Chorio-allantoic membrane (CAM) assay

Fertile Lohman-selected white leghorn eggs were incubated for 3 days at 37 °C and 55% relative humidity and rotated once every hour. On day 3, a rectangular window (1 cm $\times$ 2 cm) was made in the eggshell. The window was covered with tape to prevent dehydration. The window allowed undisturbed observation of the developing vasculature of the CAM. On day 7 a silastic ring (10 mm diameter) was placed on the CAM to allow local drug administration within the ring. Peptides $\beta\text{pep-25}$ (anginex) and $\beta\text{pep-28}$ dissolved in sterile saline (0.9% NaCl) were applied daily in aliquots of 65 μl from day 10 to day 13. On day 14 the CAMs were photographed. Photographs were scanned and stereological principles were applied according to described methods. Three concentric rings were projected on the image. The number of intersections of rings and blood vessels was determined and used as a measure of vessel density. Student's *t* test was used to determine statistical significance of observed differences.

RESULTS

The amino acid sequences for βpep peptides are given in Figure 1 along with sequences from the β -sheet domains of IL-8, PF4 and BPI. These βpep , IL-8, PF4 and BPI peptides were tested for their ability to inhibit the proliferation of growth-factor-induced (bFGF, 10 ng/ml) HUVECs using the [^3H]thymidine incorporation assay. Whereas IL-8, PF4 and BPI peptides were inactive at the highest concentration tested (25 μM), a number of βpep peptides were inhibitory at both the 25 μM (100 $\mu\text{g}/\text{ml}$) and 2.5 μM (10 $\mu\text{g}/\text{ml}$) doses (Table 1). $\beta\text{pep-25}$ (anginex) was the most active, inhibiting approx. 50% EC growth at 2.5 μM . Similar results were found for vascular endothelial growth factor-stimulated and spontaneous proliferation of ECs. This being the case, the remainder of this paper focuses on the anti-angiogenic effects and mechanism of action of $\beta\text{pep-25}$. Another βpep peptide, $\beta\text{pep-28}$, was chosen as a good negative control, because it showed no EC-growth-inhibitory potency at 25 μM and was most similar to and identical with $\beta\text{pep-25}$.

For assurance that $\beta\text{pep-25}$ and $\beta\text{pep-28}$ do indeed fold as β -sheets and to avoid the argument that indirect effects may account for activity differences, CD and ^1H -NMR spectra were acquired with both peptides. $\beta\text{pep-28}$ shows well-defined, dispersed and downfield-shifted NH and αH resonances, as expected for a β -sheet-forming peptide (Figure 2) and as observed with other βpep peptides [18,20]. Since βpep peptides in general exist in solution in a monomer/dimer/tetramer equilibrium [20,35], pulsed-field gradient NMR self-diffusion measurements (results not shown) were performed which indicate the presence

B/PI	ANIKISGKWKAAQKRFLKMSGNFDLSIEGMSISAD
PF4	RHITSLEVIKAGPHSPTAQLIATLK..NGRKISLD
IL-8	KFIKELRVIESGPHSANTEIIVKLS..DGRELSLD
β pep-1	KFIVTLRVIKAGPHSPTAQIIVELEK..NGRKISLD
β pep-2	ANIKLSVEMKLFKRHLKWKIIVKLN..DGRELSLD
β pep-4	SIQDLNVSMKLFKRQAKWKIIVKLN..DGRELSLD
β pep-5	HIKELQVKWKAQKRF LKM SIIVKLN..DGRELSLD
β pep-7	HIKELQVKMKAQKRF LKW SIIVKLN..DGRELSLD
β pep-8	ANIKLSVKWKAQKRF LKMSINVDLS..DGRELSLD
β pep-11	SIQDLNVSMKLFKRQAKWKINVKLN..DGRELSLD
β pep-14	SIQDLNVSMKLFKRQAKWKIIVKLN..DGRELSLD
β pep-15	SIQDLNVSMKLFKRQAKWKIILKLN..DGRELSLD
β pep-16	SIQDLNVSMKLFKRQAKWKIIAKLN..DGRELSLD
β pep-17	SIQDLNVSMKLFKRQAKWKILVKLN..DGRELSLD
β pep-18	SIQDLKVSMLKLFKRQAKWKIIVKLN..DGRELSLD
β pep-19	SIQKLVSMKLFKRQAKWKIIVKLN..DGRELSLD
β pep-20	SIQDLNVSMKLFKRQAKWKIIVKLN..DGRELSLD
β pep-21	SIQDLNVSLKLFKRQAKWKIIVKLN..DGRELSLD
β pep-22	SIQDLNLSMKLFKRQAKWKIIVKLN..DGRELSLD
β pep-23	SIQDLKVSMLKLFKRQAKWKIIVKLN..DGRELSLD
β pep-24	SIQFLKVS LNL DRKQAKWKIIVKLN..DGRELSLD
β pep-25	ANIKLSVQMKLFKRHLKWKIIVKLN..DGRELSLD
β pep-26	SIQDLNVSMKLFKRQAKWKIIVKLN..DGRELSLD
β pep-27	SIQDLNVSMKLFKRQAKWKAIVKLN..DGRELSLD
β pep-28	SIQDLNVSMKLFKRQAKWKIVKLN..DGRELSLD
β pep-29	SIQDLNVSMKLFKRQAKWKILKLN..DGRELSLD
β pep-30	SIQDLNVSMKLFKRQAKWKVIKLN..DGRELSLD

Figure 1 Amino acid sequences for β pep peptides, and for the β -sheet domains from PF4, IL-8 and BPI

Sequences are aligned to the longer BPI peptide, and a gap has been left in the other sequences where one residue is absent relative to the BPI sequence.

