Domain Swapping in a COOH-terminal Fragment of Platelet Factor 4 Generates Potent Angiogenesis Inhibitors

Martin Hagedorn, Lior Zilberberg, Jörg Wilting, Xavier Cannon, Giorgio Carrabba, Carlo Giussani, Mauro Pluderi, Lorenzo Bello, and Andreas Bikfalvi

Institut National de la Santé et de la Recherche Médicale EMI 0113 Moleculaire Mechanisms of Angiogenesis, Université de Bordeaux I, 33 405 Talence, France. [M. H., L. Z., X. C., A. B.]; Department of Neurosurgery, Department of Neurological Sciences, University of Milano, Ospedale Maggiore Policlinico, Istituto di Ricovero e Cura a Carattere Scientifico, 20122 Milan, Italy [G. C., E. G., M. P., L. B.]; and Universitätskinderklinik Göttingen, Abteilung Pädiatrie I, 37570 Göttingen, Germany [J. W.]

ABSTRACT

A few peptide residues in structurally important locations often determine biological functions of proteins implicated in the regulation of angiogenesis. We have shown recently that the short COOH-terminal segment PF-447–70 derived from platelet factor 4 (PF-4) is the smallest sequence that conserves potent antiangiogenic activity in vitro and in vivo. Here we show that modified COOH-terminal PF-4 peptides containing the sequence ELR (or related DLR), a critical domain present in proangiogenic chemokines, surprisingly elicit several times greater antiangiogenic potential than the original peptide. The modified peptides inhibit binding of iodinated vascular endothelial growth factor and fibroblast growth factor 2 to endothelial cell receptors, endothelial cell proliferation, migration, and microvessel assembly in the rat aortic ring model at lower doses than PF-447–70. On the differentiated chick chorioallantoic membrane, angiogenesis induced by vascular endothelial growth factor is significantly inhibited binding of iodinated VEGF or FGF-2 to cellular receptors, both essential for normal development and tumor angiogenesis (15–17).

Different strategies have been applied to block VEGF/VEGFR interactions; these include monoclonal antibodies (17, 18), tyrosine kinase inhibitors (19), inhibitory peptides (20), and soluble dominant-negative receptors (21).

PF-4 belongs to the CXC-chemokines, which, in general, are proangiogenic when the tripeptide ELR precedes the first CXC-domain, but are antiangiogenic when this motif is absent (22). Exceptions are growth-related protein β, which contains an ELR motif but inhibits angiogenesis in vitro and in vivo (23), and the ELR-negative stromal-derived factor 1, which shows proangiogenic effects in vitro and in vivo (24, 25). Administration of full-length tetrameric (ELR-negative) PF-4 restrains tumor growth and metastasis in mice models via inhibition of angiogenesis (26, 27). Survival of mice is prolonged by transducing established intracerebral glioma with an adenoviral vector encoding a secreted form of PF-4 (28). PF-4 exerts its effects most likely via interfering with FGF-2 and VEGF binding to receptors (29, 30). This activity is conserved in a COOH-terminal portion of the chemokine (9).

Domain swapping experiments between chemokines implicated in angiogenesis and hematopoiesis have revealed regions of importance for their biological functions. Replacing the NH2-terminal DLQ domain of PF-4 with the corresponding ELR motif of the angiogenic CXC-chemokines IL-8 or neutrophil-activating protein 2 confers CXCR-2-dependent neutrophil activation of these proteins to PF-4 (31, 32). On the other hand, mutation of ELR to DLQ in IL-8 greatly reduces its biological activity (33). Thus, the ELR motif plays a decisive role in defining biochemical behavior in this family of proteins.

