Structure and Inhibitory Effects on Angiogenesis and Tumor Development of a New Vascular Endothelial Growth Inhibitor*§

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Blocking angiogenesis is an attractive strategy to inhibit tumor growth, invasion, and metastasis. We describe here the structure and the biological action of a new cyclic peptide derived from vascular endothelial growth factor (VEGF). This 17-amino acid molecule designated cyclopeptidic vascular endothelial growth inhibitor (cyclo-VEGI, CBO-P11) encompasses residues 79–93 of VEGF which are involved in the interaction with VEGF receptor-2. In aqueous solution, cyclo-VEGI presents a propensity to adopt a helix conformation that was largely unexpected because only β-sheet structures or random coil conformations have been observed for macrocyclic peptides. Cyclo-VEGI inhibits binding of iodinated VEGF₁₆₅ to endothelial cells, endothelial cells proliferation, migration, and signaling induced by VEGF₁₆₅. This peptide also exhibits anti-angiogenic activity in vivo on the differentiated chicken chorioallantoic membrane. Furthermore, cyclo-VEGI significantly blocks the growth of established intracranial glioma in nude and syngeneic mice and improves survival without side effects. Taken together, these results suggest that cyclo-VEGI is an attractive candidate for the development of novel angiogenesis inhibitor molecules useful for the treatment of cancer and other angiogenesis-related diseases.

Angiogenesis takes place during embryonic development and in the adult during wound healing and the female ovulatory cycle. In pathological states, angiogenesis is observed during solid tumor growth and metastasis, diabetic retinopathy, and chronic inflammatory disorders. A number of angiogenic regulators such as vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), and angiopoietins have been identified (1–4). VEGFs are endothelial cell mitogens in vitro and also stimulate angiogenesis in vivo (2, 5, 6). VEGF exerts its biological effects through high affinity binding to two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (kinase domain receptor), which are expressed in most vascular endothelial cells (6–8). VEGF is expressed in a large number of human tumor types (9, 10). Clinical studies have documented the importance of VEGF in human cancers. As an example, VEGF is increased in the plasma of cancer patients and is correlated with response to chemotherapy (11, 12). In growing tumors, VEGF expression is up-regulated by hypoxia, growth factors, and oncogenes (6, 13).

Antagonizing VEGF has shown to inhibit tumor development in vivo. Different strategies have been designed to inhibit VEGF function. These include monoclonal neutralizing antibodies (14), dominant-negative mutants of the VEGF receptor-2 (VEGFR2) (15), antisense oligonucleotides (16), anti-VEGFR2 antibodies (17), blockers of VEGFR2 tyrosine phosphorylation (18), VEGF-toxin conjugates (19), antagonistic VEGF mutants (20), peptides that interfere with VEGF/VEGFR interactions (21–25), and decoy-soluble receptors (26).

Relevant regions of VEGF important for its binding on VEGFR2 and VEGFR1 have been investigated by structural and mutagenesis studies (27–29). Some of the residues important for the interaction between VEGF and kinase domain receptor are clustered within region 79–93 of VEGF which

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; FGF-2, fibroblast growth factor-2; PDGF-BB, platelet-derived growth factor-BB; cyclo-VEGI, cyclo-vascular endothelial growth inhibitor; CAM, chorioallantoic membrane; VEGFR, vascular endothelial growth factor receptor; BAE, bovine aortic endothelial; BCE, bovine capillary endothelial; CHO, Chinese hamster ovary; ERK1,2, extracellular signal-regulated kinases 1 and 2; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; CD, chemical shift deviation; HSQC, heteronuclear single quantum correlation spectroscopy; TFE, 2,2,2-trifluoroethanol; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MAP, mitogen-activated protein; HSPG, heparan sulfate proteoglycan; Fmoc, N-(9-fluorenylmethyl)carbonyl; NOE, nuclear Overhauser effect spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; EC, endothelial cell; TOCSY, total correlation spectroscopy; HMBP, total correlation spectroscopy; BHA, butylated hydroxyanisole; HSPGs, heparan sulfates proteoglycans.
forms a β-hairpin, Arg^{82}, Lys^{84}, and His^{86} being key residues (27). Based on these structural and mutagenesis studies, a number of cyclic and linear peptides have been synthesized. One of the most potent compound was the cyclic peptide CBO-P11 (β-Phe-Pro-Pro-His-Arg) designated herein cyclopentidic vascular endothelial growth inhibitor (cyclo-VEGFI).\(^2\) In the present report, we determined the structural features of this peptide and characterized its biological properties. We show that this peptide adopts an unexpected three-dimensional structure and is able to inhibit critical steps of angiogenesis. Moreover, this peptide inhibits the growth of both established human intracranial and syngeneic glioma in mice.