Table 1 The library of β pep peptides tested for inhibition of EC proliferation

HUVECs were cultured for 3 days with the indicated concentrations of β pep peptides. During the last 6 h cultures were pulsed with 0.5 μ Ci/well [3 H]thymidine. Results (means $\pm$ S.E.M. from three independent experiments performed in triplicate) are percentages of c.p.m. relative to control cultures without peptide.

β pep peptide	β pep concentration ...	Percentage c.p.m. relative to control cultures	
		2.5 μ M	25 μ M
β pep-1		119 $\pm$ 15	79 $\pm$ 9
β pep-2		71 $\pm$ 17	8 $\pm$ 1
β pep-4		119 $\pm$ 4	100 $\pm$ 6
β pep-5		84 $\pm$ 9	29 $\pm$ 3
β pep-7		72 $\pm$ 8	13 $\pm$ 1
β pep-8		69 $\pm$ 4	6 $\pm$ 2
β pep-11		76 $\pm$ 10	39 $\pm$ 7
β pep-16		87 $\pm$ 3	103 $\pm$ 4
β pep-20		78 $\pm$ 11	34 $\pm$ 1
β pep-21		71 $\pm$ 6	22 $\pm$ 1
β pep-24		86 $\pm$ 9	26 $\pm$ 5
β pep-25		46 $\pm$ 2	12 $\pm$ 5
β pep-30		98 $\pm$ 9	76 $\pm$ 9

of mostly tetramers and dimers for β pep-28 and β pep-25 at a peptide concentration of 2 mM. Even though CD data (results not shown) demonstrate the presence of significant populations

Figure 2 NMR data on β pep-25 and β pep-28

The overall β -strand alignment in monomers is shown for β pep-25 and β pep-28 (upper left panel). Horizontal lines between strands indicate intra- and inter-monomer α H- α H alignments. Where two residues are shown, the first is for β pep-25 and the second is for β pep-28. Lower panel: 1 H-NMR spectra (800 MHz) are shown for β pep-25 (anginex) and β pep-28 in H_2O/D_2O (9:1). The peptide concentration was 10 mg/ml in 20 mM potassium phosphate, pH 6.3, at 40 °C. Spectra were accumulated with 8192 data points over 6000 Hz sweep width and were processed with 2 Hz line broadening. Only the spectral region downfield from the water resonance is shown. In the NOESY spectrum in the upper right panel, several cross-peaks have been boxed-in and/or labelled to indicate intra- and inter-monomer α H- α H nuclear Overhauser effects (NOEs) that have been used to indicate β -strand alignments. The boxes are labelled with two numbers separated by a slash to indicate which residues in the sequence are involved.

of β -sheet in both β pep-28 and β pep-25, aggregate exchange of β pep-25 monomers/dimers/tetramers falls within the intermediate exchange regime on the chemical-shift time scale resulting in substantial resonance broadening (see Figure 2). This is particularly apparent for resonances arising from the less populated tetramer state. One very broad α H resonance envelope from the tetramer state, for example, is observed between 4.8 and 5.6 p.p.m. Therefore it is evident that β pep-25 forms weaker dimer-dimer subunit associations than β pep-28. Structurally, NOESY data on β pep-28 (Figure 2, upper right panel) show the presence of α H- α H nuclear Overhauser effects, indicative of the same anti-parallel β -sheet strand alignment observed in other β pep peptides [21]. In fact, in all other β pep peptides that give similarly good NOESY spectra, this same overall β -sheet fold is conserved. Since β pep-25 is 91% sequentially similar to and 67% sequentially identical with β pep-28, it is most probable that these two peptides also share the same overall β -sheet fold. Figure 2 (upper-left panel) gives the amino acid sequences for both β pep-25 and β pep-28, along with the β -sheet folding pattern for a monomer subunit within the tetramer state [21]. In the biological assays discussed below, the β pep peptide concentration is much lower than that used in these NMR structural studies. At these lower peptide concentrations, β pep tetramers dissociate into dimers and monomers [35]. Since CD spectra indicate that in both lower aggregate states some β -sheet structure is still present [35], this same folding pattern (Figure 2) may be conserved. However, this remains unknown.