For that reason we tried to reverse biological activities of PF-447–70, described recently as the smallest antiangiogenic portion of PF-4 (8) by inserting ELR or DLQ mutations. The modified peptides surprisingly inhibited binding of iodinated VEGF or FGF-2 to cellular receptors at several times lower concentrations than the unmodified peptide and aborted VEGF or FGF-2-induced EC proliferation but not glioma cell growth. Using a newly developed antiangiogenesis assay on the differentiated CAM, where capillary angiogenesis is induced in the stroma by VEGF, we show antiangiogenic effects of the modified peptides at a dose where the unmodified peptide was inactive. Enhanced inhibitory effects on tumor growth at lower doses than with the original peptide were observed in an intracranial glioma model. Taken together, our results show that a few amino acid residues within an antiangiogenic peptide can determine to a great extent the biological activity in vitro and in vivo. Modified PF-4

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peptides represent good candidates for the development of peptide-based antiangiogenic drugs for cancers with high expression of VEGF and FGF-2.

MATERIALS AND METHODS

Synthetic Peptides. COOH-terminal PF-4 peptides (PF-447–70, NGRK-CLDLQAPLYKIIKKLLES; PF-447–70LRR: NGRKICL-DLAPLYKIIKKLLES; PF-447–70LDR: NGRKICL-DLAPLYKIIKKLLES; and PF-447–70SNG: NGRKISL-DLAPLYKIIKKLLES) were purchased from ThermoFisher (Ulm, Germany) or were donated by Drs. Hervé Prats (Institut National de la Santé et de la Recherche Médicale U397, Toulouse, France) and stored in sterile, double-distilled water at −80°C. Recombinant human VEGF165 was produced in insect cells and purified by high-performance liquid chromatography to >95%, and calculated molecular weight was confirmed by mass spectrometry.

Growth Factors. Recombinant human FGF-2 was kindly provided by Dr. Hervé Prats (Institut National de la Santé et de la Recherche Médicale U397, Toulouse, France) and stored in sterile, double-distilled water at −80°C. Recombinant human VEGF165 was produced in insect cells and purified as described elsewhere (34, 35). Human VEGF165-encoding baculovirus was a kind gift of Dr. Jean Plouet (Institut de Pharmacologie et de Biologie Stucturale, UMR 5089, Toulouse, France).

Cells. BCE cells were a kind gift of Dr. Daniel B. Rifkin (New York University Medical Center, New York, NY). BAEC cells were from Dr. Georg Breier (Department of Molecular Biology, Max-Planck-Institut fur physiologische und klinische Forschung, Bad Nauheim, Germany). ACE cells were donated by Dr. Jean-Jacques Feige (Institut National de la Santé et de la Recherche Médicale EPI 0105, CEA-Grenoble, France). All of the ECs were grown at 37°C, 5% CO2 in DMEM, 10% NBCS, and 1% glutamine and antibiotics (Life Technologies, Inc., Cergy Pontoise, France), and were used up to passage 25. BCE and ACE cells were grown in the presence of 2 ng/ml of FGF-2. C6 rat glioma cells (a kind gift from Dr. Paul Canioni, Centre National de la Recherche Scientifique UMR 5536, Université Bordeaux II, Bordeaux, France) were grown in DMEM, 10% FBS and antibiotics. U87 glioma cells (American Type Culture Collection) were grown in MEM α medium, 10% FBS, plus antibiotics. H15 insect cells were a kind gift of Dr. Jean Plouet and were cultured without CO2 at 28°C in IPL41 insect cell medium (Life Technologies, Inc.) containing 10% FBS, t-glutamine, and antibiotics.

Binding Assays. VEGF165 and FGF-2 were labeled with Na125I using Iodogen (Pierce, Rockford, IL) as a coupling agent according to the manufacturer’s instructions (36). ACE cells were seeded at 2.5 × 10^7 density in gelatin-coated six-well plates and cultured in complete medium for 2 days. Cells were washed twice with ice-cold PBS, and incubated with 10 ng/ml 125I-labeled FGF-2 and peptides at indicated concentrations in binding medium [DMEM, 20 mM HEPES (pH 7.4), and 0.15% gelatin] for 2 h on a shaker at 4°C. 125I-labeled FGF-2 from high and low affinity binding sites was recovered and quantified as described earlier (8). 125I-labeled VEGF binding was evaluated essentially in the same manner, except that cells were detached (2% Triton, 10% glycerol, and 1 mg/ml BSA) before gamma counting. Each condition was tested in duplicates. Data are expressed as percentage of total radioactivity, and IC50 for every peptide was calculated from smooth curve fits.