**EXPERIMENTAL PROCEDURES**

**Structural Analysis of Cyclo-VEGI (CBO-P11)**

**NMR Spectroscopy**—NMR samples were prepared at a 2 mM peptide concentration in water or in mixtures of TFE-d$_{2}$/water containing 10% D$_2$O. All NMR spectra were recorded on Bruker Avance spectrometers operating at a 1H frequency of 500 MHz and were processed with the Bruker XWINNMR program. One-dimensional spectra were acquired over 16 K data points using a spectral width of 5000 Hz and a reference to internal sodium 3-trimethylsilyl-(2,2,3,3-$\delta$)-propionate at 0 ppm. Solvent suppression was achieved by presaturation during the relaxation delay (1.5 s) or with a WATERGATE sequence using pulsed field gradients (30). Protein assignments were obtained at 288 and 298 K from two-dimensional NOESY (20- and 80-ms mixing times for the amide and NOESY (150-, 200-, or 300-ms mixing times) spectra (31, 32). Typically, two-dimensional data were collected with 400-600 t$_1$ increments and 2048 data points in t$_2$ over a spectral width of 6000 Hz in both dimensions. Prior to Fourier transformation in t$_2$ and t$_1$, the time domain data were multiplied by a z$\delta$ shifted square sinebell function and zero-filled. Base-line distortions were corrected with a fifth-order polynomial function. 1H-13C HSQC experiments were acquired using pulse field gradients for coherence selection (33). The chemical shift deviations of H$_{\alpha}$ and C$_{\alpha}$ were calculated using random coil values reported in water (34). The temperature gradients of the amide proton chemical shifts were derived from a series of TOCSY spectra recorded at different temperatures (from 283 to 303 K). The resonance assignments are listed in Tables S1–S3 in the Supplemental Material.

**Structure Calculation**—Interproton distance restraints were derived from the analysis of NOESY spectra. NOE cross-peaks were categorized as strong, medium, and weak and converted into distance ranges of 1.8–2.8, 2.8–3.8, and 3.8–5.0 Å, respectively. Pseudooatoms were introduced for distances involving methyl protons and non-reolved methylene protons, and upper limits were corrected appropriately (35). The ϕ angle of L-amino acid residues was restrained to negative values because all these residues did not exhibit strong intraresidual doAN NOEs. Structures were calculated using InsightII version 981 (Biosym, San Diego, Inc.), running on SGI O2 R10000 workstations, and AMBER forcefield (36). The simulated annealing protocol first comprised 15 ps of dynamics at 1000 K during which the force constants of the distance and dihedral restraint terms were gradually increased. The non-bond interaction, defined by a simple quartic repulsive potential, was slowly increased during the next 10 ps of dynamics. Finally, the structures were cooled from 1000 to 0 K over 25 ps. The structures were then minimized by steepest descent and conjugated gradient algorithms, using a Lennard-Jones potential for the van der Waals interaction and a distance-dependent dielectric screening of 4r for the electrostatic term.

**Growth Factors and Peptides Synthesis**

Recombinant human FGF-2 was kindly provided by Dr. Hervé Prats (INSERM U397, Toulouse, France) and stored in sterile, double-distilled water at ~80 °C. Recombinant human VEGF$_{165}$ was produced in insect cells and purified as described elsewhere (37, 38). Human VEGF$_{165}$-encoding baculovirus was a kind gift of Dr. Jean Plouet (Institut de Pharmacologie et de Biologie Structurale, UMR 8098, Toulouse, France). CBO-P11 (cyclo-VEGFI) (2PQMRKHPGQQHIGE) and linear control CBO-P14 (2PQMRKHPGQQHIGE) peptides were synthesized using Fmoc/BOC-butyll butyl solid phase synthesis on an Applied Biosystems 430A automated peptide synthesizer. For preparation of protected peptide fragments, pre-loaded acid-labile 2-chlorotriyl resins (H-HisTrt$^2$-CTrt and H-Ile$^2$-CTrt resin) or HMPB-BHA resin based on BHA polystyrene functionalized with Rink’s 4-hydroxymethyl-3-methoxyphenoxybutanoic acid linker (Fmoc-Gly-HMPB-BHA resin) were utilized. Subsequent Fmoc amino acids were coupled using a 4-fold excess of amino acids activated as HOBT ester by means of DCC. The crude linear protected peptides were analyzed by reverse phase high pressure liquid chromatography on a Lichrosorb RP-18 column (Merck). The peptides were then isolated on semi-preparative high pressure liquid chromatography. Cyclization of the protected linear precursor (head-to-tail) was performed by Fmoc/BOC-butyll allyl strategy.\(^2\)