Figure 3 $\beta\text{pep-25}$ specifically inhibits growth-factor-induced EC proliferation

Proliferation of bFGF-stimulated (10 ng/ml) EC cultures was measured by quantification of [^3H]thymidine incorporation. Proliferation is expressed as mean c.p.m. ($\pm$ S.E.M.) of quadruplicate cultures from three independent experiments. (A) Dose-response curves of $\beta\text{pep-25}$ and $\beta\text{pep-28}$ on bFGF-stimulated HUVEC proliferation. Dose-response curves of $\beta\text{pep-25}$ and $\beta\text{pep-28}$ on human microvascular ECs (B), bovine capillary ECs (C), normal human fibroblasts (D) and breast cancer cell line HS578T (E). Additional growth factors were not used to stimulate fibroblast and tumour cell cultures. IP-10, interferon- γ -inducible protein-10.

Growth-inhibitory effect from $\beta\text{pep-25}$ is specific for EC

To demonstrate that $\beta\text{pep-25}$ (anginex) specifically inhibits the growth of endothelial cells, Figure 3 gives a more complete $\beta\text{pep-25}$ dose response for growth-inhibitory potency against HUVECs (Figure 3A), human microvascular ECs (Figure 3B), bovine capillary ECs (Figure 3C), normal human foreskin-derived fibroblasts (Figure 3D) and breast cancer cell line HS578T

(Figure 3E). Human endometrium-derived fibroblasts, smooth-muscle cells and phytohaemagglutinin-stimulated peripheral blood leucocytes, as well as various other cancer cell lines, were also tested (results not shown). $\beta\text{pep-25}$ was inhibitory only to the growth of EC. In this dose-response curve, $\beta\text{pep-25}$ showed a half-maximal inhibitory effect at approx. 1.5 μM , close to the value of 2.5 μM reported above. In all cases, negative control peptide $\beta\text{pep-28}$ was ineffective, as expected. In this same series

Figure 4 β pep-25 induces apoptosis in cultured human endothelial cells

The left panels show HUVECs cultured for 2 days with or without 100 μ g/ml β pep-25. Apoptosis induction was demonstrated by analysis of sub-diploid cells after DNA extraction and staining with propidium iodide, and the panel on the right shows the time-response curve of HUVECs cultured for 3 days in the presence of 1% serum ($\bullet$), β pep-25 ($\blacksquare$), β pep-26 ($\blacktriangledown$), β pep-28 ($\blacklozenge$), PF4 ($*$) or medium alone ($\blacktriangle$). The percentage of apoptotic cells was quantified as the percentage of sub-diploid cells, as described in the Experimental procedures section.

of HUVEC proliferation experiments, various angiogenesis inhibitors demonstrated half-maximal inhibitory effects: endostatin (no effect detected), PF4 (4 μ M), AGM-1470 (50 μ M) and interferon- γ -inducible protein-10 (3 μ M). The absence of any inhibitory effect from endostatin was surprising. Addition of an equimolar amount of low-molecular-mass heparin to the endostatin solution did cause a small inhibitory effect at 25 μ M, but the effect was minimal, with an approx. 20% decrease in EC growth. In addition, the kinetics of β pep-25 inhibition of EC proliferation were the same as those for PF4 and the other angiogenic inhibitors, with half-maximal inhibition at 24–36 h and maximal inhibition occurring after 2–3 days of culture (results not shown). Although results in Figure 3 were generated using bFGF-stimulated ECs, essentially the same results were obtained using vascular endothelial growth factor-stimulated ECs and spontaneous proliferation.

β pep-25 mechanism of action

β pep-25 (anginex) clearly inhibited EC growth. This inhibition could occur via two main routes: (i) cytostatically, for example, by arresting ECs in the S phase as observed for PF4 [5], or (ii) cytotoxicity by directly or indirectly inducing EC death. Since detachment from the matrix, shrinkage and cell death were observed in EC proliferation assays (Figure 4), β pep-25 functions cytotoxicity. The question then arose as to whether mortality was mediated by the induction of apoptosis. Apoptosis, most easily quantified by analysis of DNA fragmentation following DNA extraction and propidium iodide staining, was measured in HUVEC cultures with and without β pep-25 or β pep-28. Serum deprivation, which is known to induce apoptosis in ECs, was used as a positive control. Although already measurable after 24 h, profound apoptosis-mediated DNA fragmentation by β pep-25 was observed after 48 h of culture (Figure 4). Apoptosis reached maximal levels of approx. 80–90% of the cells after 3 days, as indicated in the time course shown in Figure 4. The magnitude of the response was similar to the induction of apoptosis by starvation in medium containing low serum concentration. The negative control peptide, β pep-28, failed to

Table 2 TUNEL analysis of HUVECs

Percentage of TUNEL-positive HUVECs of a representative experiment out of three is shown. NM, not measurable due to excessive progressed cell death.