Proliferation Assays. ACE cells were seeded in six-well culture plates overnight in 10% NBCS at 5000–7500 cells/well. Medium was changed to 1% NBCS and 10 ng/ml FGF-2, and peptides at indicated concentrations were added to duplicate wells. FGF-2-stimulated controls were not treated. After 48 h, medium was changed, and stimulation with FGF-2 and peptide treatment repeated. One day later, cells were counted on a Coulter counter. For VEGF165-induced cell proliferation (10 ng/ml), BAEC cells were used in conditions similar to ACE cells. IC50 were determined from smooth curve fits. U87 (C6) glioblastoma cells were seeded at 5000 (25000) cells/well in 10% FBS, treated with peptides at the same concentrations as ECs, and counted 72 h (48 h) later.

Migration Assay. Migration tests with BAE or BCE cells were performed using a method described earlier (8, 37). In brief, ECs were seeded in 35-mm culture plates and were allowed to grow to confluence. Complete medium was replaced with serum-free DMEM, and incubation was continued overnight. One linear scar was drawn in the monolayer and divided into five 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). The dishes were washed, and fresh serum-free medium containing 0.1% BSA, 10 ng/ml FGF-2 (BAE migration), or 10 ng/ml VEGF165 (BAE migration) and peptides were added. Peptide concentration was 5 μM for BAE cells and 10 μM for BCE cells. After 18 h, cells were fixed in 1% glutaraldehyde, counterstained (Giemsa), and a second set of photos was taken. Photos were superposed, and ECs that migrated across the line drawn at the border of the scar in the first photo set were counted. Each condition was tested in duplicates in two independent experiments. Means for all fields of each group were calculated, background migration subtracted, and plotted as percentage of the mean of untreated stimulated control.

Rat Aortic Ring Model. Cultures of rat aortic rings were prepared as described earlier (8). Briefly, aortics rings from male or female Sprague Dawley rats, between 200 and 500 grams, (IFFA CREDO, L’Arbresle, France or HARLAN, Gannat, France) were placed in 24-well dishes in collagen gel and serum-free MCDB131 medium. Rings were treated with peptides on days 0, 2, and 4, and photos were taken on day 7; controls received no peptide or peptide PF-4. Microvessel length and number were measured using digital image analysis software (Lucia G). Statistical analysis of the differences in vessel length and vessel number between the treatment groups was performed by the ANOVA analyses of variance followed by Student’s Newman-Keul pair wise comparison (CRUNCH Software Corporation, Oakland, CA).

CAM Antiangiogenesis 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 after punctuating the air chamber and sealed with Durapore tape. On day 13, plastic rings (made from Nunc Thermoxan coverslips) were put on the CAM. Three μg of VEGF165 was premixed with 20 μg of peptides or with the equivalent volume of sterile water alone, and deposed in the center of the plastic ring. Treatment was repeated the following day. On day 17, the CAMs were fixed in vivo with 4% paraformaldehyde for 30 min at room temperature, and the area containing the ring was cut out for additional analysis. Photos of each CAM were taken under a stereomicroscope (Nikon SMZ800) using a digital camera (Nikon Coolpix 950). Two observers scored the inhibition of VEGF165-induced angiogenesis from 0 to 2 (0 = none, 1 = medium, and 2 = high). Means of the obtained scores were analyzed by the Kruksal-Walls one-way ANOVA and pair wise comparisons of the different treatment groups (CRUNCH Software Corporation). For histological studies, samples were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.12 M sodium cacodylate buffer, postfixed in 1% osmium solution, immersed with uranyl acetate, and embedded in Epon resin (Serva, Germany). Semithin sections (0.75 μm) were cut from samples using an Ultracut S microtom (Leica, Bensheim, Germany) and stained with 1% methylene blue and 1% azure II (Fluka, Buchs, Switzerland). Photos (×200 magnification) were taken using a Leica DMR microscope.

