N° d'ordre : **2550**

THÈSE

présentée à

L'UNIVERSITÉ BORDEAUX I

ÉCOLE DOCTORALE SCIENCES DU VIVANT, GEOSCIENCES,
SCIENCES DE L'ENVIRONNEMENT

par **Dr Martin HAGEDORN**

POUR OBTENIR LE GRADE DE

DOCTEUR

SPÉCIALITÉ : **Biologie et Physiologie cellulaire**

Caractérisation et développement des inhibiteurs peptidique de l'angiogenèse provenant du facteur plaquettaire 4

Soutenue le 25 Septembre 2002

Après avis de :

M.COURTOIS, Yves	Directeur de recherche INSERM	**Rapporteurs**
Mme EICHMANN, Anne	Chargé de recherche CNRS	

Devant la commission d'examen formée de :

M. ROBERT, Jacques	Professeur à l'Université Bordeaux II	**Président**
M. COURTOIS, Yves	Directeur de recherche INSERM	**Rapporteur**
Mme EICHMANN, Anne	Chargé de recherche CNRS	**Rapporteur**
M. BIKFALVI, Andreas	Professeur à l'Université Bordeaux I	**Examinateur**
M. NURDEN, Alan	Directeur de recherche CNRS	**Examinateur**

- 2002 -

Avant-propos

Les travaux présentés dans cette thèse ont été réalisés au sein du laboratoire INSERM EMI 0113 “Mécanismes moléculaire de l’angiogenèse”, exepté les travaux portant sur les gliomes chez la souris « nude » qui ont été effectués à l’Université de Milan en Italie.

Je tiens à remercier le Professeur Andreas BIKFALVI pour m’avoir accueilli dans son laboratoire et pour avoir inspiré et dirigé cette thèse, ainsi que tous les membres du laboratoire qui m’ont aidé pendant ces travaux, en particulier Xavier CANRON.

Je tiens à exprimer au Professeur Joerg WILTING (Universitaet Goettingen, Allemagne) toute ma reconnaissance pour m’avoir consacré beaucoup de son temps à l’enseignement de l’utilisation du modèle de la membrane chorio-allantoic chez le poulet ; et pour ces conseils avisés.

Je remercie Jean Plouët (IPBS, UMR 5089, TOULOUSE) et Sylvie POUSSARD (ISTAB USC-INRA 429) pour m’avoir aidé à la production et la purification du VEGF.

Je tiens à remercier les Docteurs Anne EICHMANN, Yves COURTOIS, Alain NURDEN et le Professeur Jacques ROBERT qui m’ont fait l’honneur de participer à ce jury.

Je remercie également les organismes qui m’ont soutenu financièrement pour la réalisation de ma thèse: l’Académie Nationale de Médecine, l’Association française de diabète (ALFEDIAM/AFD), la Fondation de France (Fondation Berthe Fouassier), la Fédération des Aveugles et handicapés visuels de France (FAF), et la Fondation pour la Recherche Médicale (FRM).

RESUME en français

L'angiogenèse joue un rôle important dans les pathologies comme le cancer, la rétinopathie diabétique ou l'arthrite rhumatoïde. Elle est contrôlée par un équilibre entre des facteurs stimulants et inhibiteurs endogènes. Nous avons caractérisé dans la région C-terminale du facteur plaquettaire 4, un inhibiteur naturel, la séquence peptidique essentielle à son activité. Ce peptide montre des effets anti-angiogéniques dans de multiples tests d'angiogénèse in vitro et in vivo. Le changement d'un acide aminé dans sa séquence supprime son activité ($C^{52}S$) alors que l'introduction d'un autre la potentialise ($Q^{56}R$). Cette potentialisation a été révélée par l'inhibition de la croissance 1) des vaisseaux sanguins induite par le VEGF sur la membrane chorio-allantoïdienne de l'embryon de poulet et 2) des cellules tumorales de type gliome chez des souris nudes. Ces peptides sont des bons candidats pour le développement des drogues anti-angiogéniques pour le traitement du cancer, notamment des glioblastomes.

TITRE en anglais

Characterization and developpement of peptide angiogenesis inhibitors derived from platelet factor 4

RESUME en anglais

Angiogenesis plays a major role in pathologies like cancer, diabetic retinopathy and rheumatoid arthritis. It is controlled by a balance between angiogenic growth factors and endogenous inhibitory molecules. We have characterized a C-terminal fragment of platelet factor 4, a natural inhibitor of angiogenesis, essential for its activity. This peptide has anti-angiogenic activity in various in vitro and in vivo assays. Changing one amino acid within the primary sequence yields a completely inactive peptide (C52S), whereas another change (Q56R or D54E/Q56R) potentializes its activity. The augmentation in efficacy has been shown in 1) an anti-angiogenesis assay on the chick chorio-allantoic membrane, and 2) inhibition of tumor growth of established intracerebral glioma in nude mice. These peptides are thus good candidates for the development of anti-angiogenic drugs for the treatment of cancer, especially for glioblastoma.

DISCIPLINE - SPECIALITE DOCTORALE

Biologie et physiologie cellulaire

MOTS-CLES

angiogenesis, vascular endothelial growth factor, platelet factor 4, fibroblast growth factor, peptide inhibitors, chick chorio-allantoic membrane

INTITULE ET ADRESSE DE L'U.F.R. OU DU LABORATOIRE

Pr Andreas BIKFALVI - Université Bordeaux I, INSERM EMI 0113 'Mécanismes Moléculaires de l'Angiogenèse' Avenue des Facultés, 33405 Talence, FRANCE

Mention: Très honorable

SUMMARY

INDEX OF FIGURES AND TABLES

INTRODUCTION

Historical background: identification of the first angiogenic factors

During the last 30 years, significant progress has been made to understand the biological basis of new blood vessel growth. This process, termed “angiogenesis”, generally describes the growth of new capillaries out of pre-existing vessels. Figure 1 illustrates the rapid growth of this new field of research in comparison to cancer-related research and total scientific publications.

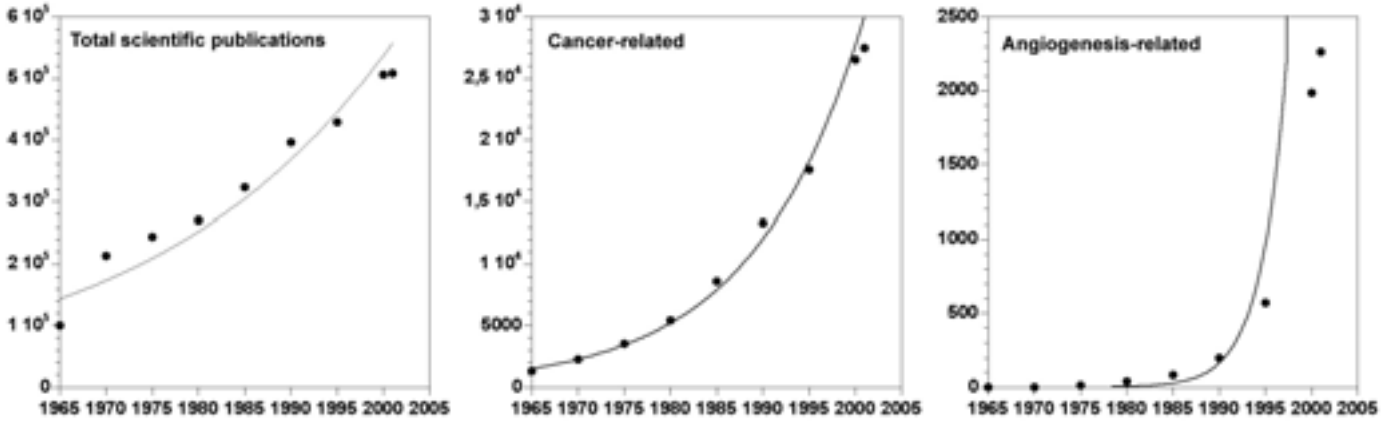

Figure 1: Emergence of angiogenesis-related research.

A Medline search (PubMed, NLM) with the keywords “angiogenesis” or “cancer” (no word for total publications) in abstract was performed in 5-year steps (except 2000 / 2001). Curve fits were calculated using KaleidaGraph software. Note that number of scientific publications increases steadily, cancer-related papers grow in a slow exponential manner, whereas angiogenesis-related research shows a rapid progression since 1980.

The observation that malignant tumor growth may be related to an alteration in blood vessel morphology is over 100 years old and has initially described by Virchov in the 19th century and Goldmann in the beginning of the 20th century. In 1945, Algire and Chalkley have shown that tumors transplanted in the skin of cats evoke a more aggressive vascular response than other transplanted tissue. Rondoni conclued in 1946, that tumor vasculature originates from capillaries of the surrounding stroma (reviewed in (1)).

Judah Folkman's working hypothesis was that a growing tumor would need to attract new blood vessels from the host body via soluble (growth) factors. He isolated the first angiogenic factor in 1971 from rat carcinoma cell fractions, termed 'tumor angiogenesis factor' (2). In the 80s, the first angiogenic proteins were isolated using their high affinity for heparin. Acidic fibroblast growth factor (FGF-1) was purified from bovine pituitary gland or bovine brain (3, 4), whereas basic FGF (FGF-2) was isolated from placenta or hepatoma cells (5, 6). Few years later, researchers identified another heparin-binding molecule from pituary glands, which was a selective mitogen for endothelial cells (7-9). This molecule, today known as vascular endothelial growth factor (VEGF), was found to be identical to a molecule already described by Senger in 1983 for its induction of a reversible hyper-permeable effect on blood vessels (10).

Mechanisms of blood vessel formation

Sprouting angiogenesis

There are several distinct ways how new blood vessels can be formed in physiological and pathological conditions. One well-known mechanism is termed "sprouting angiogenesis". It can be divided in three phases: initiation, growth and maturation. The driving force behind the sprout formation process is a metabolic deficit (hypoxia, hypoglycemia, etc.), which must be eliminated by providing nutrients via blood supply in order to maintain tissue integrity and to avoid necrosis. During the first phase, a pre-existing vessel dilates and becomes leaky in response to VEGF and NO (nitric oxide). Proteinases digest the basement membrane and endothelial cells start to migrate into the extracellular space through gaps in the vessel wall. Angiopoietin 2 (Ang2), a ligand for the Tie-2 receptor and antagonist to Ang1, the other Tie-2 ligand, may additionally provide plasticity by detaching mural cells (11). During the growth phase, ECs continue to migrate and proliferate in response to FGF-2 and VEGF. Vascular integrins like $\alpha v\beta 3$ and $\alpha v\beta 5$ are upregulated on activated ECs and mediate spreading and migration within the surrounding matrix (12). Ang1 at this stage may start to tighten up the newly formed sprout (13). During the maturation phase, mural cells are attracted to the vessel wall. This interaction requires platelet derived growth factor-B (secreted by ECs) and the presence of PDGFR-β on pericytes and vascular smooth muscle cells (14). TGF-beta and Ang1/Tie-2 signaling is also

required for PC recruitment and vessel stabilization. Proteinase inhibitors like plasminogen activator inhibitor 1 (PAI-1) protect the newly formed extracellular matrix from degradation through proteinases and are necessary for angiogenesis (15).