	Percentage of TUNEL-positive HUVECs	
	24 h	3 days
Medium	2.8	1.0
25 μ M PF4	3.6	1.5
25 μ M β pep-28	1.9	0
25 μ M β pep-25	4.9	37.4
Starvation (1% serum)	26.2	NM

induce apoptosis. As anticipated from earlier reports [5], native PF4, being cytostatic towards ECs, also did not induce apoptosis. The apoptosis-inducing capacity of β pep-25 was confirmed by TUNEL analysis (Table 2) and by apoptotic morphology (results not shown) on cytospin preparations of cultured cells. Although the number of apoptotic cells seems lower when assessed by the TUNEL assay, which is primarily due to the loss of cells in advanced stages of apoptosis in the cytospin procedure, these results confirm the induction of apoptosis by β pep-25.

Apoptosis can be induced in a number of ways. For example, direct binding to the integrin receptor $\alpha_v\beta_3$, present on the cell surface of ECs, can trigger the apoptotic cascade [36,37]. Alternatively, since ECs must adhere to and migrate on components of the extracellular matrix during angiogenesis, prevention of EC attachment to matrix components (anoikis) [38,39] will induce apoptosis. These studies indicate that β pep-25 functions using the latter pathway. Even though β pep-25 does not directly prevent attachment of HUVECs to matrix components, such as collagen, fibronectin, vitronectin or hyaluronan, which were immobilized on tissue-culture plastic plates, exposure of HUVECs to 100 μ g/ml β pep-25 for 24 h did lead to a significant suppression of adhesion to all matrix components tested. This is exemplified with vitronectin in Figure 5(A). The negative control peptide β pep-28 had no effect. These results

Figure 5 βpep-25 inhibits EC adhesion and migration

(A) HUVECs were cultured for 24 h in the absence (▲) or presence (■) of 100 μg/ml βpep-25. Harvested cells were allowed to adhere to vitronectin-coated tissue-culture plastic. Non-adherent cells were washed away and percentages of adhered cells were determined by counting under an inverted microscope. (B) Migration of ECs was determined by wound assays. Confluent HUVEC cultures were wounded. Subsequently, wound width was measured at certain time points. Migration of ECs is inhibited by βpep-25 (▲) as compared with untreated cells (■). Means ± S.E.M. from three different experiments are shown. Asterisks represent statistical difference from control experiments performed in the absence of βpep-25 ($P < 0.01$). (C) FACS analysis of endothelial adhesion molecules. HUVECs were exposed to 100 μg/ml βpep-25 or medium alone in control cultures for 24 h. Data are from one representative experiment out of four and are expressed as mean fluorescence intensity from 5000 cells.

suggest that βpep-25 induces the down-regulation of adhesion receptors, rather than directly blocking interactions between matrix and EC adhesion molecules. This was confirmed by the observation that βpep-25 acts to down-regulate EC adhesion molecules α₂β₁-, α₅β₁- and α_vβ₃-integrins and CD44 (Figure 5C).

To assess the effect of βpep-25 (anginex) on EC migration *in vitro*, the wound assay was used. On vitronectin-coated tissue-culture plastic plates, EC migration was markedly inhibited, but not completely blocked, by the presence of βpep-25 (Figure 5B). Whereas in control wounds ECs migrated to confluency within 8 h, cultures treated with βpep-25 reached confluency only after at least 24 h. Maximal inhibition of greater than 50% was observed after 2 h. Again, βpep-28 demonstrated no effect on EC migration. All βpep peptides that inhibited EC proliferation had a proportional effect on EC migration, suggesting a direct correlation between these two processes (results not shown). EC migration on tissue-culture plastic-coated plates with collagen, fibronectin, vitronectin or hyaluronan showed similar results (not shown).

βpep-25 inhibits angiogenesis *in vitro* and *in vivo*

Since angiogenesis is a complex process which, aside from EC proliferation, depends on cell migration and differentiation, the effects of βpep peptides on angiogenesis were initially investigated in an *in vitro* collagen-gel-based sprout-formation assay. The same βpep peptides that inhibit EC proliferation also inhibited sprout formation. Again, of all βpep peptides, βpep-25 (anginex) demonstrated the greatest inhibition of angiogenesis. At 250 nM (1 μg/ml), βpep-25 inhibited greater than 40% of sprout formation, increasing to 80% at 25 μM (100 μg/ml, Figure 6). PF4 is also known to inhibit sprout formation [40]. The kinetics of inhibition for βpep-25 were the same as those for PF4. These results were obtained from experiments using bovine ECs where sprout formation was induced with bFGF. Similar results were

observed when human breast cancer spheroids were used in the gel as the angiogenic stimulus [41] to induce sprout formation in human foreskin-derived microvascular ECs. In all assays where sprout formation was induced, βpep-25 was not cytotoxic for EC growing in monolayers, suggesting specificity for angiogenically activated ECs.