Intracranial Glioma Model. Groups of 10 6-week-old nude mice (Charles River Italia, Monza, Italy) were implanted intracranially with 50,000human U87 glioma cells using an open window technique (38). After 15 days, animals were implanted with 2004 Alzet osmotic minipumps (ALZET, Cupertino, CA). The pump reservoir was connected to an intracranial catheter, placed slightly posterior to the tumor cell injection site, in the same hemisphere. The pump reservoir was filled with 0.25, 0.5, or 1 mg of the peptide in PBS. Control groups received pumps containing PBS or no pumps at all. Animals were sacrificed 30 days after pump implantation. Brains were removed, immediately frozen in liquid nitrogen, and embedded in OCT. Sections (5 μm) were made and processed for histology. Tumor volumes were measured from histology sections using the ellipsoid formula (width × length/2; Ref. 38). Statistical analysis of tumor volumes was performed with a two-way ANOVA (peptide × dose) followed by analyses of simple main effects to compare the effects of the three peptides at each dose. Pairwise comparisons between peptides at each dose were performed by the Newman-Keuls post-test. Immunohistochemistry was carried out using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Anti-CD31 antibody (PharMingen, San Diego, CA) was used at 1:100 dilution. Signal was visualized with 3,3′-diaminobenzidine chromogen, and sections were counterstained with hematoxylin. Microvessel counts and density were scored as reported previously (39). Apoptotic

cells were detected with the ApopTag plus kit (Intergen, Purchase, NY) and quantified as described earlier (39). Animal studies (intracranial glioma model) were approved by the ethical committee of the University of Milano.

RESULTS

Modified PF-4-derived Peptides Inhibit Binding of VEGF and FGF-2 to Their Receptors. First, peptides were tested for their ability to interfere with binding of $^{125}$I-labeled FGF-2 and $^{125}$I-labeled VEGF165 to capillary ECs, which express receptors for both families of growth factors (36, 40). A concentration-dependent inhibition of binding was observed with modified peptides. IC$_{50}$ necessary to inhibit $^{125}$I-labeled VEGF$_{165}$ binding to its receptors were 0.4 $\mu$M for PF-4$^{47-70}$DLR, 0.56 $\mu$M for PF-4$^{47-70}$ELR, and 1.58 $\mu$M for PF-4$^{47-70}$ (Fig. 1A). IC$_{50}$ for inhibition of FGF-2 binding to low or high (in brackets) affinity sites were 0.75 $\mu$M (0.46 $\mu$M) for PF-4$^{47-70}$DLR, 1.51 $\mu$M (0.53 $\mu$M) for PF-4$^{47-70}$ELR, and 3.47 $\mu$M (2.06 $\mu$M) for peptide PF-4$^{47-70}$ (Fig. 1A, B and C). Control peptide PF-4$^{47-70}$S at the highest concentration tested in this assay did not compete for receptor binding.

Inhibition of EC but Not Glioma Cell Proliferation. EC proliferation is a key step in the angiogenic process, and strongly inducible by VEGF$_{165}$ (35) and FGF-2 (41). When BAE cells were stimulated by VEGF, PF-4$^{47-70}$DLR showed the strongest inhibitory activity (IC$_{50}$ 1.57 $\mu$M; Fig. 2A), followed by PF-4$^{47-70}$ (2.15 $\mu$M) and PF-4$^{47-70}$ELR (2.91 $\mu$M). The differences were more pronounced for FGF-2-induced ACE cell proliferation: PF-4$^{47-70}$ELR and PF-4$^{47-70}$DLR exhibited IC$_{50}$ values of 1 $\mu$M and 2.34 $\mu$M in comparison to 11.17 $\mu$M for PF-4$^{47-70}$ (Fig. 2B). In contrast, serum-induced U87 or C6 glioma cell proliferation was not affected by the peptides (C).