**Cell Culture**

Bovine capillar endothelial (BCE) cells were a kind gift of Dr. Daniel B. Rifkin (New York University Medical Center, New York). Bovine aortic endothelial (BAE) cells were from Dr. Georg Kreis (Department of Molecular Biology, Max-Planck-Institut für physiologische und Klinische Forschung, Bad Nauheim, Germany). All endothelial cells were grown at 37 °C, 5% CO$_2$ in DMEM containing 10% newborn calf serum, 2 mM t-glutamate, and 50 IU/ml penicillin, 50 IU/ml streptomycin antibiotics and were used up to passage 25. BCE cells were grown in the presence of 2 ng/ml FGF-2. CHO-VEGFR2 and CHO-VEGFR1 cells were a kind gift of Dr. J. Plouet (GDR CNRS 1927 “Angiogénèse,” Toulouse, France). CHO-VEGFR2 and CHO-VEGFR1 were grown in DMEM supplemented with 10% fetal calf serum, 2 mM t-glutamate, 1% non-essential amino acids and antibiotics (Invitrogen). Balb3T3 were grown in DMEM supplemented with 10% FBS, 2 mM t-glutamate, and antibiotics. U87 human glioma cells (ATCC) were grown in minimum Eagle’s α-medium, 10% FBS, 2 mM t-glutamate, and antibiotics. GL261 murine glioblastoma cells (a gift from Dr. David Zagzag, New York University, New York) were cultured in DMEM supplemented with 10% FBS, 2 mM t-glutamate, 1% non-essential amino acids, and antibiotics.

**Binding Assays**

VEGFR$_{165}$ was labeled with Na$I^{125}$ using IODO-GEN (Pierce) as coupling agent according to the manufacturer's instructions. BAE, CHO-VEGFR2, and CHO-VEGFR1 cells were seeded at 2.5 × 10$^5$ density in gelatin-coated 6-well plates and cultured in complete medium for 2 days. Cells were washed twice with ice-cold PBS and incubated with 10 ng/ml 1$^25$I-VEGF and peptides at indicated concentrations in binding medium (DMEM, 20 mM Hepes, pH 7.4; 0.15% gelatin) on a shaker at 4 °C. After 2 h, cells were washed 3 times with PBS and solubilized by the addition of 2% Triton, 10% glycerol, and 1 mg/ml bovine serum albumin prior to γ-counting. Each condition was tested in duplicate and repeated at least two times. Data are expressed as percentage of total radioactivity.

**Proliferation Assays**

BCE and BAE cells were seeded in 24-well culture plates overnight in 10% newborn calf serum at 7500 cells per well. Medium was changed to 1% newborn calf serum, 10 ng/ml growth factor, and peptides at indicated concentrations were added to duplicate wells. After 48 h, medium was changed, and stimulation with growth factor and peptide treatment were repeated. One day later, cells were counted on a Coulter counter. Balb3T3 were seeded at a density of 10,000 cells/well in complete medium. Medium was changed to 0.5% fetal calf serum and 20 ng/ml human recombinant PDGF-BB (Sigma), and peptides at indicated concentrations were added to duplicate wells. Cells were counted 2 days later. U87 and GL261 cells were seeded at 1 × 10$^3$ cells/well 1% FBS, treated with peptides at the same concentrations as ECs, and counted 72 h later. Each condition was tested in duplicate and repeated at least two times.

**ERK-1 (p44)/ERK-2 (p42) Phosphorylation Assay**

BAE cells were plated in 35-mm plates. Subconfluent cultures were serum-deprived for 24 h. Peptides were then added for 6 min in the presence of 5 ng/ml PDGF-CB (Sigma). Cells were serum-starved for 10 min on ice in Nonidet P-40/SDS lysis buffer (50 mM Hepes, pH 7.4; 75 mM NaCl; 1 mM EDTA; 1% Nonidet P-40; 0.1% SDS) containing a mixture of protease inhibitors. The insoluble material was removed by centrifugation for 20 min at 12,000 × g at 4 °C. The cleared supernatant was stored at ~80 °C. Protein concentration was measured by using the Bradford method (Bio-Rad). The cytosolic and membrane extracts were resolved by SDS-PAGE on 12% gels under reducing conditions and electrot trans-ferred onto a nitrocellulose membrane. The blocked membranes were then incubated with primary antibodies (phospho-specific mitogen-activated protein kinase p42/p44 or mitogen-activated protein kinase 35365
p42/p44 were obtained from New England Biolabs, Ozyme, France), washed, and incubated with secondary horseradish peroxidase-conjugated mouse or rabbit antibodies (Dako SA, Trappes, France). Detection of antibodies was performed using the ECLplus Western blot detection system (Amersham Biosciences). Each condition was tested at least two times.

Migration Assay

Migration test with BAE cells was performed using a method described earlier (39). In brief, ECs were seeded in 350-mm culture plates and were allowed to grow to confluence. Complete medium was replaced with serum-free DMEM, and incubation was continued overnight. A linear scar was drawn in the monolayer and divided into seven equal fields. A set of digital photos was taken of each scar, and the denuded area was marked using digital image analysis software (Lucia G, www. lim.ca). The dishes were washed and incubated with fresh serum-free medium containing 0.1% bovine serum albumin, 10 ng/ml VEGF

CAM Assay

Fertilized chicken eggs (Gallus gallus) (E.A.R.L. Morizeau, Dangers, France) were incubated at 37 °C and 80% humidified atmosphere. On day 4 of development, a window was made in the eggshell and sealed with Durapore® tape. On day 13, plastic rings (made from Nunc Thermanox® coverslips) were put on the CAM. 3 μg of VEGF