To study the inhibition of angiogenesis *in vivo*, the chick embryo CAM assay was used. This assay, which measures developmental angiogenesis, is performed routinely prior to use of an agent in *in vivo* tumour growth models. CAMs were studied in intact fertilized eggs where windows of 1 cm × 2 cm were made on day 3 of development. A flexible silicon ring, into which compounds to be tested could be pipetted, was placed on the CAM at day 7, and treatment was initiated. In CAMs treated with βpep-25 (3 μg/ml) daily between days 10 and 13 of development, a profound inhibition of microvessel formation was observed, whereas larger, pre-existing vessels were apparently unaffected. Figure 7 shows the development of CAM vasculature at day 14 after fertilization and treatment with βpep-25 or vehicle alone. Vessel counts (see the Experimental section) after treatment with βpep-25 were 29.3 ± 3.5 compared with 54.6 ± 4.1 (means ± S.E.M.) for treatment with vehicle alone. Inhibition of vessel density was 46.3% (S.D. ± 5.9%, $n = 5$; $P < 0.002$). Treatment with βpep-28 or vehicle alone resulted in normal vascular development similar to that observed with untreated CAMs.

DISCUSSION

The design of novel peptides is a powerful tool in the development of biologically active molecules. Anginex (βpep-25) is one such designer peptide with a clear biological function as a cytokine-like agent that specifically inhibits EC proliferation and angiogenesis. Because anginex induces apoptosis and causes cell death in ECs, it is cytotoxic. Anginex is specific for angiogenically

Figure 6 *In vitro* angiogenesis is inhibited by β pep-25

Unstimulated bovine microvascular ECs (**A**) form tubes in a collagen gel upon stimulation with bFGF (**B**). This bFGF-induced stimulation is inhibited by β pep-25 (**C**), but not by β pep-28 (**D**). Moderate inhibition was found with β pep-24 (**E**). (**F**) Demonstrates that sprout formation is concentration-dependent (the panel shows the results of one representative experiment out of four). Inhibition of sprouting at 25 μ M (100 μ g/ml), relative to PF4, is quantified in (**G**). Asterisks represent statistical significance (mean sprouting $\pm$ S.E.M., $n = 6$, * $P < 0.05$, ** $P < 0.01$).

activated ECs. Resting ECs, as found in the confluent monolayer in the sprout-formation assay, are apparently unaffected by the presence of anginex. This is important for any agent that may eventually be used therapeutically, because it suggests that anginex should not act on quiescent ECs in normal vasculature *in vivo*.

Mechanistically, anginex inhibits EC adhesion to and migration on protein components of the extracellular matrix, an event that subsequently induces apoptosis in the ECs. Anginex, therefore, is cytotoxic towards ECs. This apoptotic process is specific for angiogenically activated EC and suggests a role for endothelial (cell-surface) adhesion molecules that are up-regulated during EC proliferation. Electron-microscopic evidence demonstrates that the peptide is present on the matrix-binding ('abluminal') side of ECs after 24 h of culture (results not shown). Integrins are one possibility for these endothelial adhesion molecules. For example, $\alpha_v\beta_3$ -integrin production is induced upon growth stimulation in ECs and is therefore expressed specifically in vessels of angiogenically stimulated tissues such as tumours [37]. Expression of CD44 and a number of other matrix-binding integrins, mainly β_1 -integrins, are also found to be elevated on angiogenically activated EC [42,43].

Figure 7 Angiogenesis in the chick CAM is inhibited by β pep-25

Development of vasculature in chick embryo CAMs (vehicle alone in **A**) is inhibited by 3 μ g/ml β pep-25 (**B**). CAMs treated with the control peptide β pep-28 developed similarly to untreated CAMs (vehicle alone). Solutions of peptide (65 μ l, 3 μ g/ml) were added on days 10–13. Photographs represent CAM development on day 14. Scale: the silicon ring has a diameter of 10 mm.

However, since it was not possible to block the binding of anti-integrin ($\alpha_v\beta_3$, $\alpha_2\beta_1$ and $\alpha_5\beta_1$) antibodies with anginex and vice versa, none of these integrins could be identified as the receptor for anginex. Binding to integrins often requires an RGD motif on the peptide ligand. Anginex, however, does not contain the RGD tripeptide, but does contain the reverse tripeptide DGR. It is unknown whether this reverse sequence can promote RGD-type activity. Moreover, DGR is also present in the negative control peptide β pep-28, supporting the conclusion that this sequence does not contribute to the angiostatic effect from anginex. RGD is not the only integrin-binding sequence. For example, an endogenous non-catalytic fragment of matrix metalloproteinase-2 called PEX that is also devoid of the RGD motif was shown to bind to $\alpha_v\beta_3$ -integrin [44]. Therefore the absence of RGD does not preclude binding to integrins. In any event, since anginex affects adhesion to and migration on a

number of extracellular matrix components, the peptide may in fact be interacting with more than one endothelial adhesion molecule.