![Fig. 1.](image1) ACE cells were incubated with 10 ng/ml of $^{125}$I-labeled VEGF$_{165}$ (A) or $^{125}$I-labeled FGF-2 (B and C) and peptides at indicated concentrations. Control peptide PF-4$^{47-70}$S at the highest dose tested (20 $\mu$M) had no effect. IC$_{50}$ were calculated from smooth curve fits. Peptide PF-4$^{47-70}$DLR inhibited $^{125}$I-labeled VEGF binding to VEGFRs at approximately three times lower concentrations and PF-4$^{47-70}$DLR at four times lower concentrations than peptide PF-4$^{47-70}$ (A). FGF-2 binding to low affinity receptors (LA FGR) was affected in a similar way: PF-4$^{47-70}$ELR inhibited binding nearly five times stronger than that of PF-4$^{47-70}$ (B). FGF-2 binding to high affinity receptors (HA FGR) was inhibited at approximately four times lower concentrations by peptides PF-4$^{47-70}$ELR and PF-4$^{47-70}$DLR than by peptide PF-4$^{47-70}$ (C).

![Fig. 2.](image2) Proliferation was stimulated with VEGF$_{165}$ using BAE cells (A) or FGF-2 and ACE cells (B). PF-4$^{47-70}$, PF-4$^{47-70}$ELR, and PF-4$^{47-70}$DLR inhibited cell proliferation in a concentration-dependent manner; peptide PF-4$^{47-70}$S did not show any effects at all concentrations tested. Horizontal line at 0 indicates conditions where 100% inhibition of VEGF- or FGF-2-induced proliferation is reached. IC$_{50}$s were determined from smooth curve fits and were in the micromolar range, with the following order of activity (highest to lowest): PF-4$^{47-70}$DLR, PF-4$^{47-70}$, PF-4$^{47-70}$ELR for VEGF-induced proliferation and PF-4$^{47-70}$DLR, PF-4$^{47-70}$ELR, PF-4$^{47-70}$ for FGF-2-induced proliferation. In contrast, serum-induced U87 or C6 glioma cell proliferation was not affected by the peptides (C).
Inhibition of Micro- and Macrovascular EC Migration. Without stimulation, some random background migration occurred (Fig. 3A). BAE cells migrated into the denuded scar area when stimulated with VEGF<sub>165</sub> (Fig. 3B) and, in a very similar way, when FGF-2 was added to BCE cells (data not shown). We tested the modified peptides at a concentration where PF-4<sup>-47</sup>–<sup>-70</sup> was inactive or much less active (5 μM on BAE cells and 10 μM on BCE cells). PF-4<sup>-47</sup>–<sup>-70</sup>ELR strongly inhibited EC migration (Fig. 3, E and G); PF-4<sup>-47</sup>–<sup>-70</sup>DLR induced complete inhibition of VEGF-induced migration (Fig. 3, F and G). PF-4<sup>-47</sup>–<sup>-70</sup>S showed no effect (Fig. 3C) and PF-4<sup>-47</sup>–<sup>-70</sup> showed only a small one (Fig. 3, D and G). One representative BAE migration experiment is shown; modified peptides showed a similar increase in activity compared with PF-4<sup>-47</sup>–<sup>-70</sup> in FGF-2-induced BAE migration (data not shown).

Blocking of Microvessel Assembly in the Rat Aortic Ring Model. In the absence of peptides, aortic rings develop an extensive network of sprouting microvessels after 5–7 days (Fig. 4A). PF-4<sup>-47</sup>–<sup>-70</sup>S at the highest concentration tested in this assay did not show any effects on vessel length or number (Fig. 4, E and F). A strong reduction of the mean microvessel length was observed only for peptide PF-4<sup>-47</sup>–<sup>-70</sup>ELR at 10 μM compared with the same dose of PF-4<sup>-47</sup>–<sup>-70</sup> (P < 0.05) or PF-4<sup>-47</sup>–<sup>-70</sup>ELR [P < 0.002; Newman-Keul posthoc test after ANOVA analysis of all groups: F(7.79) = 11.23; P < 0.001; Fig. 4, D–F].