CAM Assay

Immunochemistry

Immunohistochemistry was performed on 5-μm sections. Immunohistochemistry on 5-μm sections was carried using the Vectastain Elite kit (Vector Laboratories). Primary antibodies include anti-CD31 (1:100 dilution, BD Biosciences) and anti-Ki-67 (1:100 dilution, Dako). Detection was carried out using DAB chromogen. Sections were counterstained with hematoxylin. Negative control slides were obtained by omitting the primary antibody. Ki-67 staining was quantified by counting the number of positively stained cells of 100 nuclei in 20 randomly chosen fields (40, 41). Microvessel count and density were scored as reported previously (40, 41). Apoptotic cells were detected with ApoTag® plus kit (Genenco International, New York) with 1% methyl green as a counterstain. Apoptosis and proliferative indices were quantified by determining the percentage of positively stained cells for all microvessels in 20 randomly chosen fields per section at ×200 magnification (40).

RESULTS

Secondary and Three-dimensional Structure of Cyclo-VEGI

Secondary Structure—Standard NMR methodology was used to obtain sequence-specific assignments of proton resonances in water and in TFE/water mixtures at two different ratios, 15:85 or 30:70 (v/v). TOCSY spectra were used to identify spin systems, and NOESY spectra were used to obtain inter residue connectivities (42). The 13C resonances of Cα carbons were assigned from 13C-1H HSQC spectra. Weak additional resonances could be observed in the NMR spectra recorded in water and also in TFE/water mixtures, albeit to a lesser extent. In the absence of chemical heterogeneity, a likely explanation of the observed chemical shift heterogeneity would be cis-trans isomerism of peptide bonds preceding proline. The major form corresponds to a trans conformation of the peptide bonds preceding the two Pro residues, as evidenced by the observation of strong αi–(i - 1) sequential NOEs. The proportion of minor forms was too weak to give rise to NOE cross-peaks. However, the presence of alternative spin systems of Gln/Glu, Phe, His, and Lys in TOCSY spectra, i.e. amino acids around Pro residues, suggests that both ε-Phe1–Pro2 and ε-Lys3–Pro4 peptide bonds are likely to isomerize. The lower proportion of minor species in mixtures of TFE/water indicates that the stabilization of folded structures (see below) decreases the amount of cis isomers, as observed for cyclic gramicidin S.

The chemical shift deviations (CSDs) of Hα and Cα resonances, calculated as the difference between observed chemical
shifts and corresponding random coil values, carry information about the secondary structure (43). A stretch of residues exhibiting upfield shifts of H protons and downfield shifts of C carbons is characteristic of a helix, whereas the observation of downfield shifts of H and upfield shifts of C indicates the presence of β-sheet structures. Fig. 1 shows the H and C CSDs in water and in TFE/water mixtures. The peptide in water displays very weak CSDs along its whole sequence, indicating that it is largely unstructured in aqueous solution. The addition of TFE (from 15 to 30%) progressively induces an upfield shift of H and a downfield shift of C resonances for residues 1–6, supporting the formation of a helical structure. Because the corresponding H CSDs are slightly negative in water, this suggests that the 1–6 segment has a small helical propensity in water and that helical conformations explored in this region are stabilized by the addition of TFE. Strong upfield shifts of amide protons chemical shifts are also induced by the addition of TFE for residues 3–8. Interestingly, these variations are opposite to those observed for cyclic gramicidin S under the same conditions and are correlated to the formation of a β-sheet structure. Furthermore, residues 3–8 exhibit weak temperature gradients of their amide proton chemical shifts (∆δH/∆T). Altogether, these chemical shift data are consistent with the formation of a helical structure in segment 1–8. No significant CSD variations could be observed in the other parts of the sequence, suggesting that the peptide remains unstructured, apart from the 1–8 region.

Three-dimensional Structure—The cyclic peptide shows few NOEs in water, besides intrresidual and sequential connectivities. In contrast, the peptide in 30% TFE exhibits NOEs characteristic of helical structure in segment 1–8, including strong dNN (i,i + 1) and medium daN (i,i + 1) sequential connectivities, together with several daN (i,i + 2), daN (i,i + 3), and daN (i,i + 4) medium range connectivities. The structure of the cyclic peptide in 30% TFE was calculated by restrained molecular dynamics, using a set of 17 intrresidual, 63 sequential, and 20 medium range distance restraints. The best 20 calculated structures are shown in Fig. 2. They have low energies and few distance violations, indicating that they are in good agreement with NMR experimental restraints. The overall structure of the cyclic peptide is poorly defined, as evidenced by the large root mean square deviation of backbone atoms (3.4 Å). However, the superimposition of structures using backbone atoms of residues 1–8 or 1–6 shows that the backbone is better defined in this region, with corresponding root mean square deviations of 1.6 and 1.1 Å, respectively. Segment 1–8 adopts a helical conformation. Unexpectedly, residues D-Phe1–Pro2 do not form a type II β-turn but are rather part of the helical 1–8 segment, as shown by medium range correlations between D-Phe1–Ile4 and Pro2–Met5. Similarly, residues Pro2–His do not form a stable β-turn. The conformations of other residues are not well defined but are not completely random coil due to the cyclic topological constraint.