The protein BPI functions similarly to anginex [13]. In itself, this initially may have been expected since the design of β pep peptides [20] was based in part on an amino acid sequence derived from the β -sheet domain of BPI (residues 82–108). However, that same BPI sequence, made synthetically, is inactive as an anti-angiogenic agent. Moreover, comparison of the anginex and BPI sequences (Figure 1) shows them to be homologous, yet quite different. In fact, anginex is sequentially closer to the β -sheet domain from PF4 also used in part in the design of β pep peptides. Although the PF4 β -sheet domain sequence is also inactive, parent protein PF4 functions cytostatically, and not cytotoxicity like anginex, by arresting EC in the S phase [5]. This, in turn, indicates that anginex and PF4 also function differently on the molecular level and emphasizes the idea that even though sequences are homologous, and even partly identical, bioactivities can be different. It may be that the activity of these peptides depends more on folded conformation than on sequence. For example, anginex forms β -sheet structure in aqueous solution, whereas the BPI peptide is unstructured random coil [18]. Quarternary structure is another factor that can modulate activity. Anginex, like various cytokines, e.g. α -chemokines and TNF, self-associates to form dimers and β -sandwich tetramers [21]. PF4 also forms dimers and tetramers in solution, yet PF4 is probably anti-angiogenically active as a monomer, since, at its normal concentration in the serum, biophysical studies indicate that PF4 would mostly be dissociated into monomers [45]. For IL-8, dimers which define the highest aggregation state, are considerably more stable than those for PF4; yet both monomers and dimers of IL-8 are biologically active [46,47]. Under physiological conditions, anginex, like PF4, should exist mostly in the monomer state [35].

Identification of specific amino acid residues and their spatial relationships, which are crucial to promoting anti-angiogenic effects at the level of molecular interactions, is one of the main goals among structural biologists and pharmaceutical chemists working to develop more effective anti-angiogenic agents. Nevertheless, in anti-angiogenic proteins such structure–activity relationships, i.e. specific residues and conformations which impart activity, are sorely needed, and even the analysis of high-resolution molecular structures of a number of anti-angiogenic proteins, e.g. endostatin [14], PF4 [15] and BPI [17], have not provided this information. Working with a small peptide like anginex, however, allows more rapid assessment of some structure–activity relationships. By comparing these highly homologous β pep sequences (Figure 1) in terms of their relative ability to inhibit EC proliferation, insight into which amino acid residues are required for activity may be gleaned. First of all, since C-terminal residues 26–33, which were taken in large part from the β -sheet sequences of PF4 and IL-8, are identical in all β pep peptides, active and inactive, these residues are probably irrelevant to activity. Secondly, even though the N-terminal half of the most active β pep peptides is most like the β -sheet sequence from BPI, its structure can be changed without being detrimental to activity. For one of the most active peptides, β pep-8, residues identical to those in BPI are ANIK ... S ... KWKAQKRFLKMS ... N ... DLS However, highly active β pep-2 and especially β pep-25 share much less sequence identity with BPI peptide 82–108, because the mid-portions of their sequences have been reversed relative to that found in β pep-8 and BPI: KWKAQKRFLKMS. Therefore only the N-terminal stretch of residues, ANIK ... S ... , and the KWK tripeptide remains. The KWK tripeptide can also be excluded from imparting activity since it is

present in most β pep peptides, and the ANIK ... S sequence can be excluded because similarly active β pep-7, -11, -21 and -24 have completely different N-terminal sequences. Lastly, even though β pep-23 is sequentially most like inactive β pep-4, its anti-proliferative activity is quite good. In effect, therefore, nothing remains of the actual BPI β -sheet amino acid sequence (residues 82–108) used in part in the design of β pep peptides. Moreover, this same BPI sequence produced synthetically (Figure 1) is inactive at inhibiting EC proliferation, indicating that the BPI-derived sequence alone is not sufficient to promote this activity. Rather, it is the novel combination of amino acids within the β pep sequence that is paramount to activity. One additional reason for the presence of activity in these β pep peptides is conformation. The BPI-derived sequence (residues 82–108) is completely random coil in solution, whereas all β pep peptides fold, to varying extents, as amphipathic β -sheets [18]. The novel β pep peptide design [20], therefore, has imposed certain sequential and conformational constraints in the context of a small peptide, which act to promote anti-proliferative and apoptotic activities against ECs.

In the final analysis, residues in β pep-25 which emerge as being apparently crucial to anti-proliferative activity are Leu-5, Val-7, Lys-10, Lys-17, Ile-20, Val-22 and Leu-24. Assuming that the β -sheet fold of β pep-25 as shown in Figure 2 is the bioactive conformation, it is interesting to note that all five hydrophobic residues are proximal and are on the hydrophobic face of the amphipathic β -sheet. The two lysines are positioned on the opposite amphiphilic face, lying diagonally from one another on strands 1 and 2. Although this does not prove that this is the bioactive conformation or that these amino acid residues are the most important, it is suggestive. As structure–function relationships in other anti-angiogenic proteins and peptides become known, it will be interesting to see if this is a common feature among anti-angiogenic proteins.

Conclusions

A number of β pep peptides can specifically inhibit proliferation in ECs. Anginex (β pep-25) is the most potent of these, and functions by inhibiting EC adhesion and migration, thereby inducing apoptosis. This cytotoxicity, specific for angiogenically activated ECs, forms the basis for the angiostatic activity of the peptide. Comparison between active and inactive β pep peptide sequences has suggested that several hydrophobic and two lysine residues may be crucial in promoting these activities. This novel peptide anginex, therefore, makes for a potentially good anti-angiogenic therapeutic agent that may be capable of controlling various pathological disorders like tumour growth, rheumatoid arthritis, restenosis and diabetic retinopathy.