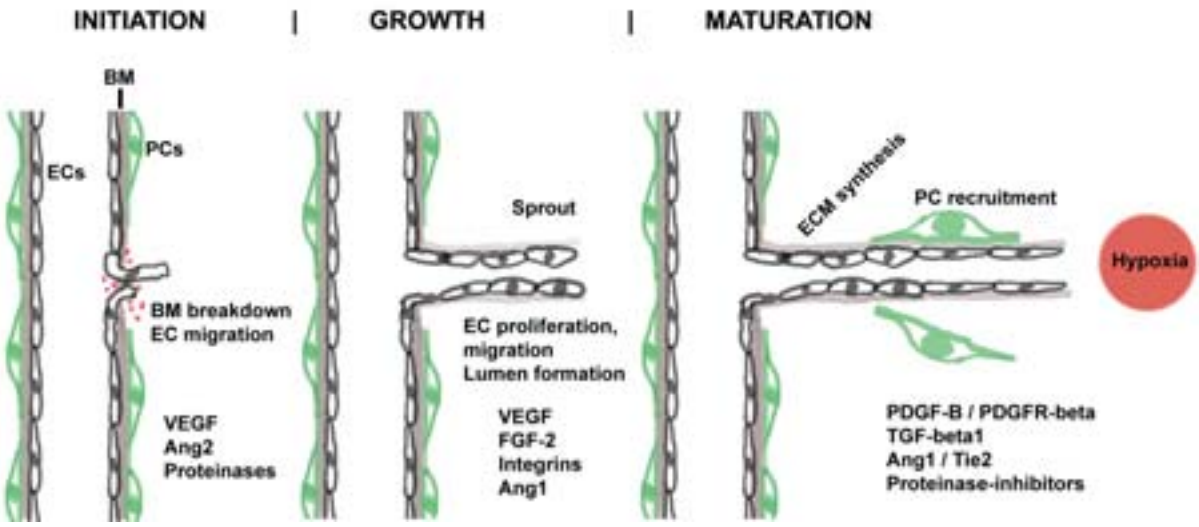

Figure 2: Sprouting angiogenesis.

Sprouting angiogenesis towards hypoxic stimuli can be divided in three phases: initiation, which requires basement membrane breakdown; growth, which is characterized by EC proliferation and migration, and finally maturation, with ECM synthesis and mural cell recruitment.

Intussusceptive angiogenesis

Intussusceptive microvascular growth (IMG) is characterized by the forming of intra-capillary pillars, which fuse and cause the capillary to divide into two new ones. It is of great importance during the formation of the vascular network in the kidney and the lung, but also occurs during tumor angiogenesis. The vasculature of the chick chorio-allantoic membrane is mainly expanded by intussusceptive growth between day 8 to day 12 of development (16). Recent findings suggest that a variant of IMG, intussusceptive branching remodeling (IBR), where pillars fuse at the bifurcation point of two vessels, is an optimized and fast way used by blood vessels to adapt to constantly changing hemodynamic forces during development (17).

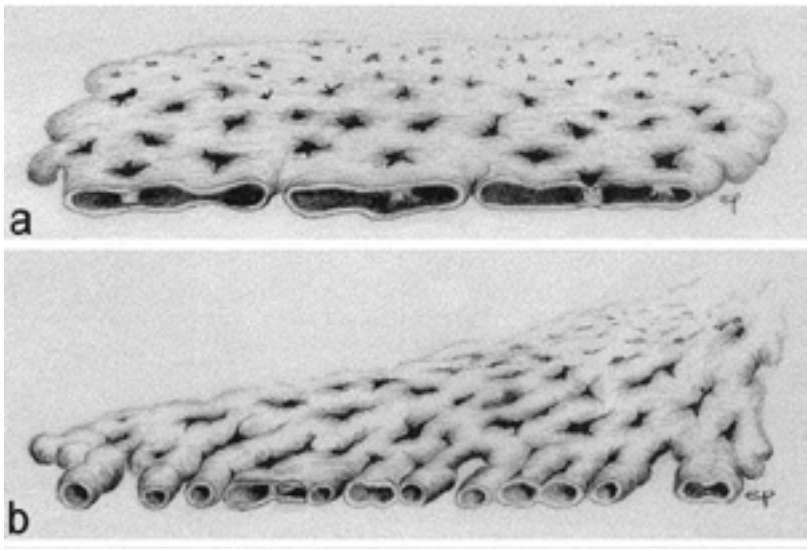

Figure 3: Intussusceptive angiogenesis.

Intussusceptive angiogenesis, a form of non-sprouting angiogenesis, is used to expand vascular networks in the lung, kidney or, here, in the chick chorio-allantoic membrane. It is characterized by the forming of intra-capillary pillars, which fuse and give rise to new capillaries (figure from (18)).

Pre- and postnatal vasculogenesis

The term vasculogenesis generally refers to differentiation of endothelial cell precursors (angioblasts) from mesoderm and the formation of a primitive vascular plexus (19). Mesodermal cells differentiate into angiogblasts, and associate into structures called blood islands, lined by angioblasts and filled with hemopoietic precursor cells. Important molecules implicated in these differentiation steps are VEGF and its receptor 2 (VEGFR-2), the latter being one of the earliest markers of cells committed to differentiation towards the endothelial cell lineage (for review see (19)). PECAM-1 (CD31), CD34 and VE-cadherin are also expressed by angioblasts and play distinct roles during cell adhesion, and later, lumen formation and vascular permeability. Fibronectin and its receptor $\alpha 5\beta 1$ integrin are vital during early vascular development (20). Interestingly, the αv integrin subunit present in integrins important for pathological angiogenesis (21) does not seem to play a major role during vasculogenesis in mice. αv null mice die *in uteri* due to placentation defects but 20% are born alive and die because of hemorrhages in brain and the intestines later on (22).

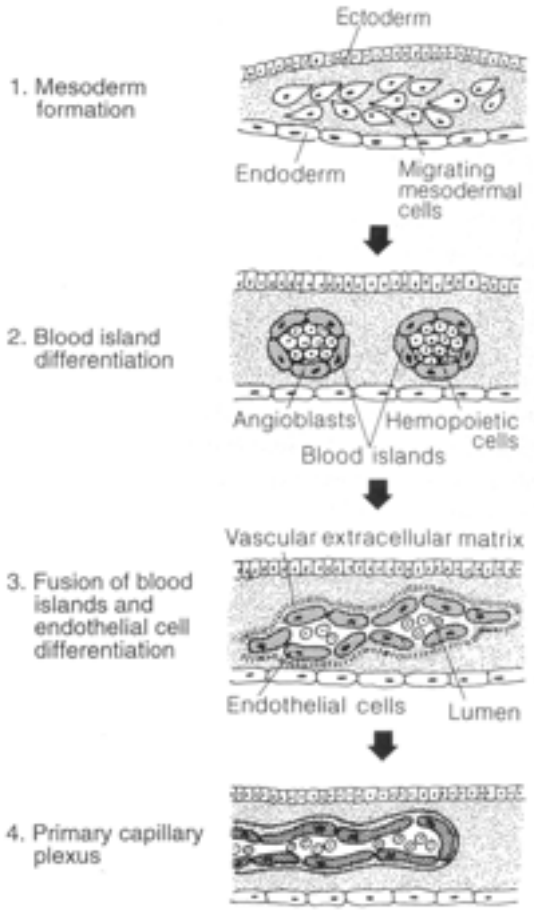

Figure 4: Vasculogenesis.

Events occurring during vasculogenesis (taken from Flamme & Risau, 1995, (19)): After differentiation of mesodermal cells into angioblasts and hemopoietic cells, blood islands containing first endothelial cells form, fuse and differentiate into a primary capillary plexus.

Endothelial cell progenitors also play a role during vascularisation processes in the adult. Angioblasts have been detected in peripheral blood of adult (23) and intraperitoneal injections of VEGF in mice increase the number of CD34-, VE-cadherin- and VEGFR-2-positive cells in the blood stream. There is some evidence that VEGF and PlGF mediate mobilization of angioblasts from bone marrow to circulation (24, 25). Circulating angioblasts can integrate into blood vessels at sites of wound healing, in ischemic tissue in the heart and the hindlimb and in transplanted tumors (26). Recent studies suggest the use of endothelial progenitors as pro-angiogenic treatment for ischemic vascular diseases (27, 28). Another group successfully used pluripotent cells for anti-angiogenic therapy: modified bone marrow-derived cells, which express a truncated soluble form of VEGFR-2, are incorporated in tumor vessels and can restrict tumor growth when grafted into the bone marrow of mice bearing neuroblastoma or Wilm's tumors

(29). These data suggest a role of postnatal vasculogenesis in physiological and pathological angiogenesis and open up new perspectives to include endothelial cell precursors in pro- or anti-angiogenic therapy strategies.

Arterial – venous differentiation

After the primary vascular plexus has formed by vasculogenesis, it is further remodeled and expanded by interdigitation, branching and sprouting, hence angiogenesis. Recently, molecular mechanisms have been discovered which may determine the arterial or venous fate of developing blood vessels from a very early time point on. Wang et al. described the selective expression of the ephrin-B2 membrane ligand on arterial endothelial cells and its receptor Eph-B4 on venous ECs. Their ephrin-B2 null mice revealed that proper interaction of this receptor-ligand pair is required for remodeling of veins as well as for arteries in the primary capillary plexus in the yolk sac and the head (30). Other proteins expressed in arteries are the notch ligand delta-like 4 (DLL4) (31) and the transcription factor gridlock (32). The latter is required for development of the aorta in zebrafish and the DLL4 - notch1/4-receptor system may be important for angiogenesis during development (33).

A murine isoform of VEGF, $VEGF_{188}$, plays also a role in arterial-venous patterning in the retina. Mice selectively expressing $VEGF_{188}$ have normal venules, but impaired arterial development. The same authors also found neuropilin-1 (NRP1), a co-receptor for some VEGF isoforms, predominantly expressed on arterioles (34).

During early embryonic chick development, neuropilin-2 (NRP2) is found on blood vessels on the venous side, whereas NRP1 is expressed on arteries (35). NRP1-expressing cells from grafted quail arteries can integrate into veins and arteries in the chick during early stages, but from embryonic day 11 on, they prefer to associate with arteries. The same could be observed for ECs from grafted quail vein fragments; they preferentially home into veins when grafted into chick after day 11. During early phases of development a certain “plasticity” regarding the arterial or venous outcome of ECs is thus preserved and lost later on. Isolated ECs grafted into chick embryos do not show any preference for arterial or venous colonization (36). Vessel wall components may negatively influence plasticity of ECs in regard to arterial or venous differentiation.

Using the same technique of quail-chick chimera, another group has reported that grafted arterial endothelial cells defined by the ephrin-B2 marker keep up the expression when integrated in chick arteries but lost expression when they integrated in veins. Vein-derived ephrin-B2-negative ECs, which integrated in arteries, acquired the ephrin-B2 marker (37). This study reveals a decisive role of the vessel microenvironment for the selective expression of arterial-venous markers.

Angiogenesis modulators

Fibroblast growth factors (FGFs)

Fibroblast growth factors are a large family of related growth factors. More than 20 different members have been identified by this writing, FGF-23 has recently been cloned and shown to be expressed preferentially in the ventrolateral thalamic nucleus (38). Fibroblast growth factors play important roles during development, angiogenesis, tissue remodeling, and carcinogenesis. Conditioned medium of normal mouse mammary cells transfected with a retroviral vector carrying FGF-4 cDNA, induced angiogenesis in vitro in human umbilical vein endothelial cells (HUVECs) via an autocrine induction of VEGF (39). Recombinant adenovirus expressing human FGF-5 has been shown to improve blood flow in the heart, associated with angiogenesis, in a model of stress-induced myocardial ischemia (40). Pro-angiogenic effects also have been described for FGF-8: mouse mammary tumor cells transfected with the murine splicing variant FGF-8b give rise to highly vascularized tumors in nude mice. Pro-angionic effects in the CAM-assay as well as in vitro assays are also reported (41).

However, FGF-1 and –2 are the two members of the fibroblast growth factor family, which are most intensively studied in the field of angiogenesis.

A single copy gene for FGF-2 encodes for 18, 22, 22.5 and 24 kDa variants; the three latter isoforms are produced by initiation of translation at three CUG codons located upstream from the classical AUG initiation site (42). The FGF receptors consist of four transmembrane tyrosine kinases which give rise to multiple isoforms as a result of alternative splicing (43, 44).