Statistical analysis of peptide effects on microvessel number showed that groups differed significantly from each other [one-way ANOVA: F(7.79) = 28.37; P < 0.001]. Inhibition (40%) was observed in cultures treated with 5 μM PF-4<sup>-47</sup>–<sup>-70</sup> and 63.5% at 10 μM compared with PF-4<sup>-47</sup>–<sup>-70</sup>S. Mean vessel number was reduced by 33% at 5 μM (40% at 10 μM) in cultures treated with PF-4<sup>-47</sup>–<sup>-70</sup>ELR compared with control peptide PF-4<sup>-47</sup>–<sup>-70</sup>S. Fifty percent inhibition was reached at 5 μM, and 90% inhibition was achieved at 10 μM in cultures treated with PF-4<sup>-47</sup>–<sup>-70</sup>ELR (Fig. 4F). Thus, in this assay, only peptide PF-4<sup>-47</sup>–<sup>-70</sup>DLR showed a clear increase in inhibitory activity in comparison to PF-4<sup>-47</sup>–<sup>-70</sup> Posthoc pairwise comparison between PF-4<sup>-47</sup>–<sup>-70</sup>DLR and PF-4<sup>-47</sup>–<sup>-70</sup> or PF-4<sup>-47</sup>–<sup>-70</sup>ELR proved the difference in activity to be significant [P < 0.001; Newman-Keul: F(7.79) = 11.23].

Inhibition of VEGF-induced Angiogenesis on the CAM. Three μg of recombinant human VEGF<sub>165</sub> applied in the center of a plastic ring on the surface of the CAM induced strong capillary formation (Fig. 5B). The effect of VEGF is also present around the site of application because of diffusion. Water alone had no effect (Fig. 5A). When premixed with the growth factor and deposited on the CAM, a clear antiangiogenic effect of peptide PF-4<sup>-47</sup>–<sup>-70</sup>ELR and PF-4<sup>-47</sup>–<sup>-70</sup>DLR was visible inside the ring (Fig. 5, C and D). PF-4<sup>-47</sup>–<sup>-70</sup>ELR also causes a hyperplasia of the chorionic epithelium, comparable with hyperosmolar effects (Fig. 5C, inset).

Control peptide PF-4<sup>-47</sup>–<sup>-70</sup> did not inhibit VEGF-induced capillary growth in the CAM stroma (Fig. 5E). At the low dose tested, PF-4<sup>-47</sup>–<sup>-70</sup> failed to stop capillary growth in this assay (Fig. 5F) and did not differ from the control peptide (P < 0.05; after analysis of differences between all treatment groups, Kruskal-Wallis test: H
stroma, filled with erythrocytes. VEGF165 induces a strong capillary layer stays within the chorionic epithelium ( tiers B, arrows, inset in F). Control peptide PF-447 had several times greater tumor volumes, and the two groups did not differ significantly from peptide-treated animals at all doses (P > 0.0001). The modified peptides PF-447-70 S and PF-447-70 DLR both showed an equally strong antiangiogenic effect at the dose tested compared with PF-447-70 S (**P < 0.002 for both peptides; Fig. 5G). In semi-thin sections of control CAMs treated with sterile water, the capillary layer stays within the chorionic epithelium (Fig. 5A, arrows, inset); large conduit vessels are present in the stroma, filled with erythrocytes. VEGF165 induces a strong de novo growth of capillaries, which are present throughout the stroma (arrows, inset in B). Control peptide PF-447-70 S does not neutralize the capillary angiogenesis induced by VEGF165 (inset in E). PF-447-70 at the low dose tested (2 × 20 μg) also does not inhibit capillary angiogenesis (inset in F). PF-447-70 ELR and DLR both markedly decreases angiogenesis nearly to control levels (insets in C and D).