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REFERENCES

- 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* **88**, 277–285.
- Boehm, T., Folkman, J., Browder, T. and O'Reilly, M. S. (1997) Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature (London)* **390**, 404–407.
- Molema, G. and Griffioen, A. W. (1998) Rocking the foundations of solid tumor growth by attacking the tumor's blood supply. *Immunol. Today* **19**, 392–394.

- 4 Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.* **1**, 27–31
- 5 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
- 6 Tolsma, S. S., Volpert, O. V., Good, D. J., Frazier, W. A., Polverini, P. J. and Bouck, N. (1993) Peptides derived from two separate domains of the matrix protein thrombospondin-1 have anti-angiogenic activity. *J. Cell Biol.* **122**, 497–511
- 7 Sato, N., Goto, T. and Haranaka, K. (1986) Actions of tumor necrosis factor on cultured vascular endothelial cells: morphologic modulation, growth inhibition and cytotoxicity. *J. Natl. Cancer Inst.* **76**, 1113–1121
- 8 Palombella, V. J. and Vilcek, J. (1989) Mitogenic and cytotoxic actions of tumor necrosis factor in BALB/c 3T3 cells. The role of phospholipase activation. *J. Biol. Chem.* **264**, 18128–18136
- 9 Robaye, B., Mosselmans, R., Fiers, W., Dumont, J. E. and Galand, P. (1991) Tumor necrosis factor induces apoptosis in normal endothelial cells *in vitro*. *Am. J. Pathol.* **138**, 447–453
- 10 Luster, A. D., Greenberg, S. M. and Leder, P. (1995) The IP-10 chemokine binds to a specific cell surface heparan sulfate site shared with platelet factor 4 and inhibits endothelial cell proliferation. *J. Exp. Med.* **182**, 219–231
- 11 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* **79**, 315–328
- 12 Pike, S. E., Yao, L., Jones, K. D., Cherney, B., Appella, E., Sakaguchi, K., Nakhasi, H., Teruya-Feldstein, J., Wirth, P., Gupta, G. and Tosato, G. (1998) Vasostatin, a calreticulin fragment, inhibits angiogenesis and suppresses tumor growth. *J. Exp. Med.* **188**, 2349–2356
- 13 Van der Schaft, D. W. J., Toebes, E. A. H., Haseman, J., 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
- 14 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
- 15 Mayo, K. H., Roongta, V., Barker, S., Milius, R., Ilyina, E., Quinlan, C., La Rosa, G. and Daly, T. (1995) NMR solution structure of the 32 kD tetrameric platelet factor-4 ELR-motif N-terminal chimera: a symmetric tetramer. *Biochemistry* **34**, 11399–11409
- 16 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
- 17 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* **276**, 1861–1864
- 18 Mayo, K. H., Haseman, J., 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
- 19 Gray, B. H., Haseman, J. R. and Mayo, K. H. (1995) B/PI-derived synthetic peptides: synergistic effects in tethered bactericidal and endotoxin neutralizing peptides. *Biochim. Biophys. Acta* **1244**, 185–190
- 20 Mayo, K. H., Ilyina, E. and Park, H. (1996) A recipe for designing water-soluble, β -sheet-forming peptides. *Protein Sci.* **5**, 1301–1315
- 21 Ilyina, E., Roongta, V. and Mayo, K. H. (1997) NMR structure of a *de novo* designed, peptide 33-mer with two distinct compact β -sheet folds. *Biochemistry* **36**, 5245–5250
- 22 Mire Sluis, A. R., Gaines Das, R. and Thorpe, R. (1997) Implications for the assay and biological activity of interleukin-8: results of a WHO international collaborative study. *J. Immunol. Methods* **200**, 1–16
- 23 Priestle, J. P., Schar, H. P. and Grutter, M. G. (1989) Crystallographic refinement of interleukin-1-beta at 2.0 Å resolution. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9667–9671
- 24 Jones, E. Y., Stuart, E. D. and Walker, N. P. C. (1989) Structure of tumour necrosis factor. *Nature (London)* **338**, 225–228
- 25 Eck, M. J., Ultsch, M., Rinderknecht, E., De Vos, A. M. and Sprang, S. R. (1992) The structure of human lymphotoxin (tumor necrosis factor-beta) at 1.9 Å resolution. *J. Biol. Chem.* **267**, 2119–2122
- 26 Daopin, S., Piez, K. A., Ogawa, Y. and Davies, D. R. (1992) Crystal structure of transforming growth factor-beta 2: an unusual fold for the superfamily. *Science* **257**, 369–373
- 27 Bax, A. and Davis, D. G. (1985) MLEV-17-based two-dimensional homonuclear magnetization transfer spectroscopy. *J. Magn. Reson.* **65**, 355–360