Knock-out studies of the FGF-2 gene have not revealed a specific function, but FGF-2 deficient mice exhibit an altered migration of neurons to the cortex at birth

and are hypotonic when adult (45-47), another group reported delayed skin wound healing in FGF-2 null mice (48). On the other hand, mice deficient for FGFR-1 are not viable and show abnormal organization of early embryonic events (47), thus no further conclusion regarding a specific role for this receptor during vasculogenesis or angiogenesis can be drawn from these experiments. Mice expressing a dominant-negative form of FGFR-1 develop retinal degeneration due to photoreceptor cell loss and show abnormal choroidal angiogenesis (49). In humans, mutations in the FGFRs are closely linked to craniofacial dysostosis syndromes (50).

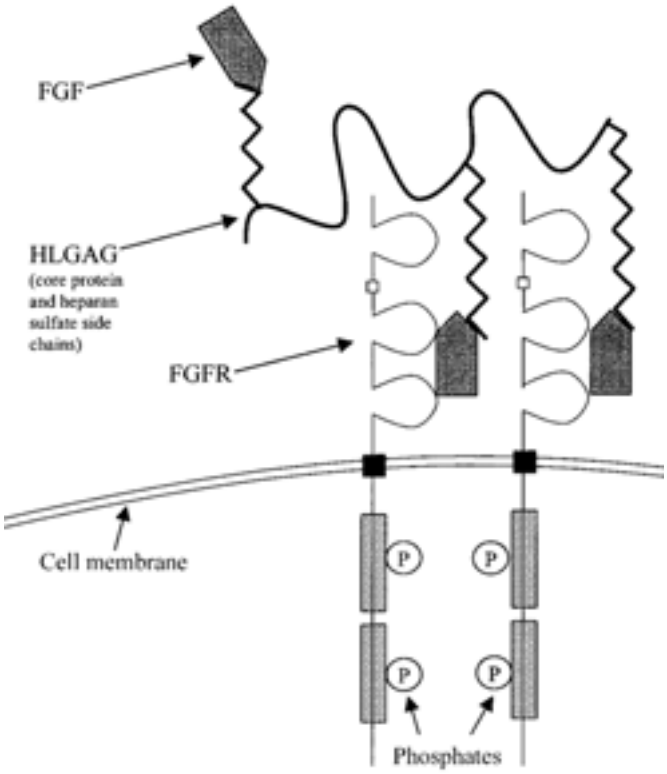

Figure 5: Interaction of FGF-2 with tyrosine kinase receptors and glycosaminoglycans.
(Taken from Powers et al. (51)): FGFs activate their receptors by inducing receptor dimerization, an event which is facilitated by HLGAGs (heparin-like glycoaminoglycans).

An important role in tumor angiogenesis has also been clearly established for FGF-2. Compagnie et al. have generated a recombinant adenovirus encoding for soluble FGFRs, which strongly inhibited the induction and maintenance of tumor angiogenesis and tumor growth in nude mice transplanted with cell lines derived from pancreatic β-cell carcinoma. The same virus strongly suppressed tumor

growth in the RIP1-Tag2 transgenic mouse model (52). In this model, 100% of animals normally develop pancreatic β-cell tumors (53).

Numerous antibodies against different regions on FGF-2 have been raised and display anti-tumor efficacy in vivo (54, 55). In particular, antibodies against the heparin-binding domain seem to have strong activity in vivo (55). This is confirmed by an interesting study where peptides containing the FGF-2 heparin-binding domain or the receptor-binding domain were used to vaccinate mice. Only mice vaccinated with the heparin-binding domain peptide showed a reduced angiogenic response in a gelfoam sponge model of angiogenesis, and experimental metastasis was inhibited by more than 90% in two tumor models (56). When C6 glioma cells were transfected with a dominant-negative form of FGFR-1 or -2 and implanted in immunodeficient mice, smaller and less vascularized tumors developed (57). These studies clearly show that FGF-2 and its receptors have important roles in pathological angiogenesis.

FGF-2 signaling

Inhibition of FGFR signaling has become an interesting target for drug developers. A pyrimidine derivate (PD166866) with nanomolar affinity for the kinase domain of FGFR-1 efficiently block microvessel outgrowth of cultured human placental arteries (58). A tyrosine kinase inhibitor of FGFR1, PD173074, has potent anti-angiogenesis properties *in vitro* and *in vivo*; oral treatment of mice with breast cancer leads to prolonged survival and enhanced efficacy of photodynamic therapy (59). The molecule displays also selectivity for VEGFR-2 (at much higher doses) and corneal angiogenesis induced by FGF-2 or VEGF can be effectively inhibited (60). High-resolution structure analyses have revealed that it binds to the ATP-binding cleft of FGFR1 (60).

Another kinase inhibitor, SU6668, which targets not only FGF-R but also PDGFR and VEGFRs is a very active agent against established human tumors like melanoma or glioma transplanted in athymic mice (61, 62). Other potential targets for anti-angiogenic therapies are phosphatidylinositol-3' kinase (PI3K) and protein kinase C isoforms (63, 64). To achieve full inhibition of FGF-2-induced endothelial cell proliferation, both PI3K and ERK1/2 pathways have to blocked: each pathway alone mediates 50% of FGF-2 mitogenic activity (65).

Increased insight in structure-activity relationships of synthetic small molecules with tyrosine kinases may generate highly selective and effective new drugs in the future.

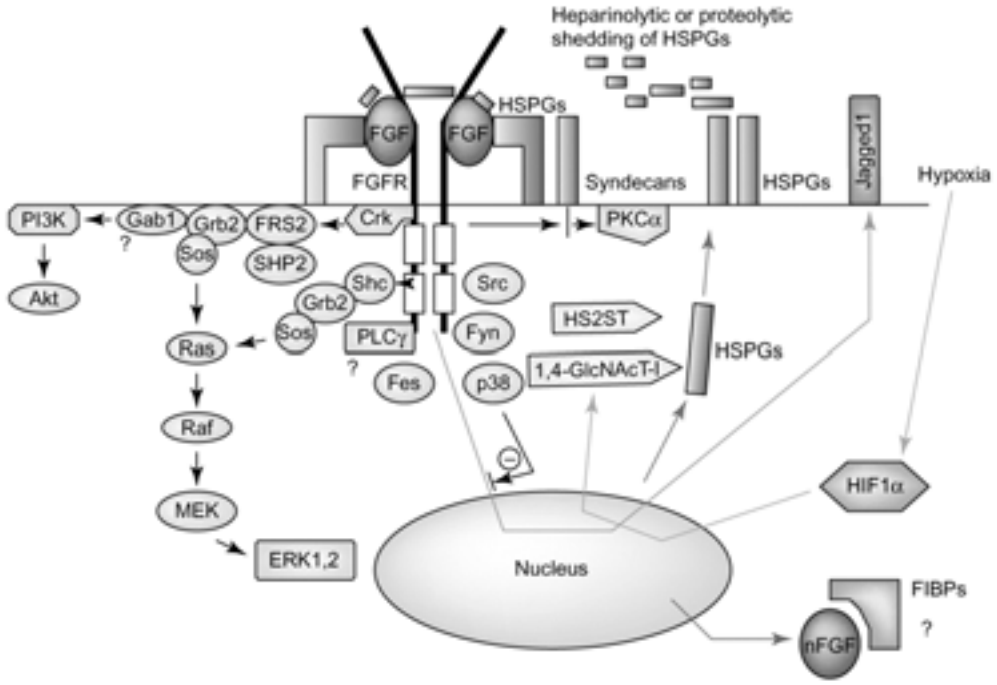

Figure 5: FGF-2-induced signaling cascades

FGF-2 binding leads to receptor dimerization and phosporylation of specific tyrosine residues, which trigger signal pathways inducing different biological responses. Signal molecules like Src and Fyn mediate tubulogenesis, whereas activation of ERK1/2 via the Ras-Raf-MEK pathway induces EC migration. PI3K is connected to the receptor via docking proteins like Grb2 and Crk and interacts with Akt to support endothelial cell survival. HIF1a may enhance binding of FGF-2 by increasing HSPGs on the cell surface (diagram from (66).

Abbreviations: *ERK, extracellular-signal-regulated kinase; FIBP, FGF-binding protein; FRS2, FGFR substrate-2; GlcNAcT-I, N-acetylglucosamine transferase I; HIF1α, hypoxia inducible factor-1α; HS2ST, heparan sulfate 2- Osulfotransferase; HSPG, heparan sulfate proteoglycan; MAPK, mitogen-associated protein kinase; MEK, MAPK kinase; nFGF, nuclear FGF; PI3K, phosphatidyl inositol 3 kinase; PKC-α, protein kinase C-α; PLC-γ, phospholipase C–γ.*

Vascular endothelial growth factors (VEGFs)

VEGFs are some of the few endothelial cell specific growth factors discovered to date, even though evidence for a relation of this factor with nerve growth and physiology is emerging (for review see (67)). Six VEGF isoforms ($VEGF_{121, 145, 165, 183, 189, 206}$) are generated by alternative splicing and exhibit different biochemical

properties; variant $VEGF_{165}$ represents the most abundant form and is one of the strongest angiogenic growth factors (68, 69). Beside the originally described VEGF (VEGF-A) five other forms of VEGF have been identified: VEGF-B (70), VEGF-C (71), VEGF-D (72, 73), VEGF-E (74) and the related placenta growth factor (PlGF) (75). Recently, a novel organ-specific form of a heparin-binding vascular endothelial growth factor, named EG-(endocrinal gland) VEGF has been cloned and found to specifically promote growth of endothelial cells found in steroidogenic endocrinal glands (76). EG-VEGF is not structurally related to the other VEGFs, but it shares biological activities with them: induction of EC proliferation, migration and fenestration.

Other biological entities have evolved molecules similar to VEGF. For example, VEGF-E is encoded by the Orf parapoxvirus, which induces angiogenic lesions in infected subjects. This form of VEGF binds to and activates VEGFR-2 like its mammalian counterpart (74). Another VEGF-like molecule called “increasing capillary permeability protein” (ICPP) has recently been isolated from snake venom. It shares about 50% homology with human VEGF and has biological activities similar to VEGF, which could be abrogated by a VEGFR-2 tyrosine kinase inhibitor (77).

VEGF receptors VEGFR-1 (synonym flt-1) and VEGFR-2 (KDR/flk-1) are expressed in endothelial cells of blood vessels, whereas the expression of VEGFR-3 (flt-4) is restricted to lymphatic endothelium (69). Accessory receptors are neuropilin-1 and –2, which can enhance biological activities of VEGFR-2 and $VEGF_{165}$ (78).

Targeted gene inactivation experiments have revealed the importance of the VEGF growth factor family for normal development. Knock-out mice with only a single allele of the VEGF gene die during early embryonic stages due to impaired blood-island formation (79, 80). Inactivation of VEGFR-1 results in a deadly form of abnormal blood vessel assembly (81); and mice deficient for VEGFR-2 have no yolk-sac blood islands and severely reduced number of hemangioblasts (82). Targeted gene inactivation of VEGFR-3 leads to embryonic lethality due to malformation of large vessels (83).