Inhibition of Intracerebral Tumor Growth. Animals treated with control peptide PF-447-70 S, with PBS, or those without pumps developed large tumors (Fig. 6A, top panel). Animals receiving different doses of peptide PF-447-70 DLR or PF-447-70 showed a strong reduction of tumor volumes (Fig. 6A, lower panel). Immunohistochemical analysis with CD31 antibody revealed that inhibition of tumor growth in animals treated with PF-447-70 DLR or PF-447-70 is associated with a decreased vessel density (Fig. 6B) and an increased number of apoptotic cells (Fig. 6C). Statistical analysis of tumor volumes of animals treated with the equal dose of active peptides (PF-447-70 and PF-447-70 DLR) revealed a significant difference in antitumor efficacy and showed a clear advantage of peptide PF-447-70 DLR, especially at the lowest dose tested, 0.25 mg (**P < 0.005).

DISCUSSION

PF-447-70 is the smallest antiangiogenic fragment of PF-4 with strong activity in vitro and in vivo (8). The peptide contains a DLQ motif, essential for inhibition of myeloid progenitor proliferation by full-length PF-4 (33). When we replaced it with the ELR tripeptide, present in proangiogenic chemokines like IL-8 or neutrophil-activating protein 2, we expected a peptide with proangiogenic activity. In contrast, antiangiogenic activity was enhanced compared with PF-447-70 in a number of assays. An even stronger effect was observed with PF-447-70 DLR (Q56 to R), which had initially been designed as a control for PF-447-70 ELR, because the ELR to DLR mutation in the N-terminal of IL-8 greatly diminishes its function (33). FGF-2 and VEGF165 binding to tyrosine kinase receptors on ECs is strongly suppressed by PF-447-70 ELR and DLR. Endostatin also inhibits VEGF165 binding to VEGFR-2, but the effect seems to depend greatly on preincubation of endostatin with cells before addition of iodinated VEGF165 (42). Disturbing VEGF and FGF-2 functions at the receptor level leads to impairment of fundamental angiogenic events such as EC proliferation and migration. Modified peptides inhibited both micro- and macrovascular EC proliferation and migration induced by VEGF165 or FGF-2 in the low micromolar range. Comparable results have been obtained with a designed peptide inhibitor termed “Angi- inex,” a 33-mer β-sheet-forming peptide containing sequences from PF-4, IL-8, and bactericidal-permeability increasing protein (43). However, Anginex doses necessary for inhibition of EC migration were several times higher than those of PF-447-70 ELR or DLR, and only FGF-2-induced angiogenesis has been studied. COOH-terminal PF-4 peptides inhibit EC proliferation below 1% serum controls from a certain concentration on, which suggests that residual VEGF and/or FGF-2 (or other mitogenic factors) present in serum were also inhibited. Peptide PF-447-70 DLR was a strong inhibitor of vascular sprouting, but growth of fibroblastic cells was not affected, which suggests that the peptide antagonizes preferentially factors supporting EC growth and organization. PF-447-70 ELR did not inhibit microvessel sprouting very well in this assay. Because the ELR motif is crucial for receptor activation of the proangiogenic chemokine IL-8 (44), it is possible that PF-447-70 ELR stimulates chemokine receptors on resid-