- 28 Wider, G., Macura, S., Anil-Kumar Ernst, R. R. and Wuthrich, K. (1984) Homonuclear two-dimensional ^1H NMR of proteins. Experimental procedures. *J. Magn. Reson.* **56**, 207–234
- 29 Marion, D. and Wuthrich, K. (1983) Application of phase sensitive two-dimensional correlated spectroscopy (COSY) for measurements of ^1H - ^1H spin-spin coupling constants in proteins. *Biochem. Biophys. Res. Commun.* **113**, 967–974
- 30 Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1985) Rapid recording of 2D NMR spectra without phase cycling. Application to the study of hydrogen exchange in proteins. *J. Magn. Reson.* **89**, 393–399
- 31 Yang, Y., Mayo, K. H., Daly, T. J., Barry, J. K. and La Rosa, G. J. (1994) Subunit association and structural analysis of platelet basic protein and related proteins investigated by ^1H NMR spectroscopy and circular dichroism. *J. Biol. Chem.* **269**, 20110–20118
- 32 Relou, I. A. M., Damen, C. A., Van der Schaft, D. W. J., Groenewegen, G. and Griffioen, A. W. (1998) The effect of culture conditions on endothelial cell phenotype and responsiveness. *Tissue Cell* **30**, 525–530
- 33 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
- 34 Barendsz-Janson, A. F., Griffioen, A. W., Muller, A. D., Van Dam-Mieras, M. C. E. and Hillen, H. F. P. (1998) *In vitro* tumor angiogenesis assays: plasminogen lysine binding site 1 inhibits *in vitro* tumor-induced angiogenesis. *J. Vasc. Res.* **35**, 109–114
- 35 Mayo, K. H. and Ilyina, E. (1998) A folding pathway for β -pep-4 peptide 33mer: from unfolded monomers and β -sheet sandwich to well-structured tetramers. *Protein Sci.* **7**, 358–368
- 36 Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G. and Cheresch, D. A. (1994) Integrin $\alpha v \beta 3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* **79**, 1157–1164
- 37 Brooks, P. C., Clark, R. A. F. and Cheresch, D. A. (1994) Requirement of vascular integrin $\alpha v \beta 3$ for angiogenesis. *Science* **264**, 569–571
- 38 Re, F., Zanetti, A., Sironi, M., Polentarutti, N., Lanfrancone, L., Dejana, E. and Colotta, F. (1994) Inhibition of anchorage-dependent cell spreading triggers apoptosis in cultured human endothelial cells. *J. Cell Biol.* **127**, 537–546
- 39 Frisch, S. M., Vuori, K., Ruoslahti, E. and Chan Hui, P. Y. (1996) Control of adhesion-dependent cell survival by focal adhesion kinase. *J. Cell Biol.* **134**, 793–799
- 40 Griffioen, A. W., Damen, C. A., Mayo, K., Barendsz-Janson, A. F., Martinotti, S., Blijham, G. H. and Groenewegen, G. (1999) Angiogenesis inhibitors overcome tumor induced endothelial cell anergy. *Int. J. Cancer* **80**, 315–319
- 41 Barendsz-Janson, A. F., Griffioen, A. W., Muller, A. D., Van Dam-Mieras, M. C. E. and Hillen, H. F. P. (1998) *In vitro* tumor angiogenesis assays: plasminogen lysine binding site 1 (LBS-1) inhibits *in vitro* tumor induced angiogenesis. *J. Vasc. Res.* **35**, 109–114
- 42 Griffioen, A. W., Coenen, M. J. H., Damen, C. A., Hellwig, S. M. M., Van Weering, D. H. J., Vooy, W., Blijham, G. H. and Groenewegen, G. (1997) CD44 is an activation antigen on human endothelial cells; involvement in tumor angiogenesis. *Blood* **90**, 1150–1159
- 43 Klein, S., Giancotti, F. G., Presta, M., Albelda, S. M., Buck, C. A. and Rifkin, B. B. (1993) Basic fibroblast growth factor modulates integrin expression in microvascular endothelial cells. *Mol. Biol. Cell* **4**, 973–982
- 44 Brooks, P. C., Silletti, S., von Schalscha, T. L., Friedlander, M. and Cheresch, D. A. (1998) Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. *Cell* **92**, 391–400
- 45 Chen, M.-J. and Mayo, K. H. (1991) Human platelet factor 4 subunit association-dissociation thermodynamics and kinetics. *Biochemistry* **30**, 6402–6411
- 46 Leong, S. R., Lowman, H. B., Liu, J., Shire, S., Deforge, L. E., Gillece-Castro, B. L., McDowell, R. and Hebert, C. A. (1997) IL-8 single-chain homodimers and heterodimers: interactions with chemokine receptors CXCR1, CXCR2 and DARC. *Protein Sci.* **6**, 609–617
- 47 Rajarathnam, K., Sykes, B. D., Kay, C. M., Dewald, B., Geiser, T., Baggiolini, M. and Clark, L. (1994) Neutrophil activation by monomeric interleukin-8. *Science* **264**, 90–92

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