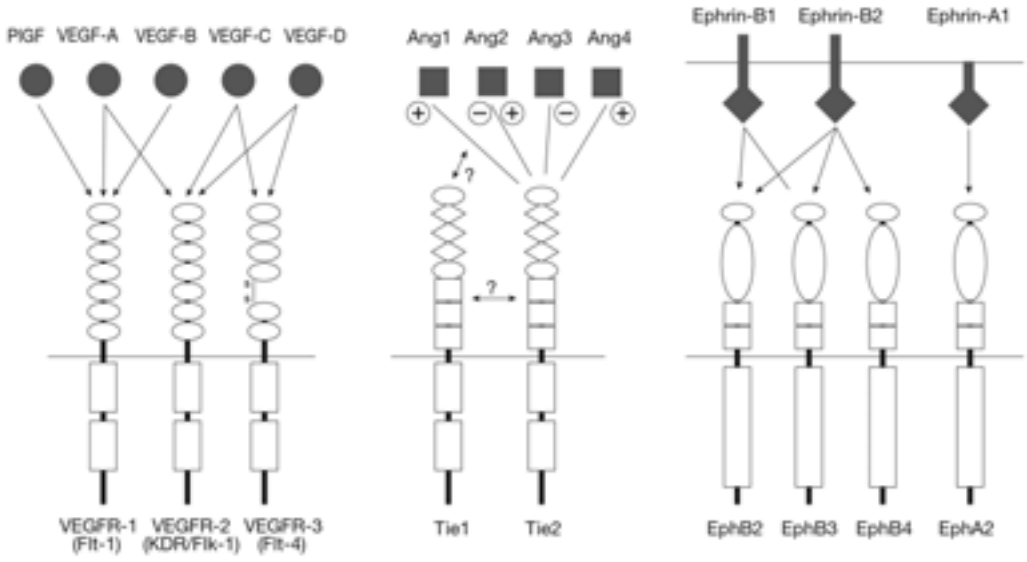

Figure 6: Vascular growth factors and their receptor specificity.

(Taken from Yancopoulos et al., (84)): The three families of vascular growth factors known to date. VEGFs bind to tyrosine kinase receptors with different affinities and specificities. VEGFR-2 is the main receptor for $VEGF_{165}$ and responsible for most of the growth and permeability actions of the growth factor. VEGFR-1 has a higher affinity for $VEGF_{165}$ but a much lower tyrosine kinase activity, suggesting a role as a decoy receptor. VEGFR-3 is predominantly expressed in lymphatic ECs and is activated by VEGF-C. Angiopoietin-1 and -2 bind to the Tie-2 receptor, both receptor-ligand interactions are crucial for normal development. Little is known about Ang3 and -4 and the role of Tie-1. Amongst the large Eph receptor family, ephrin-B2 ligand and its EpHB4 receptor play specific roles in during vascular development, possibly by determining the arterious or venous fate of a vessel.

A recent study has shown that VEGF-A has a unique role in tumor angiogenesis, which cannot be replaced by other growth factors or even other VEGFs. In a modified RIP1-Tag2 mouse model of pancreatic β-cell cancer, where the VEGF-A gene was inactivated in pancreatic β-islets, only small “hollow” tumors with very limited vasculature developed upon transgene activation (85). These experiments show that members of the VEGF / VEGFR system have distinct, irreplaceable roles during development and tumor angiogenesis and suggest the absence of redundancy in this ligand-receptor system.

One variant of VEGF, VEGF-C (71), is a ligand for VEGFR-3 which is predominantly expressed on lymphatic endothelial cells and it co-localizes in the lymphatic network with this receptor (86). VEGF-C induces lymphangiogenesis in vivo if over-expressed in transgenic mice (87) or deposited on the chick-

chorioallantoic membrane (88). Recent findings suggest that VEGF-C might play a pivotal role in tumor metastasis. When transgenic animals overexpressing VEGF-C under the control of the insulin promoter (RIP-VEGF-C) were crossed with the RIP1-Tag2 mice, which develop a non-metastatic form of pancreatic β-cell cancer, metastasis occurred to regional lymph nodes via the newly formed lymphatic vessels surrounding the β-islets (89).

Given the unique roles of the VEGFs and their receptors during vasculogenesis and physiological angiogenesis, it is of obvious interest to explore anti-VEGF strategies in pathological conditions such as cancer, retinopathy and rheumatoid arthritis, all diseases, whose progression is angiogenesis-dependent (90).

VEGF signaling

An obvious challenge for drug discovery is to determine the role of intra-cellular signaling molecules during the different phases of angiogenesis. VEGF-A binds to two receptor tyrosine kinases, VEGFR-1 and –2. Little is known about the physiological importance of VEGFR-1 signaling. Two ligands for this receptor, PlGF and VEGF-A, do not induce migration or proliferation of normal or VEGFR-1-transfected endothelial cells, but migration of monocytes seems to be mediated by PlGF or VEGF binding to VEGFR-1 (91, 92) Apparently, signaling activity of VEGFR-1 is not required for normal development; transgenic mice lacking the intracellular tyrosine domain are viable (93). Recent work of Carmeliet and colleagues points towards an important function of VEGFR-1 during pathological angiogenesis. Mice deficient for PlGF, a high affinity ligand for VEGFR-1, fail to exhibit a normal response towards numerous angiogenic stimuli such as healing wounds, ischemia and tumors. Bone marrow of normal mice transplanted into PlGF (-/-) mice restored angiogenic responses, suggesting a role of the PlGF/VEGFR-1 receptor system in recruiting angioblasts (25).

Binding to VEGFR-2 leads to receptor auto-phosphorylation and initiation of a complex signaling cascade involving specific enzymes and adaptor proteins like Grb2, Nck and Shc. The signals converge to the Raf-MEK-MAPK pathway, resulting in gene transcription. Specific inhibitors of MAPK kinases (e.g. PD98059) are able to abolish VEGF pro-angiogenic effects and are thus potential anti-cancer drugs. It should be noted however, that the very efficient inhibitor of VEGF signaling, SU5416, which blocks angiogenesis in various *in vitro* and *in vivo* studies (94) has now been retrieved from clinical studies due to inefficacy

(95). To achieve good anti-angiogenic response in humans, it might be necessary to target more than one signaling pathway. This is of importance, since VEGF alone is able to induce endothelial cell proliferation using pathways (e.g. PKCs), which are not used by FGF-2 or epidermal growth factor (96). Very promising results come from synthetic molecules with a simultaneous inhibitory activity against different PKC isoforms and other tyrosine kinase receptors: administered orally to mice protects against new blood vessel growth in a hypoxia model of retinal neovascularization (64, 97).

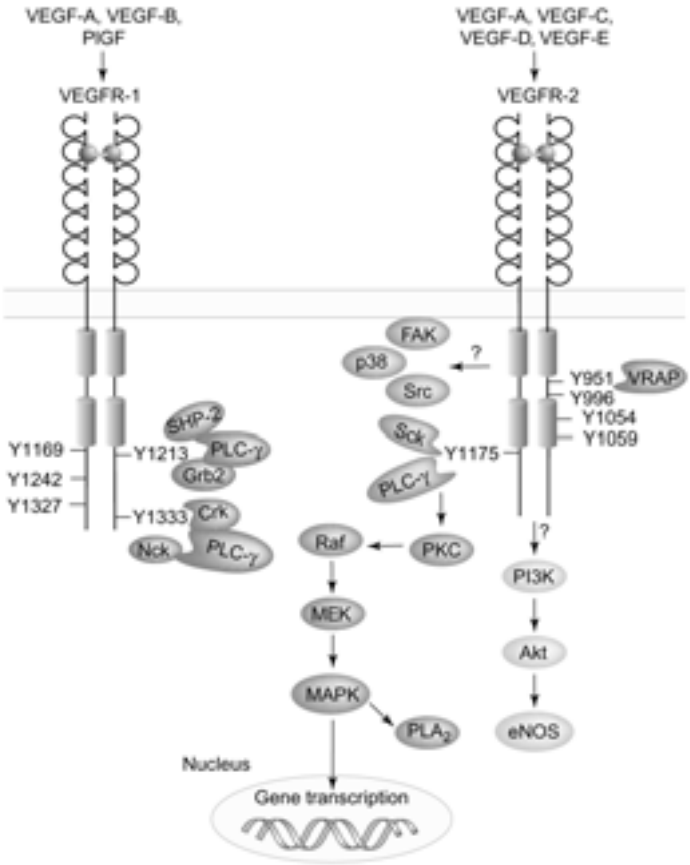

Figure 7: Signaling pathways of the VEGF receptors 1 and –2.

PlGF, VEGF-1 and –B constitute ligands for VEGFR-1, whereas VEGF-A, -C, -D and –E bind to VEGFR-2 (schema taken from (98)). Tyrosine residues (Y) implicated in activation of signaling cascade are shown. Several signaling molecules can interact directly with Y1213 and Y1333 of VEGFR-1. Downstream effector molecules can mediate specific physiological events. For example, activation of PLA_2 or eNOS is associated with permeability effects of VEGF, whereas Akt and PI3K mediate survival functions.

Abbreviations: *eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PI3K, phosphoinositide 3-kinase; PLA 2 , phospholipase A 2 ; PLC ,phospholipase C ; PKC, protein kinase C; Sck, Shc-like protein; SH2, src – homology domain 2; SHP-2, SH2 phosphatase 2; VRAP, VEGF receptor-associated protein.*

Endogenous angiogenesis inhibitors

The first anti-angiogenic activities were isolated from shark cartilage extracts (99, 100). Shark cartilage had been chosen as a potential source for anti-angiogenic molecules because of the natural absence of blood vessels in this tissue and the peculiar fact that sharks almost never develop cancer. Today, numerous single molecules with anti-angiogenic effects have been isolated from cartilage from different species. These include troponin I, a component of human cartilage (101), U995, a peptide inhibitor (102), and Neovastat, which has already entered phase III clinical trials. Protamine was also one of the first angiogenesis inhibitors discovered (103). Like platelet factor 4 (104), it has high affinity for heparin, an important co-factor for angiogenic growth factors like FGF-2 and VEGF (for reviews see (105)).

Folkman's group has made an interesting observation several years ago: When the primary tumor of mice bearing Lewis lung cell carcinoma was removed, metastases started to grow rapidly. This led to the suggestion, that the primary tumor secretes a (anti-angiogenic) factor, which restrains the growth of metastases. They could isolate a protein from serum and urine from these mice, which was named angiostatin (106). Amino acid sequencing revealed that angiostatin is a fragment of plasminogen, a precursor of the anticoagulant protein plasmin. Using a similar approach, the same group discovered later a protein called endostatin (107), which was isolated in vitro from cell culture supernatants of hemangioendothelioma. Endostatin causes regression of primary tumors in animal models. Interestingly, it is also a fragment of a larger protein, collagen XVIII. It is thus possible that numerous proteins of various functional biological systems (eg. clotting cascade, basement membrane) undergo controlled cleavage by yet unknown mechanisms to generate molecules that participate in the endogenous regulation of angiogenesis.

The first evidence of an anti-angiogenic protein directly linked to the process of carcinogenesis was given by the discovery of thrombospondin-1 (108). Loss of the wild-type allele of the p53 tumor suppressor gene, a hallmark of malignant cell growth, results in reduced expression of thrombospondin-1 and subsequent acquisition of an angiogenic phenotype (109). This could thus be a mechanism by which cancer cells circumvent the physiological anti-angiogenic state of

quiescent adult endothelium. The other way round, this is also true: a human fibrosarcoma cell line (HT1080), which had retained the active p53 tumor suppressor gene, prevents the growth of experimental B16/F10 melanoma metastases in the lungs of nude mice due to the production of high levels of thrombospondin-1 (110). TSP-1 most likely exerts its anti-angiogenic effects in vivo by inducing apoptosis mediated by the CD36 receptor in microvascular endothelial cells (111).

Beside these first endogenous inhibitors, numerous molecules have been discovered, which are parts of larger proteins with functions not primarily related to angiogenesis.