Fig. 6. Locally administered peptide PF-447-70 or PF-447-70 DLR strongly reduced tumor volumes of established intracerebral tumors in nude mice at all three doses (A, bottom panel). PF-447-70 DLR displayed a greater antitumor activity than PF-447-70 at all doses. This difference was more pronounced at the lowest dose (0.25 mg) than at the two higher doses (two-way ANOVA followed by the Newman-Keuls post-test: 0.25 mg: +P < 0.005; 0.5 mg: +P < 0.02; 1 mg: +P < 0.05). PBS-treated animals or animals without pump (controls) had several times greater tumor volumes, and the two groups did not differ significantly from each other (Student’s t test; t = 30, degrees of freedom = 18; NS, A, top panel). Control peptide PF-447-70 did not have any influence on tumor growth and volumes differed significantly from peptide-treated animals at all doses (P < 0.0001). Reduced microvessel numbers and elevated apoptosis indices suggest inhibition of angiogenesis as the pathophysiological mechanism of action in animals treated with PF-447-70 or PF-447-70 DLR (B and C).
ional leukocytes and ECs (45) present in the aortic wall, which may partially override the direct inhibitory effects on endogenous VEGF and FGF-2. Endostatin completely inhibits vascular sprouting in the aortic ring assay at 500 μg/ml (46), whereas PF-4/70-DLR shows maximal activity between 14 and 28 μg/ml (5–10 μM), thus, at 18–35 times lower concentrations. This strong activity is probably because the peptide interferes with both, VEGF and FGF-2, by inhibition of binding to their receptors.

We tested effects of COOH-terminal PF-4 peptides for their ability to interfere with VEGF-induced angiogenesis on the differentiated day 13 CAM (47), to our knowledge the first attempt to study angiogenesis inhibitors in this modified and rigorous type of assay. The typical brush-like formation of capillaries in the stroma of the CAM induced by human recombinant VEGF165 is strongly reduced by PF-4/70-DLR and –ELR. In some eggs, PF-4/70-DLR and –ELR also showed some minor activity, but this may result from interindividual variations, the high sensitivity of the assay system, or the difficulty to exactly quantify differences between low inhibitory effects. Metastasin, a recently described angiogenesis inhibitor, was active in the day 10 CAM assay at a two times higher dosage. Additionally, angiogenesis was induced with VEGF at a 300-fold lower dose as in our assay (48). Cyclic peptide antagonists for αVβ3-integrin showed strong antiangiogenic effects in the day 10 CAM at 300 μg; again, the angiogenic response was induced by VEGF or FGF-2 at doses <1 μg (49). These comparisons indicate that modified COOH-terminal PF-4 peptides inhibit VEGF-induced angiogenesis in vivo in a very efficient way. Both peptides start with the NGR tripeptide, a motif, which has been shown recently to be a homing sequence to angiogenic blood vessels (50). This might also contribute to their strong in vivo activity. Glioma growth is strongly promoted via up-regulation of angiogenesis by VEGF and FGF-2. U87 glioma cells produce high levels of these growth factors in culture (51). Two-hundred-fifty μg of peptide PF-4/70-DLR were already sufficient to reduce tumor growth to one-half compared with PF-4/70-treated animals. This effect is paralleled by an increased reduction of blood vessels within the tumor, and elevated tumor and/or EC cell apoptosis. Increased apoptosis in tumors is commonly observed after treatment with angiogenesis inhibitors (52–54). Similar results have been obtained previously by systemic treatment of U87 glioma-bearing mice with the antiangiogenic molecule PEX alone or in combination with low-dose chemotheraphy. When PEX was included in the treatment, animals survived significantly longer and showed no measurable side effects (38, 39).

Binding of FGF-2 or VEGF165 to tyrosine kinase receptors is facilitated by heparan sulfate proteoglycans on the cell surface and is enhanced by heparin-binding peptides of PF-4 consisting of two double-lysine clusters (56), operating in part via heparan sulfate proteoglycans on the cell surface and is facilitated by heparan sulfate proteoglycans on the cell surface and is enhanced by heparin-binding motifs of PF-4. These motifs are also present in the internal R49. Similar observations have been made with peptides mimicking the surface of endostatin: an increase of arginine residues enhances their antiangiogenic effects probably because of an increased affinity for heparin (58).

It has been shown recently that unmodified PF-4/70-DLR associates directly with FGF-2, independently of glycosaminoglycan binding (7), leading to a conformational change of the growth factor. It is possible that the increase in activity observed with the modified peptides is also because of an increase in affinity for FGF-2 and perhaps VEGF too. Taken together, modified COOH-terminal pep-

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ANGIOGENESIS INHIBITION BY MODIFIED PF-4 PEPTIDES


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