Inhibition of VEGF Binding to Its Receptors

We first looked at the ability of cyclic-VEGI to interfere with binding of 125I-labeled VEGF165 to Chinese hamster ovary (CHO) cells expressing VEGFR2. Cyclo-VEGI inhibited 125I-labeled VEGF165 binding to VEGFR2 in a dose-dependent manner (Fig. 3A) with a half-maximal inhibition (IC50) of 1.3 μM. The linear control peptide P14 with the same amino acid sequence as cyclo-VEGI did not compete for receptor binding even at the highest concentration tested (Fig. 3A). To investigate whether cyclo-VEGI inhibits binding of VEGF165 to VEGFR2 specifically, we tested the effect of cyclo-VEGI on the binding of 125I-labeled VEGF165 to CHO cells expressing VEGFR1. Interestingly cyclo-VEGI also inhibited binding of VEGF to VEGFR1 (IC50 value of 0.7 μM, Fig. 3B). We next evaluated its effect on 125I-labeled VEGF165 binding to high affinity VEGF receptors...
in BAE cells. As shown in Fig. 3C, cyclo-VEGI effectively inhibits the binding of 125I-labeled VEGF165 to high affinity receptors with an IC50 of 12 μM. Linear control P14 at the highest concentration tested (50 μM) had no effect (Fig. 3C).

**Inhibition of the Proliferation of Endothelial Cells but Not of Glioma Cells**

We next investigated whether cyclo-VEGI inhibits VEGF-induced proliferation of BAE cells. When BAE cells were stimulated by 10 ng/ml 125I-VEGF165 and peptides at indicated concentrations, Cyclo-VEGI inhibited VEGF binding in a dose-dependent manner, whereas the control peptide P14 did not, even at 100 μM on CHO-VEGFR2 cells (A), or 50 μM on CHO-VEGFR1 (B), and BAE (C) cells. The half-maximal inhibition (IC50) values were 1.3, 0.7, and 12 μM for CHO-VEGFR2, CHO-VEGFR1, and BAE cells, respectively. Data represent means ± S.D. of duplicate experiments. The figure depicts a representative experiment from two independent experiments.

**FIG. 3**. Inhibition of 125I-VEGF165 binding to CHO-VEGFR2 (A), CHO-VEGFR1 (B), and BAE (C) cells by cyclo-VEGI. CHO-VEGFR2, CHO-VEGFR1, and BAE cells were incubated with 10 ng/ml 125I-VEGF165 and peptides at indicated concentrations. Cyclo-VEGI inhibited VEGF binding in a dose-dependent manner, whereas the control peptide P14 did not, even at 100 μM on CHO-VEGFR2 cells (A), or 50 μM on CHO-VEGFR1 (B), and BAE (C) cells. The half-maximal inhibition (IC50) values were 1.3, 0.7, and 12 μM for CHO-VEGFR2, CHO-VEGFR1, and BAE cells, respectively. Data represent means ± S.D. of duplicate experiments. The figure depicts a representative experiment from two independent experiments.

FIG. 4. Effect of cyclo-VEGI on cell proliferation. Proliferation was stimulated with 10 ng/ml VEGF165 using BAE cells (A), 10 ng/ml FGF-2 using BCE cells (B), and 20 ng/ml PDGF-BB using Balb3T3 cells (B) and 1% serum using U87 and GL261 cells (C). A, cyclo-VEGI inhibited BAE cell proliferation in a concentration-dependent manner (IC50 of 5.8 μM). 100% inhibition of proliferation was achieved with 20 μM cyclo-VEGI. The linear control P14 did not show any effect. B, cyclo-VEGI inhibited FGF-2-induced proliferation of BCE cells in a dose-dependent manner with an IC50 of 18 μM. Peptide P14 had no effect. Cyclo-VEGI did not show any effect at all concentrations tested on PDGF-BB-induced Balb3T3. C, cyclo-VEGI did not show any effect at all concentrations tested on U87 and GL261 cells proliferation. Dose dependence experiments were performed in duplicates in two independent experiments; data of one experiment are shown. D, inhibition of VEGF-stimulated MAP kinase phosphorylation. Serum-deprived bovine aortic endothelial cells (1st lane) were stimulated with 10 ng/ml VEGF165 (2nd lane) in the presence of 20 μM cyclo-VEGI (3rd lane), or 20 μM of linear control P14 (4th lane). Cell lysate blot was probed with phosphospecific ERK1 and ERK2 antibodies, and the total amount of MAPs protein loading was assessed by probing with MAP antibodies. Blots depict a representative experiment from two independent experiments.