FULL PROTEIN	ANTI-ANGIOGENIC FRAGMENT
Plasminogen	Kringle 1-4: angiostatin (106)
Plasminogen	Kringle 5 (112)
Prothrombin	Prothrombin kringle-1 & kringle-2 domains (113, 114)
Fibrinogen	Fibrinogen E fragment (115)
Thrombin	aAT (carboxyl-terminal loop) (116)
Tissue factor	Cytoplasmatic tail (117)
Collagen XVIII	Endostatin (C-terminus, 185 aa) (107)
Collagen XV	Restin (118)
Collagen IV (α2 chain)	Canstatin (119)
Calreticulin	Vasostatin (120)
Prolactin, growth hormone variant, placental lactogen	N-terminal 16-kDa fragments (121, 122)
MMP-2	PEX (123)
Fibronectin	III1-C (Anastellin) (124)

Table 1: Anti-angiogenic molecules derived from other proteins.

This table shows a summary of anti-angiogenic peptides and protein fragments that are part of larger proteins, whose primary biological functions are not related to angiogenesis.

Publication I describes in more detail the major groups of molecules implicated in the regulation of angiogenesis and how the insight gained by fundamental research has led to the development of anti-angiogenic strategies to fight diseases like cancer, rheumatoid arthritis and retinopathies.

PUBLICATION I

“Target molecules for anti-angiogenic therapy: from basic research to clinical trials”

by Martin Hagedorn and Andreas Bikfalvi

Published in Critical Reviews in Oncology and Hematology 34 (2000) 89–110

ELSEVIER

Critical Reviews in Oncology/Hematology 34 (2000) 89–110

Critical Reviews in Oncology Hematology

www.elsevier.com/locate/critrevonc

Target molecules for anti-angiogenic therapy: from basic research to clinical trials

Martin Hagedorn, Andreas Bikfalvi [1]*

Laboratoire des Facteurs de Croissance et de la Différenciation cellulaire (Growth Factor and Cell Differenciation Laboratory), Bâtiment de Recherche Biologie Animale, Avenue des Facultés, Université de Bordeaux I, 33405 Talence, France

Accepted 19 October 1999

Contents

Abstract

There is growing evidence that anti-angiogenic drugs will improve future therapies of diseases like cancer, rheumatoid arthritis and ocular neovascularisation. However, it is still uncertain which kind of substance, out of the large number of angiogenesis inhibitors, will prove to be a suitable agent to treat these human diseases. There are currently more than 30 angiogenesis inhibitors in clinical trials and a multitude of promising new candidates are under investigation in vitro and in animal models. Important

Abbreviations: CAM, chicken allantoic membrane; flk, fetal liver kinase; flt, fms-like tyrosine kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GST, glutathione-*S*-transferase; HUVEC, human umbilical vein endothelial cell; LLC, Lewis lung carcinoma; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; PDGF, platelet derived growth factor; Tiel, tyrosine kinase with immunglobulin and epidermal growth factor homology domain; TIMP, tissue inhibitor of matrix metalloproteinase; THP, tumor homing peptide; VEGF, vascular endothelial cell growth factor; VEGFR, vascular endothelial cell growth factor receptor; VN, vitronectin.

* Corresponding author. Tel.: +33-556-848703; fax: +33-556-848705.

E-mail address: a.bikfalvi@croissance.u-bordeaux.fr (A. Bikfalvi)

[1] Website: http://www.lfcdc.org.

PII: S1040-8428(00)00056-1

therapeutic strategies are: suppression of activity of the major angiogenic regulators like vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF); inhibition of function of αv-integrins and matrix metalloproteinases (MMPs); the exploitation of endogenous anti-angiogenic molecules like angiostatin, endostatin or thrombospondin. Given the wide spectrum of diseases which could be treated by anti-angiogenic compounds, it is important for today's clinicians to understand their essential mode of action at a cellular and molecular level. Here we give an in-depth overview of the basic pathophysiological mechanisms underlying the different anti-angiogenic approaches used to date based on the most recent fundamental and clinical research data. The angiogenesis inhibitors in clinical trials are presented and promising future drug candidates are discussed.

Keywords: Angiogenesis; Endothelial cell; Clinical trial; Angiogenesis inhibitor

1. Introduction

Angiogenesis is defined as the growth of new blood vessels out of pre-existing ones. It plays a key role in numerous human diseases like malignant tumors, rheumatoid arthritis, retinal and choroidal neovascularisation and various skin diseases [1]. Under physiological conditions, angiogenesis is only observed during wound healing and at periodic time points in female reproductive organs as well as in pregnancy and embryonic development. The growth of the primitive vascular network which forms during development (vasculogenesis) emerges from multipotential mesenchymal progenitor cells (for reviews see [2,3]). It has recently been suggested that vasculogenesis may also participate in vascular development in adult tissues [4].

The concept of anti-angiogenic therapy has been formulated nearly 30 years ago by Judah Folkman who suggested that cutting the blood supply of a tumor may actually destroy it [5]. During the following three decades researchers gained essential insight into the biological mechanisms of angiogenic vessel growth. A lot of progress has been made to determine how new, proliferating endothelial cells differ from their non-proliferating 'dormant' counterparts and how their growth is controlled.

This research has led to the discovery of numerous regulatory molecules which influence endothelial cell physiology in vitro and angiogenesis in vivo. They can be divided into several groups, consisting of growth factors, extracellular matrix molecules and membrane-bound proteins like integrins and growth factor receptors. The most recent research focuses on different tyrosine protein kinases which transmit signals from specific growth factor receptors or integrins into the cell. Some already known drugs like Taxol® [6], Thalidomide [7] or Interferon-α [8] are seeing a new area of therapeutic use since it was discovered that they have anti-angiogenic properties. Other drugs already on the market can become anti-angiogenic when coupled to specific molecules which target them specifically to proliferating endothelial cells [9,10].

In this review we will focus on the mechanisms of action of the most important angiogenesis inhibitors and stimulators on a molecular and cellular level and describe how the discoveries in basic research have been used to design new drugs for humans. These include: VEGF and its receptors, Ties and angiopoietins, FGF-2, αv-integrins, angiogenin, vasostatin, platelet factor 4, thrombospondin, MMP inhibitors and uPA/PAI-1, angiostatin, endostatin, interleukins, as well as non-specific inhibitors and already known drugs with anti-angiogenic properties (see Fig. 1).

2. Target molecules in anti-angiogenesis therapy

2.1. Vascular endothelial growth factors (VEGFs) and their receptors

Among the most substantial mediators of angiogenesis are the VEGFs and their receptors. VEGF (formerly Vascular Permeability Factor, VPF or Vasculotropin, VAS) was isolated independently by two groups in 1989 from media conditioned by bovine pituitary folliculostellate cells and by human tumor cells (U937) [11,12]. VEGF is an endothelial cell-specific mitogen, secreted as a 45 kDa protein consisting of two subunits which does not induce cell proliferation in other cell types. There are five human isoforms with different numbers of amino acids ($VEGF_{121, 145, 165, 189, 206}$) [13], generated by alternative splicing of the mRNA from a single gene comprising eight exons. $VEGF_{121}$ and $VEGF_{165}$, are the only soluble isoforms and also the most abundant, with $VEGF_{165}$ being the most powerful stimulator of endothelial cell proliferation [14]. To date, five different members of the VEGF family have been discovered: VEGF [11], VEGF-B [15], VEGF-C [16], VEGF-D [17,18] and VEGF-E [19].

Knocking out a single allele of the VEGF gene in mice results in embryonic lethality, suggesting an essential role of VEGF in vascular development [20]. An intact VEGF system still remains mandatory for survival for up to 4 weeks after birth. Injection of a VEGFR-1-IgG chimera into newborn mice during that time causes death because of liver and renal failure due to increased apoptotic endothelial cell death in their

developing organs. At later stages animals survive without visible defects [21].

Clinical studies have shown the importance of VEGF in human disease. High pre-treatment serum VEGF levels in patients with small cell lung cancer are correlated with a low response to conventional chemotherapy and a poor survival rate [22]. VEGF is also a valuable marker for tumor angiogenesis in breast cancer patients [23].

Different strategies have been designed to inhibit VEGF function. Specific VEGF antibodies are a way of stopping the angiogenic effects of this growth factor. Injecting a monoclonal VEGF-antibody into nude mice bearing various tumors of human origin significantly suppresses tumor growth and reduces tumor weight of treated animals up 96% [24,25]. A humanized antibody against VEGF, rhuMAb-VEGF has recently been designed [26,27] and undergoes testing in cancer patients in Phase II clinical trials.

Another approach is the coupling of a toxin to VEGF itself. When active parts of diphtheria toxin (DT390) are linked to $VEGF_{165}$ or $VEGF_{121}$, the chimeric molecule exerts highly selective toxic effects on endothelial cells, leaving other cell types untouched. It disrupts neovascularisation in the chicken allantoic membrane assay (see Table 1), and it slows down the growth of the tumors in a murine Kaposi's sarcoma model [28].

Blocking the interaction of VEGF with its receptors has been shown to be another option for anti-angiogenic treatment. VEGFR-1 (flt-1), VEGFR-2 (flk-1) and VEGF-3 (flt-4) are almost exclusively expressed on endothelial cells. Knock-out mice lacking one of these receptors die in early embryonic state, underlining the unique importance for the vascular system (see Table 2). Interestingly, transgenic mice lacking only the tyrosine kinase domain of VEGFR-1 are alive and develop normally [29]. This can be explained by the fact that VEGFR-1 has only a very weak kinase activity but a ten-fold higher binding capacity for VEGF than VEGFR-2. It may be that VEGFR-1 acts as a co-receptor or as an extracellular absorbing 'sponge' for VEGF thus preventing against excess signaling by VEGFR-2.

Purified soluble VEGFR-1 binds VEGF with high affinity and blocks VEGF-induced endothelial cell proliferation [30]. Transfection of tumor cells with a cDNA

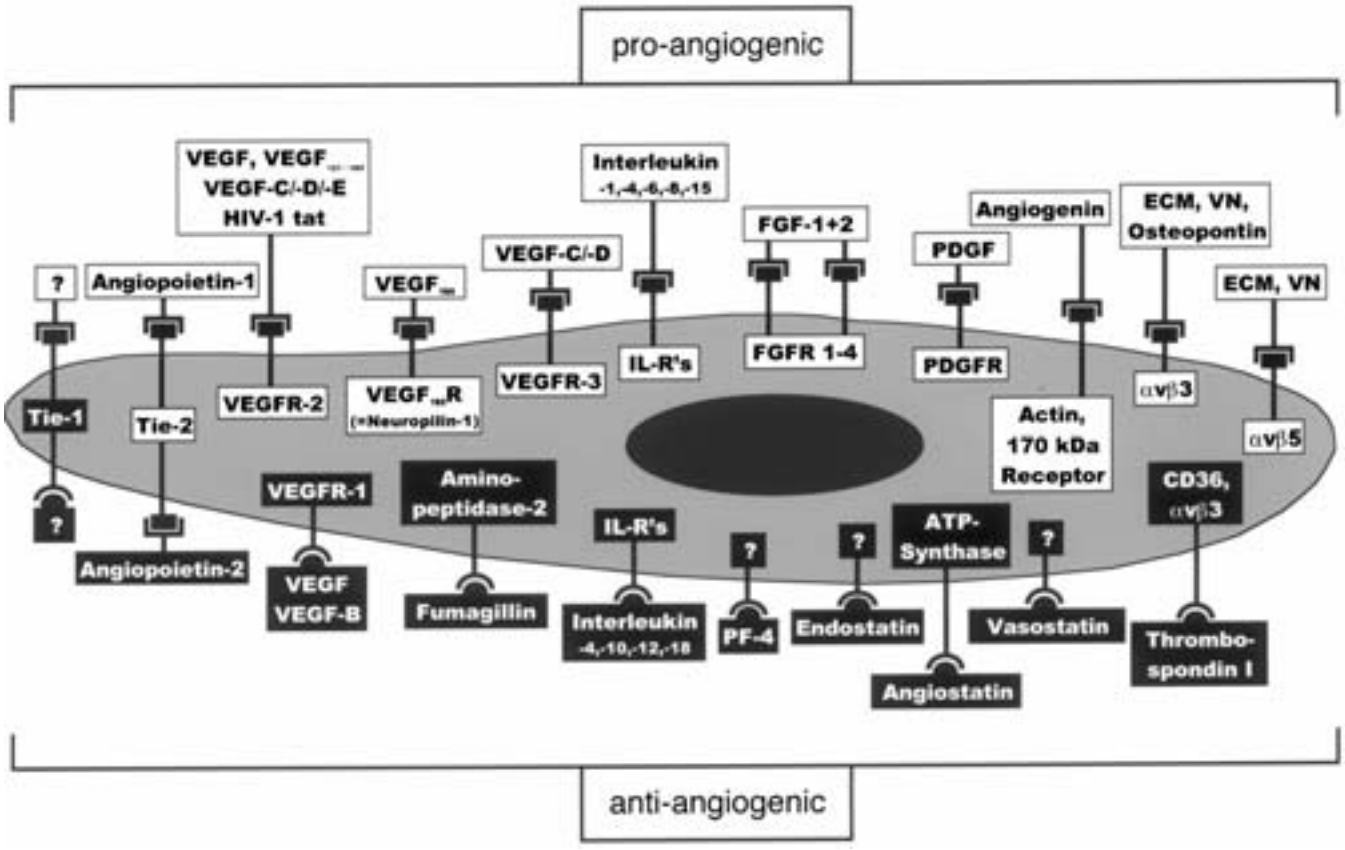

Fig. 1. Major regulators of angiogenesis and their receptors. This simplified scheme of an endothelial cell illustrates the major regulators of angiogenesis and their receptors. On the upper part of the cell angiogenesis promotors are shown, on the lower part inhibitory molecules. VEGF-C acts mainly on lymphatic endothelial cells and is the only lymph-angiogenic factor known so far [222]. $VEGF_{165}R$ (= neuropilin-1) is a co-receptor for $VEGF_{165}$ which enhances the activity of VEGFR-2 [36]. Tie-2 function depends on the ligand, overexpression of angiopoietin-1 is pro-angiogenic and angiopoietin-2 is the natural antagonist to angiopoietin-1 but not a typical inhibitor of angiogenesis.

Table 1
Models of angiogenesis

Model	(Patho) physiological mechanism
In vitro	
Endothelial cell culture systems	Formation of lumen-like 2 dimensional structures, observation of endothelial cell proliferation and migration [163]
Ex vivo	
Human placental blood vessel fragments in fibrin gel	Microculture of vessel-fragments in fibrin gels without the need to add growth factors [164]
Rat aorta explants	Rat aorta rings in serum-free collagen gel or clotted plasma [165,166]
In vivo	
Cornea micropocket model	Implantation of a pouch containing an angiogenic molecule into the stroma of the cornea of various animals with subsequent neovascularisation of the physiologically avascular cornea, allows quantification of angiogenic response [167–170]
Rat subcutaneous air sac model	Induction of a transparent air sac on the back of the animal by injection of air subcutaneously, has the advantage to be less painful for the animal than the cornea assays [171]
Tumor angiogenesis	Inoculation of tumor cells in mice either by injection in the tail vein (lung colonization), subcutaneously, or directly in a specific organ leads to development of new vessels in the growing tumor and metastases [5,105,172]
Oxygen induced models of retinal neovascularisation	Exposure of rodent pups to high oxygen concentrations at a critical time point of their development yields severe retinal neovascularisation [173]
Murine subcutaneous granulomatous tissue	Normal wound healing angiogenesis after a incision wound in the skin [174]
Angiogenesis in vivo using basement membrane extracts (Matrigel)	Angiogenesis occurs in response to subcutaneous injection of matrigel [175].
Transgenic mice with pancreatic islet cell primary tumors (RIP Tag mice)	Induction of a virus oncogene, SV40 large T antigen (Tag) under the transcriptional control of the insulin gene promotor (RIP) leads to angiogenesis-dependent islet cell tumors in all animals [176].
CAM assay	Growth of blood vessels on the chick chorio-allantoic membrane [177–179].

encoding the native soluble truncated VEGFR-1 significantly inhibits their implantation and growth in the lungs of nude mice following i.v. injection. More importantly, the survival rate was significantly higher of mice injected intracranially with human glioblastoma cells stably transfected with soluble VEGFR-1 cDNA than in control mice [31].

VEGF also plays an important role in retinal neovascularisation [32]. Low tissue oxygen concentrations are observed in tumors as well as in several retinal diseases and are known to induce VEGF expression (for reviews see [33]). A chimeric fusion protein made of the extracellular domain of VEGFR-1 and IgG inhibits retinal neovascularisation up to 77% after a single intravitreal injection [34]. These results underline the possibility of using soluble VEGF receptors as a therapeutic anti-angiogenic approach.

New peptides which interfere with the binding site of VEGF with VEGFR-2 or VEGFR-1 have been found through the use of random peptide phage display technology. Biopanning on the receptor binding domain of VEGF yielded some disulfide-constrained 20 amino acid peptides which bind directly to an active region participating in receptor recognition thereby inhibiting VEGF-induced proliferation [35].

$VEGF_{165}$ has also its own, isoform-specific receptor and binds to it via a region of 44 amino acids, encoded by exon seven. The receptor has been found to be identical to neuropilin-1 [36]. Binding of $VEGF_{165}$ to neuropilin-1 enhances indirectly the mitogenic activity of VEGFR-2 in HUVEC cells. A GST-fusion protein containing the 44 amino acid region is able to inhibit HUVEC cell proliferation up to 60% [14]. The mitogenic activity of the protein is completely dependent on a cysteine residue at a specific position (Cys^{146}).

Another group has studied peptides derived from the $VEGF_{165}$-binding region of the VEGFR-2. The 18-residue peptide inhibits $VEGF_{165}$ dependent cell growth and migration in vitro at micromolar concentrations [37]. These approaches are a promising starting point for further development of new anti-angiogenic drugs because they shed light on critical interactions between angiogenic growth factors and their receptors.

When VEGF binds to VEGFR-2, the pro-angiogenic signal is transmitted via a receptor tyrosine kinase to downstream effector proteins. Inhibition of VEGFR-2 signaling may consequently be a way to stop angiogenesis. Such inhibitors have been identified by in vitro screening of random chemical compound libraries. The panning yielded some molecules with the general structure of 3-[(five-membered heteroaryl ring)methylidenyl]indolin-2-ones which are highly specific blockers of VEGFR-2 tyrosine kinase autophosphorylation [38] (see also Table 3). One of these compounds, SU5416, strongly inhibits growth of subcutaneous tumors in mice, converting the tumors to pale white, avascular nodes [39]. SU5416 has been tested in a Phase I clinical trial without severe adverse effects and preliminary data indicate that it may have clinical activity in patients with colorectal, lung and renal cell cancers as well as AIDS-related Kaposi's sarcoma [40]. The SU5416 drug is now in phase II clinical trials.

2.2. The Tie-angiopoietin system

Beside the VEGFRs, only two other endothelial cell-specific surface receptors with tyrosine kinase activity are known so far. Since their discovery in the early nineties, Tie-1 [41–43] and Tie-2 [44] were found to hold unique functions in vascular biology as revealed by gene knockout experiments. Null-mice lacking Tie-1 die at various time points in uteri because of edema and hemorrhage resulting from a general vascular instability [45,46]. Tie-2 knockouts develop normal vessel patterns but die around embryonic day 10 with heart failures, probably due to deranged endocardial/myocardial interactions and disturbed integration and stabilization of blood vessels in their mesenchymal environment [47] (see Table 2). Interestingly, a mutation in the kinase domain of the Tie-2 receptor, resulting in an arginine-to-tryptophan substitution, has been found to be responsible for an inherited form of venous malformations in two unrelated families. The mutation leads to an increased activity of the Tie-2 receptor and patients have reduced smooth muscle cells in their venous walls [48]. As a conclusion, Tie-2 needs to be activated in a very restrained fashion because either overfunctioning or its absence results in vascular abnormalities.

Little is known about the biological functions of the Ties in the adult organism, where their expression is sporadic. Tie-1 has been found in wound healing tissue and during menstruation [49,50]. Tie-2 is also present on endothelial cells in human breast cancer and glioblastoma tissues [51,52], suggesting a connection to tumor angiogenesis.

Taking this assumption as a starting point, Lin et al. showed that they could reduce tumor growth more than 75% in mice by injecting a single dose of the extracellular domain of the Tie-2 receptor. The soluble receptor also completely inhibited corneal neovascularisation induced by pellets containing tumor cell-conditioned media [53]. In a more recent publication, the same group

Table 2
Phenotypes after genetic manipulation of important angiogenesis regulators

Targeted gene	Phenotype
Tie-1	Animals deficient in Tie-1 die directly after birth because of a failure to establish structural integrity of vascular endothelial cells, resulting in pulmonary edema and localized hemorrhages in skin and other organs [45]
Tie-2 (tek)	Animals deficient for Tie-2 die around E10.5 due to abnormal vascular network formation. Absence of sprouting and branching of additional cords from the primary vascular plexus, lack of recruitment of periendothelial support cells [45]
Angiopoietin-1	Gene knockout is lethal at embryonic day 12.5 because of disturbed interactions between the endothelium and surrounding matrix and mesenchyme, similar to the Tie-2 knockout [56].Targeted overexpression of angiopoietin-1 in the skin of mice produces living animals with larger, more numerous and more highly branched vessels [180]
Angiopoietin-2	Transgenic mice overexpressing angiopoietin-2 die at E9.5-10.5 due to similar defects as observed in angiopoietin-1 transgenic or Tie-2 knockout mice [57]
VEGF	Loss of a single VEGF allele is lethal in the mouse embryo between days 11 and 12 [20,181]
VEGF-Receptor 1 (flt-1)	Endothelial cells were developed but assembled into abnormal vascular channels with overgrowth of endothelial cells, embryos die in uteri at mid-somite stage (E8.5) [182]Mice lacking only tyrosine kinase domain of the receptor have normal endothelial/hematopoietic development, but VEGF-mediated macrophage migration is suppressed [29]
VEGF-Receptor 2 (flk-1)	Homozygotes for this mutation die in utero between 8.5 and 9.5 days post-coitum because of an early defect in the development of haematopoietic and endothelial cells, complete absence of organized blood vessels [183]
VEGF-Receptor 3 (flt-4)	Null mice have large vessels, abnormally organized with defective lumens and fluid accumulation in the pericardial cavity resulting in cardiovascular failure at embryonic day 9.5. [184]
αv-Integrin	80% born dead, 20% alive. The liveborn αv-null mice have intracerebral and intestinal hemorrhages and cleft palates [185]
FGF-2 (bFGF)	Delayed wound healing of excision skin wounds mice lacking FGF-2, significant reduction in neuronal density in most layers of the motor cortex [68]
TGF-β 1	Severe defects in yolk sac vasculogenesis, inadequate capillary tube formation and weak vessels with reduced cellular adhesiveness [186]
PDGF-B	Null mice lack microvascular pericytes, development of numerous capillary microaneurysms that ruptured at late gestation [187]