**Specific for VEGF165**, we tested the activity of cyclo-VEGI on PDGF- or FGF-induced cell proliferation. When Balb3T3 cells were stimulated with 20 ng/ml PDGF-BB, cyclo-VEGI did not show any effect even at the highest concentration tested (50 μM) (Fig. 4B). Surprisingly, cyclo-VEGI decreased FGF-induced BCE proliferation, however three times less potently (IC50 of 18
cyclo-VEGI peptides at all the concentrations tested (Fig. 4). Proliferation of two glioma cell lines, human U87 and mouse GL261, was not inhibited by VEGF165 (Fig. 4B). Recombinant human VEGF165 alone induced significant angiogenesis (Fig. 6, B and I). Some stimulation is also present around the site of application because of diffusion of the growth factor. Vehicle alone (water) in the plastic ring had no effect (Fig. 6, A and I). When premixed with VEGF165 and deposited on the CAM, a clear anti-angiogenic effect of cyclo-VEGI was visible inside the ring (Fig. 6, C and I). The linear control P14 did not inhibit VEGF-induced capillary growth in the CAM (Fig. 6, D and I). 50 µg of cyclo-VEGI applied alone on the CAM without VEGF165 did not show any effect or toxicity (data not shown).

The capillary structure in the stroma was also examined by immunohistology and confocal microscopy. Vessels were visualized by staining endothelial cells with fluorescein isothiocyanate (FITC) conjugated to S. nigrum lectin. This lectin interacts preferentially to sialic acid attached to terminal galactose by α-2,6-link and was shown to bind endothelial cells in the CAM. The extensive characterization of the CAM vasculature using this method will be described elsewhere.³ VEGF165 induced a strong increase in vessel density, a reduction of the intercapillary space, and structures suggestive of ongoing fusion events between small vessels (Fig. 6F). These chaotic vascular structures were not present when cyclo-VEGI was added together with VEGF165 in the plastic ring (Fig. 6G). However, some neovessels remained, as evidenced by a slightly higher microvessel density compared with control CAM treated with water alone (Fig. 6, G and E). CAMs treated with control peptide P14 with VEGF165 had a similar morphology than CAMs treated with VEGF165 alone (Fig. 6H).

Inhibition of Glioma Growth in Vivo

The ability of cyclo-VEGI to reduce glioma growth in vivo was initially investigated by short term studies. In these experiments, nude mice implanted intracranially with human glioblastoma cells were treated intraaperitoneally with 2 mg/kg/day of cyclo-VEGI. Treatment was started 12 days after tumor cell implantation in order to treat well established tumors. Twenty eight days later, the treatment was stopped, and the animals were sacrificed. Histological analysis of the brains from treated and untreated animals showed that treatment with cyclo-VEGI was associated with a 70% reduction of U87 tumor growth (Fig. 7A, lower panel). A similar significant inhibition (78%) was observed in immunocompetent BALB/c mice implanted with GL261 murine glioblastoma cells (Fig. 7A, top panel).

In a second set of experiments we investigated the ability of cyclo-VEGI to reduce U87 glioma growth when administered continuously and systemically by subcutaneous minipumps in nude mice. In these experiments, the pump reservoirs were filled with 0.45 mg/kg/day of the inhibitor. Analysis of tumor growth

³ M. Hagedorn, M. Balke, A. Schmidt, W. Bloch, H. Kurz, S. Javerzat, B. Rousseau, J. Wieting, and A. Bikkafalvi, submitted for publication.
volumes showed that continuous systemic administration of peptide cyclo-VEGI resulted in a 72% inhibition of intracranial glioma growth (Fig. 7B, top panel).

The same experimental design was used to perform long-term experiments. In these studies, the pump reservoirs were changed with a new one containing the same amount of the
inhibitor 28 days after implantation. Animals belonging to the control group showed a 50% survival of 32 days. Animals treated with cyclo-VEGI had a 50% survival of 75 days. No side effects were registered during the entire duration of the treatment (Fig. 7B, lower panel).

**Histological Analysis**

We next performed histological analysis of U87 glioma sections from the short-term and long-term experiments (using the subcutaneous minipumps). To quantify angiogenesis in these tumors, we stained endothelial cells for the expression of CD31. Treatment of U87 tumors with cyclo-VEGI reduced microvessel density by 68% and 62% when compared with PBS-treated controls for short-term and long-term experiments, respectively (Fig. 7C, top panel). A more detailed analysis revealed that, in control tumors, 55% capillary-like structures, 35% telangiectatic or dilated vessels, and 10% glomeruloid structures were present. In cyclo-VEGI-treated tumors, 85% capillary structures, 15% telangiectatic or dilated vessels, and no glomeruloid structures were observed. This indicates that vessels tend to be normalized by cyclo-VEGI treatment. Apoptotic indices quantified in situ by labeling fragmented DNA using the terminal deoxynucleotidyltransferase-mediated nick end labeling method were increased in tumors treated with cyclo-VEGI compared with PBS-treated controls (Fig. 7C, middle panel). An examination of proliferating cells within the tumor using Ki-67 nuclear antigen staining revealed no differences in the proliferating indices between PBS-treated controls and any cyclo-VEGI-treated groups (Fig. 7C, lower panel).