Table 3
Promising new angiogenesis inhibitors in experimental evaluation

Name of molecule	Mechanism of action	Source/type of molecule
Angiostatin [188]	Unknown, induction of endothelial cell apoptosis?	Fragment of plasminogen
PEX [150]	Inhibition of αvβ3/MMP-2 interactions	MMP-2 fragment
HWGF-containig peptides [189]	Selective blocking of MMP-2 and MMP-9	Synthetic peptides discovered by in-vitro phage display
SC-68448 [190]	Inhibition of αvβ3/vitronectin interaction at nanomolar concentrations	Peptidomimetic molecule
Cyclic RGD peptides [132]	Blocking of αvβ3-action	Cyclic peptide RGDfK
THP-dox (THP = tumor homing peptide) [10]	Peptide–doxorubicine conjugate targets the new blood vessels of tumors and penetrates into the tumor stroma	Synthetic peptides discovered by in-vivo phage display coupled to doxorubicine
Platelet factor 4 and derived peptides [72,77,79]	In part via blocking of FGF-2 dimerization and VEGF action	Natural occurring protein synthesized by platelets
PD 166866 [70]	FGF-Receptor 1 tyrosine kinase inhibitor	Synthetic molecule (Pyrimidine-derivate)
PD 173074 [71]	FGF-Receptor 1 tyrosine kinase inhibitor	Synthetic molecule (Pyrimidine-derivate)
NX 1838, NeXstar Pharmaceuticals, Boulder, CO, USA, www.nexstar.com	Inhibits binding of $VEGF_{165}$ to VEGFR-2	DNA Aptamer
Anti-VEGFR-2 antibody [191]	Inhibits binding of VEGF and signaling of VEGFR-2	Monoclonal antibody
ZD4190, AstraZeneca PLC, (formerly Zeneca and Astra AB), www.zeneca.com	VEGFR tyrosine kinase inhibitor	Synthetic molecule
Soluble Tie-2 [53]	Blocking of Tie-2 receptor mediated angiogenesis	Soluble receptor
Vasostatin [113]	Unknown	Fragment of calreticulin
Thombospondin (TSP-1) [117,192]	Unknown, but in part via blocking of FGF-2	Natural protein secreted by platelets
TSP-1 derived peptides [122,123]	Unknown	Short peptides containing the angio-inhibitory region of TSP-1
SC-7, Cell Therapeutics, Inc., Seattle, WA, USA, www.cticseattle.com	Binds extracellular copper and inhibits glysyl oxidase, which is required for collagen crosslinking, an early step in blood vessel assembling	Aryl cyclam

extended their findings by showing that effective suppression of melanoma and mammary carcinoma growth occurs if the Tie-2 receptor is delivered to mice using an adenoviral vector [54].

The natural ligands for the Tie-2 receptor are called angiopoietins (by this writing no ligand for Tie-1 has been published). Angiopoietin-1 binds and activates Tie-2 [55]. During development it can not be replaced by another molecule since the angiopoietin-1 knockout is lethal, showing very similar defects to those mice lacking its receptor, Tie-2. The signals emerging from the angiopoietin-1/Tie-2 interaction seem to be responsible for the assembly of non-endothelial cells for the vessel wall, such as pericytes and smooth muscle cells [56]. The other Tie-2 ligand, angiopoietin-2, does not activate Tie-2, suggesting an antagonist function to angiopoietin-1. Indeed, transgenic mice with a vasculature-targeted overexpression of angiopoietin-2 show similar defects as observed in the angiopoietin-1 and Tie-2 knockouts, underlining its role as a competitor of angiopoietin-1 in the developing vascular system [57]. Angiopoietin three and four have been cloned very recently. Their contribution to the Tie-2 regulatory system in vivo is yet not known, but angiopoietin-4 is only expressed in the lung whereas angiopoietin-3 has a wide tissue distribution. Given the low stringency screening methods involved in the discovery of angiopoietin-3 and -4 and the repetitive cloning of only the four forms during the screens make it likely that all forms of angiopoietin have now been identified [58].

These findings together underline the feasability to use the Tie/angiopoietin system to control angiogenesis and vasculogenesis. But before these important molecules can be used for theraphy, their effects on adult human vasculature and their interaction with other angiogenesis-influencing molecules during physiological and repair-associated angiogenesis have to become more clearly.

2.3. Fibroblast growth factors (FGFs)

One of the most important stimulators of angiogenesis are the fibroblast growth factors, first identified in 1975 [59]. The two most extensively investigated proteins of this family are FGF-1 (acidic FGF) [60] and FGF-2 (basic FGF) [61]. FGF-2 exists in four forms, one low molecular weight (18 kDa FGF-2) and three high molecular weight forms (HMWs FGF-2), resulting

from alternative initiations of translation. The four forms are found in different intracellular compartments and trigger different physiological functions [62].

FGF-2 acts in a paracrine and autocrine manner and is produced either by tumor cells, macrophages or released by the extracellular matrix. It can upregulate other important angiogenic factors like VEGF [63] or plasminogen activator [64] and inhibits endothelial cell apoptosis by Bcl-2-dependent and independent mechanisms [65], showing an essential role for endothelial cell growth and survival.

Blocking of FGF-2 results in inhibition of angiogenesis in vitro and in vivo [66] and implantation of adenovirus transfected fibroblasts expressing secreted FGF-2 (FGF-2 fused to the FGF-4 signal sequence) into mice skin results in production of functional blood capillaries [67]. Knocking out the FGF-2 gene in mice does not impair embryonic growth or vascular development. However, repair-associated angiogenesis is possibly impaired in FGF-2-knockout mice since they exhibit a delayed skin wound healing response [68] (see also Table 2). The absence of a visible phenotype in the FGF-2-knockout does not exclude an involvement of this growth factor in developmental angiogenesis because of redundancy between FGF family members.

FGFs confer their pro-angiogenic signal to the cell nucleus via a family of four transmembrane receptor tyrosine kinases (for review see [69]).

Blocking the intrinsic kinase activity of FGF-receptors is a promising new target for drug development. PD 166866, a pyrimidine derivate, specifically blocks signaling of FGFR-1 and inhibited microvessel outgrowth from cultured human placental arteries at nanomolar concentrations [70]. The interactions of another pyrimidine-based compound (PD 173074) have been characterized further by crystallography. Binding of the molecule to FGFR-1 results in interfering with the ATP-binding site in the kinase domain. This leads to potent in vitro and in vivo inhibition of neovascularisation [71]. The FGF/FGFR interaction is also a target for the anti-angiogenic effects mediated by platelet factor 4 (PF-4). PF-4 inhibits binding of FGF-2 to its receptor by abolishing the dimerization of the growth factor a necessary step for further progression of angiogenic signals [72].

2.4. Platelet factor 4 (PF-4)

Platelet factor 4 belongs to the CXC cytokine superfamily which consists of chemotactic polypeptides less than 10 kDa in size that either support or suppress angiogenesis. Members of this family are angiogenic if they contain an ELR peptide motif at their N-terminus. CXC-polypeptides lacking this sequence are angiostatic [73,74]. Platelet factor 4 does not have the ELR motif and has anti-angiogenic properties whereas ELR-containing interleukin-8 is angiogenic (for reviews see [75]).

It has been known for a while that platelet factor 4 inhibits angiogenesis [76] and that recombinant human PF-4 impedes growth factor-stimulated endothelial cell proliferation [77]. But the mechanisms by which PF-4 achieves its endothelial cell specificity and angiostatic effects has not been known until more recently. $VEGF_{165}$ is hindered to bind to VEGFR-2 in the presence of PF-4 and $VEGF_{121}$-induced endothelial cell proliferation is abrogated by PF-4 [78]. Our group has shown that PF-4 complexes with FGF-2 and inhibits endogenous or heparin-induced FGF-2 dimerization. Furthermore, the chemokine blocks FGF-2 internalization and binding to its receptor, suggesting that the factor stops angiogenesis at least in part via inhibition of FGF-2 [72]. We also have been able to link the inhibitory action of platelet factor 4 to a region localized at the C-terminus of the molecule. The C-terminal fragment blocks both FGF-2 and VEGF-mediated angiogenesis in vitro by interfering with ligand-receptor interactions [79].

PF-4 lacking the heparin binding-domain is as angiostatic as the natural protein when it is injected intralesionally into mice bearing B-16 melanoma or HCT-116 human colon carcinoma and strong inhibition of tumor growth occurs. The chemokine does not slow down the proliferation of tumor cells in vitro, suggesting the anti-tumor effects might be due to inhibition of tumor angiogenesis [80]. It also limits significantly lung metastasis and primary tumor growth if injected intravenously or subcutaneously in various murine tumor models [81]. Further evidence for the angiostatic effects of PF-4 comes from gene therapy experiments. When a secretable form of PF-4 is delivered to endothelial cells via retro-or adenoviral vectors, it inhibits cell proliferation in vitro and tumor growth in vivo, diminishing the number of blood vessels in the tumors [82].

Beside the anti-angiogenic effects, PF-4 has the interesting feature of selectively attaching to endothelial cells in vitro and to new blood vessels in vivo. Fluorescein-labeled PF-4 binds to proliferating endothelial cells of different origins (veins and arteries) and is quickly internalized. It also stains angiogenic blood vessels in a hamster cheek pouch model as revealed by intravital microscopy [83]. Two other studies suggest that the decoration of the vessel walls by PF-4 reflects a selective binding to regions of active angiogenesis [84,85].

Taken together, the existing in vitro and in vivo data show that PF-4 is a potent inhibitor of angiogenesis. It might act as a natural antagonist of FGF-2 and VEGF induced neovascularisation. A first Phase I clinical trial with nine patients suffering from advanced colorectal carcinoma revealed that recombinant human PF-4 is well tolerated and even though no effect has been observed at the doses tested so far [86]. However, it is not possible in Phase I studies to draw any conclusions about the efficacy of the anti-tumor activity of any

agent (see Table 4 for a summary of the aims of clinical trial phases).

Anti-angiogenic therapy using natural inhibitors, like platelet factor 4 or its active peptide-derivates, is likely to become an important direction in anti-angiogenesis therapy.

2.5. Interleukins

Interleukins have been known for a long time for their immunomodulatory activities but their role in angiogenesis is just now becoming a hot topic in cancer research.

Some of the interleukins have anti-angiogenic properties (interleukin-10,-12,-18) others seem to be pro-angiogenic (interleukin-1,-6,-8,-15), some have both effects (interleukin-4). The mechanisms by which interleukins achieve their effects on endothelial cells are quite different and not fully understood. For example, interleukin-10 inhibits in-vitro angiogenesis induced by prostate cancer cells through stimulation of the release of a tissue inhibitor of matrix metalloproteinases (TIMP-1) and by decreasing the secretion of MMP-2 and MMP-9. These effects could be reversed by an antibody against IL-10 receptor or TIMP-1 [87].

In contrast, interleukin-8 stimulates the angiogenic potential of melanoma cells through upregulation of MMP-2 expression at the transcriptional level. This contributes to the more aggressive behavior of injected, IL-8-transfected melanoma cells in vivo [88]. This finding is paralleled by another study which proved that IL-8 receptors are present on endothelial cells of small vessels in all samples from patients with breast cancers, but only in 50% of the samples from normal breast tissue [89].

Interleukin-12 blocks corneal neovascularisation in normal and immundeficient mice, suggesting that the presence of immuncompetent T-cells is not a prerequisite for the anti-angiogenic features of IL-12 [90]. IL-12 acts via the release of interferon-γ and consequently interferon-inducible protein 10 (IP-10), a member of the CXC chemokine family, which itself can block the activity of a major angiogenic growth factor, FGF-2 [90–92]. In addition, downregulation of MMP-9 and increase of TIMP-1 may also contribute to the angioinhibitory effects of IL-12 [93]. Interestingly, interferon-α also inhibits angiogenesis and has been successfully used to treat children with life-threatening hemangioma [8].

Interleukins may be useful tools to treat angiogenesis-related diseases and several clinical trials are underway to test the therapeutic effect in humans.

2.6. Angiogenin

Angiogenin is a 14 kDa single chain protein which has been isolated from human adenocarcinoma cells. It induces angiogenesis in the rabbit cornea in the picomolar range [94,95].