**DISCUSSION**

Structural and mutagenesis studies have shown that the residues within the amino acid sequence 79–93 of VEGF are involved in its interaction with VEGFR2 and that Arg28, Lys34, and His46 are key residues. The x-ray structure of VEGF-A revealed that this sequence is located within a β-sheet structure formed by two anti-parallel strands (β5 and β6) and connected by a type II β-turn (27–29, 47). We designed linear and cyclic peptides based on this sequence and tested them for inhibition of VEGF165 binding to VEGFR2. It appears that a 17-amino acid cyclic peptide (CBO-P11), herein designated cyclo-VEGI, inhibits very efficiently the binding of VEGF to its receptor VEGFR2.2 In this article, we undertook a series of systematic studies to determine the structural and biological properties of this peptide.

In aqueous solution, the cyclic peptide presents a weak propensity to adopt a helix structure in the 1–8 domain. The addition of TFE stabilizes helical conformations in this region, without any noticeable effect on the structure of the other parts of this peptide. TFE is known to strengthen intramolecular hydrogen bonds in peptides. The stabilization of the secondary structure by TFE has been observed for cyclic analogs of gramicidin S containing 4n + 2 residues, but intramolecular hydrogen bonding occurs between two strands in this latter case, resulting in the stabilization of β-sheet structures (48). The property of this cyclic peptide to adopt helical conformations was largely unexpected because mostly β-sheet structures or random coil conformations have been observed for macrocyclic peptides (49).

Pro has two opposite roles as inducer of helical structure when located at the N terminus or helix breaker or bender when found in the middle of a helical domain. In this cyclic peptide, Pro2 and Pro9 have these two opposite effects. Indeed, Pro2 induces the formation of helix, whereas Pro9 breaks the 1–8 helical domain. The heterochiral ω-Phe-Pro sequence is known to adopt either a type II’ β-turn or a γ-turn in small cyclic peptides. In this macrocyclic peptide, Pro2 is not part of a type II’ β-turn and initiates helix formation. Thus, despite the presence of a motif known to stabilize a type II’ β-turn in ω-hairpins, the peptide does not adopt a β-sheet structure. However, it is not in a random coil state, because region 1–8 of the peptide explores helical conformations. This topology could open new possibilities in the design of scaffolds for protein engineering, because further chemical modifications, such as C” methylation, could be used to stabilize the α-helical domain. The residues Arg28, Lys34, and His46 localized in the ω-hairpin 79–93 of VEGF are implicated in the binding to the VEGFR2 receptor. In this VEGF cyclic peptide, they are found at the end of the helical domain. Their side chains are not well defined, and it is likely that they retain enough conformational flexibility in the cyclic peptide to explore the orientation required for binding to the VEGF receptors and to prevent VEGF binding.

Although VEGF-R1 binds VEGF with 50-fold higher affinity than VEGF-R2, most of the VEGF angiogenic properties like mitogenesis and migration of endothelial cells are mediated by interaction with VEGF-R2 (6, 50). Thus, our initial goal was to design peptides that specifically interfere with binding of VEGF to its receptor VEGF-R2. Indeed, cyclo-VEGI did interfere with VEGF165 binding to CHO-VEGF-R2 in a dose-dependent manner. The linear control peptide P14 with the same amino acid sequence as cyclo-VEGI did not compete for receptor binding even at the highest concentration tested (Fig. 3A). This indicates that the inhibitory effect of cyclo-VEGI is structure-dependent. This result is in agreement with the results of other groups who showed that residues 82–90 in the loop between β-strands 5 and 6 of VEGF are critical for binding to and activation of VEGF-R2 (51, 52). This loop is part of the cyclo-VEGI peptide we have designed. Surprisingly, our peptide also inhibited the binding of VEGF165 to VEGF-R1. One possible explanation for this finding might be that the receptor binding domain determinants of VEGF165 for VEGF-R1 and VEGF-R2 share similar residues. Indeed, crystallographic studies of VEGF bound to the second Ig-like domain of VEGF-R1 and mutagenesis analysis of the binding surface of VEGF for VEGF-R2 revealed that some but not all residues in this loop domain are important for the binding to both receptors, VEGF-R1 and VEGF-R2 (27, 28).

Treatment of BAE cells with micromolar concentration of cyclo-VEGI strongly inhibited VEGF165-induced cell proliferation. Although selectivity was observed for inhibition of VEGF-induced proliferation of endothelial cells in comparison to the structurally related growth factor PDGF-BB, cyclo-VEGI was also found to impair FGF-2-induced endothelial cell proliferation. Nevertheless, the activity of cyclo-VEGI appears to be severalfold lower toward FGF-2 than toward VEGF. This effect of cyclo-VEGI on FGF-2-induced proliferation was rather unexpected but may constitute an advantage for several reasons. First, it has been shown previously that FGF-2 can induce production of VEGF via an autocrine feedback loop and that both growth factors can synergize with respect to their ability to induce angiogenesis (53, 54). Second, this might prove beneficial in vivo, because tumor cells can produce both VEGF and FGF-2 and their inhibition has a synergistic effect in the impairment of tumor growth (55, 56). This is also illustrated by the observation that a modified peptide derived from platelet factor-4, which inhibits FGF-2 and VEGF165-induced endothelial proliferation, had a strong inhibitory effect on human intracranial established glioma growth (21). The reasons for the effect of cyclo-VEGI on FGF-2 activity are not clearly understood. HSPGs are molecules found on the cell surface of almost all mammalian cells and have been shown to modulate the biological activity of growth factors (57). FGF-2 and VEGF165 function in concert with cell surface-bound HSPGs to promote...
binding to their specific receptors and to induce their biological responses (58). We cannot rule out the possibility for cyclo-VEGI to bind to the cell surface heparan sulfate proteoglycans (HSPGs) because of its basic residues, thus preventing the binding of VEGF<sub>165</sub> and FGF-2 to their receptors and subsequently inhibiting proliferation of endothelial cells. However, the linear control peptide P14, which has the same sequence as cyclo-VEGI, does not have any antagonist effect on FGF-2 or VEGF<sub>165</sub>-induced proliferation even at the highest dose of 100 μM. This finding suggests that cyclo-VEGI inhibits FGF-2-induced endothelial cell proliferation by another mechanism, probably involving specific structural properties of this cyclic peptide. Preliminary experiments indicate that cyclo-VEGI also impairs FGF-2 binding to endothelial cells, although at higher cyclo-VEGI concentrations. Further studies are under way to solve this issue.

To substantiate further the antagonist activity of cyclo-VEGI, we tested its activity on VEGF-induced signaling and migration. MAP kinases play a central role in controlling growth signals from growth factor tyrosine kinases such as VEGF (59). In agreement with the effect of cyclo-VEGI on VEGF-induced endothelial cell proliferation, we also observed an inhibition of MAP kinase activation by cyclo-VEGI in endothelial cells upon VEGF stimulation. Migration of endothelial cells, an important pre-requisite for angiogenesis, was also affected by cyclo-VEGI in a dose-dependent manner. Cyclo-VEGI inhibited EC migration below 0% serum control which suggests that residual VEGF was still present in the media. This may be due to the presence of residual VEGF<sub>165</sub> attached to HSPGs on endothelial cells or of VEGF produced by endothelial cells themselves (60, 61).

We next tested effects of cyclo-VEGI for its ability to interfere with VEGF<sub>165</sub>-induced angiogenesis on the differentiated day 13 CAM (62). The typical brush-like formation of capillaries in the stroma of the CAM induced by human recombinant VEGF<sub>165</sub> is strongly reduced by cyclo-VEGI peptides at 50 μg and not by linear control peptide P14. Cyclic peptide antagonists for α<sub>5</sub>β<sub>3</sub> integrin showed strong anti-angiogenic effects in the day 10 CAM at 300 μg. Angiogenesis was also induced by VEGF at lower doses than in our assay (1 versus 6 μg) (63). These comparisons indicate that cyclo-VEGI inhibits VEGF-induced angiogenesis in vivo in a very efficient way.

Gliomas constitute the most frequent class of primary brain tumors and are among the most malignant cancers, often resulting in the death of affected patients within months (64). In our model, established human intracranial glioma in nude mice decreased significantly by ~70% in size when treated with cyclo-VEGI in short and long term experiments. Moreover tumor-bearing mice treated with cyclo-VEGI lived significantly longer than those in the control group. Highly vascularized brain tumors such as gliomas produce high levels of VEGF in culture (56, 65). The decreased number of vessels for the cyclo-VEGI-treated tumors is consistent with a direct inhibition of VEGF signaling (as observed in our in vitro experiments) which in turn impairs tumor growth. This is also in agreement with an indirect (i.e. anti-angiogenic) antitumor effect rather than a direct antiproliferative effect on the tumor cells as evidenced by the absence of effect of cyclo-VEGI on the proliferation of tumor cells in vitro and in vivo. Histological analysis of cyclo-VEGI-treated gliomas showed characteristic findings observed in tumors treated with other potent anti-angiogenic compounds like endostatin and angiostatin. Although the proliferation index remained unchanged in tumor tissue irrespective of treatment, cyclo-VEGI decreased microvessel density which was accompanied by an increase in the apoptotic index as observed previously for other angiogenesis inhibitors (66, 67).

Taken together, these results indicate that cyclo-VEGI exhibits unique structural features and inhibits angiogenesis and tumor development in vivo. Furthermore, its inhibitor activity affects multiple angiogenesis pathways. Cyclo-VEGI is a promising candidate for the development of new cyclic angiogenesis inhibitor molecules useful for the treatment of cancer or other angiogenesis-related diseases.

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