Angiogenin is a physiological component of human serum, circulating at concentrations around 360 ng/ml. The activity of plasma angiogenin is probably controlled by plasminogen which specifically enhances elastase-mediated proteolysis of angiogenin, thereby abolishing its angiogenic function [96]. In patients with pancreatic cancer the angiogenin levels nearly double and correlate with a poor prognosis [97]. This establishes a role for angiogenin in human malignancies.

The angiogenic properties of angiogenin are the result of interactions with several other molecules: First, actin present on the cell surface binds angiogenin and the complex then activates tissue plasminogen activator (tPA) which generates plasmin [98,99]. This is followed by degradation of extracellular matrix and basement membranes, a prerequisite for endothelial cell migration. Endothelial cell proliferation may then be mediated by a 170 kDa angiogenin receptor expressed only on subconfluent endothelial cells with no detectable surface actin–angiogenin complexes [100]. Angiogenin is further processed in the cell and translocates into the nucleus, which seems also to be necessary for angiogenin to become angiogenic.

Blocking of angiogenin with a monoclonal antibody can impair subcutaneous tumor growth of colon adeno-

Table 4
Definition of clinical trials

Trial	Properties
Pre-clinical	Evaluation of pharmacological effects and short term toxicity (normally 2 weeks to three months) through in-vitro and in-vivo animal testing: Genotoxicity, drug metabolism, excretion pathways in at least two species of animals (rodent and non-rodent)
Phase I	Closely monitored first introduction of the new drug to healthy or diseased volunteers (20–80 subjects). Determining metabolism, dose-side effect relations, early evidence of effectiveness possible, but not required
Phase II	Closely monitored introduction of the drug to patients, clinical study with usually several hundred patients to obtain preliminary data on effectiveness of the drug for a particular indication.
Phase III	Expanded controlled and uncontrolled trial (several hundred/thousand patients) after evidence of effectiveness in Phase II studies. Benefit-risk relationship of the drug.

carcinoma in athymic mice in a dose-dependent manner [101]. In 40–50% of the cases, growth of human breast carcinoma xenografts in athymic mice could be completely inhibited by a humanized version of the monoclonal anti-angiogenin antibody cAB 26-2F [102]. Other strategies to abolish angiogenin-induced angiogenesis include the use of DNA aptamers (aptamers are small synthetic DNA or RNA molecules complementary to the RNA/DNA one wants to target) which have been selected from a large aptamer library. The identified molecules co-translocate with angiogenin into the cell nucleus of human endothelial cells and thereby inhibit angiogenesis [103].

Interestingly, the aminoglycosid antibiotic neomycine also blocks nuclear translocation of angiogenin, whereas other aminoglycosids do not show any similar activity. Adding as little as 20 ng neomycine per egg completely disrupts angiogenesis in the chicken allantoic membrane assay [104].

Anti-angiogenic therapy using angiogenin as a target may become an important tool because angiogenin mediates neovascularisation by mechanisms distinct from VEGF and FGF-2.

2.7. Angiostatin, endostatin and other endogenous inhibitors

Angiostatin is perhaps one the most exciting discoveries in the field of angiogenesis and cancer in the last years. Based on the clinical observation that removal of a primary tumor can result in rapid, angiogenesis-dependent growth of metastasis elsewhere in the body suggested that the primary tumor releases anti-angiogenic factor(s) which suppress metastatic growth. Lewis lung carcinoma (LLC)-bearing mice from which the primary tumor has been removed were injected intraperitoneally with serum or concentrated and dialyzed urine from mice still presenting a primary LLC. Both serum and urine from these mice were able to almost completely suppress metastatic cell growth. Serum and urine were also able to specifically inhibit proliferation of endothelial cells of different origin, but fail to show this effect on other cell types such as tumor cells. The anti-angiogenic activity was isolated from mouse urine and serum and it copurifies with a 38 kDa protein which turned out to be a fragment of plasminogen [105].

The mechanism by which angiostatin accomplishes its anti-angiogenic effect is not exactly known but it probably kills endothelial cells by inducing apoptosis [106]. Systemic administration of human or recombinant human angiostatin potently inhibits the growth of human and murine primary carcinoma in mice, showing nearly a complete inhibition of tumor growth without detectable toxicity or resistance, a result which has never been obtained before with other anti-cancer drugs [107,108]. Angiostatin makes endothelial cells resistant to angiogenic stimuli and induces 'dormancy' of metastases.

A second molecule has been isolated by the same group using a similar approach. Culture medium of a murine hemangioendothelioma cell line inhibits proliferation of capillary endothelial cells. This effect has been shown to be angiostatin-independent and the anti-proliferative activity could be identified as a 20 kDa fragment of collagen XVIII, termed endostatin [109]. Endostatin strongly inhibits endothelial cell proliferation in vitro and angiogenesis in chicken allantoic membrane assays. Furthermore, it almost completely stops the growth of metastases originating from LLC carcinoma as well as primary tumors (melanoma, fibrosarcoma, hemangioendothelioma, renal cell carcinoma) [109,110]. The crystal structure of endostatin has been resolved and the result suggests that it might inhibit angiogenesis by binding to the heparan sulphate proteoglycans implicated in growth factor signaling [111]. Phase I trials have now been initiated for endostatin to evaluate the usefulness of this promising new angiogenesis inhibitor in humans.

Beside angiostatin and endostatin there are more endogenous angiogenesis inhibitors which share the common feature being a cleaved part of larger already known molecules. Restin is a 22 kDa human collagen XV fragment which has been found by homology screening with endostatin [112]. It inhibits the migration of endothelial cells in vitro and systemic administration of restin suppressed the growth of tumors in a xenograft renal carcinoma model. A polyclonal antibody against endostatin cross-reacted with restin suggesting similar pathways of inhibition.

Vasostatin is a 180 amino acid NH_2-terminal domain of calreticulin (a protein involved in calcium homeostasis), which selectively inhibits proliferation of endothelial cells and suppressed tumor growth of human Burkitt lymphoma and human colon carcinoma in nude mice. Vasostatin may have applications in cancer therapy because it is easy to produce and appears to be a stable protein [113]. Human prolactin, growth hormone, placental lactogen and growth hormone variant are angiogenic factors whereas their 16-kDa N-terminal fragments are anti-angiogenic [114].

The fact that vascular stimulatory and inhibitory regions lie within one molecule suggests that this is part of a tight control mechanism. Specific cleavage of small parts of larger, ubiquitous proteins may thus be a general mechanism by which the body supports or restrains angiogenesis. It remains to be determined to which extent these mechanisms play a physiological/pathophysiological role in humans or to which extent

these fragments could be used as therapeutics for humans in the future.

2.8. Thrombospondin (TSP)

Extracellular matrix molecules play an important role in maintaining tissue integrity and endothelial cell viability. However, thrombospondin, one such extracellular matrix molecule, first identified in 1979 from platelets [115] is also a very powerful inhibitor of endothelial cell adhesion, motility and proliferation [116] and angiogenesis in vivo [117]. It is a relatively large protein consisting of three identical disulfide-linked 180 kDa chains which exists in two isoforms, TSP-1 and TSP-2.

Interestingly, the anti-angiogenic importance of TSP-1 can directly be linked to malignant pathology, since expression of TSP-1 is downregulated in fibroblasts when loss of the wild-type allele of the p53 tumor suppressor gene occurs, a typical feature of malignant cell growth [118]. Low expression of TSP and altered p53 function has been correlated in samples from human bladder cancer with higher vascularized tumors and consequently higher disease recurrence and lower survival rates [119].

Injecting human skin carcinoma cells transfected with an additional copy of chromosome 15, the gene locus of TSP-1, into mice leads to the development of small avascular cysts instead of tumors. This effect could be reversed by the injection of TSP-1 antisense oligos, underlining the importance of TSP-1 in tumor angiogenesis [120]. TSP-1 is also implicated in the pathogenesis of retinal neovascularisation since its mRNA expression correlates positively with the outgrowth of new blood vessels on the retina in a murine model of retinal neovascularisation. This upregulation is VEGF-mediated and may be part of a negative feedback mechanism for VEGF [121].

TSP-1 is a large multifunctional protein and therefore bears risks if used as a therapeutic agent in humans (e.g. immunogenicity). Efforts have been made to map the anti-angiogenic features of TSP-1 to smaller portions of the molecule. Indeed, a 50 kDa proteolytic fragment of TSP-1 contains the complete inhibitory activity of the intact TSP-1. Several peptides originating from this part ranging from seven to 19 amino acids in length have been found to inhibit angiogenesis in the rat cornea assay as well as vessel growth into wound granulation tissue in mice [122]. The efficacy of those peptides has been improved remarkably by replacing one L-amino acid with its D-enantiomer. A seven amino acid peptide derivate was able to inhibit the development of corneal neovascularisation completely even if injected intraperitoneally [123]. TSP-1 and derived peptides hold therefore a substantial potential to be refined into anti-angiogenic drugs for humans.

2.9. Integrins ($\alpha v\beta 3$ and $\alpha v\beta 5$)

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits with large ectodomains and short cytoplasmic tails. They control cell motility, differentiation and proliferation via interactions with extracellular matrix molecules. Nearly half of the more than 20 integrins known so far recognizes the peptide sequence RGD in their extracellular ligand and blocking of this site with synthetic RGD-peptides inhibits this interaction.

Integrins αvβ3 and αvβ5 are upregulated on proliferating endothelial cells in angiogenic blood vessels during wound healing [124], in retinal neovascularisation [125] as well as in tumor vasculature [126]. Their activity can be triggered differently by two angiogenic growth factors: angiogenesis induced by FGF-2 requires the expression of αvβ3 whereas VEGF-induced angiogenesis appears to require αvβ5 [127]. However, more recent data show that plating human endothelial cells on the αvβ3 ligand vitronectin enhances tyrosine-phosphorylation of VEGFR-2 stimulated by VEGF-A_{165}. This could be inhibited by an anti-β3 monoclonal antibody. Furthermore, VEGFR-2 co-immunoprecipitates with the β3 integrin subunit, but not with β1 or β5 [128]. This suggests that αvβ3 integrin participates significantly in the activation of VEGFR-2 by VEGF-A_{165}.

Both integrins can be detected in blood vessels in samples of various human neovascular eye diseases using the specific monoclonal antibodies LM609 (αvβ3) and P1F6 (αvβ5) [125]. Blocking of αvβ3 integrin using specific peptides, antibodies or accutin, a disintegrin, induces endothelial cell apoptosis [129,130] thus leading to regression of retinal and tumoral neovascularisation or inhibition of neointima formation after coronary arterial stent injury in animal models [126,131–133].

It is not known to which ligands αvβ3 and αvβ5 integrins bind in angiogenesis in vivo, but one candidate might be osteopontin, a RGD-containing protein which is expressed in injured blood vessels in a timely coordinated pattern with αvβ3 [134]. Another ligand could be thrombospondin which has been found to mediate cell attachment to endothelial cells via β3-integrins, since attachment could be blocked by adding the monoclonal anti-αvβ3 antibody LM609 to the culture medium [135].

FGF-2 mitogenic and adhesive activities require cell surface αvβ3 integrin because endothelial cell proliferation and attachment mediated by soluble FGF-2 can be completely blocked by the LM609 antibody [136].

This finding and the fact that αvβ3 associates also with MMP-2 [137] (see MMP-chapter) makes this integrin a particularly interesting target for anti-angiogenic therapy: blocking of αvβ3 could impair multiple significant regulatory pathways of angiogenesis at a time.

Table 5
MMP-classification

Number	Enzyme	Substrate	Remark
Secreted MMPs			
MMP-1	Interstitial collagenase	Fibrillar collagens	–
MMP-2	Gelatinase A, type IV collagenase	Non-fibrillar collagens	Knockout mice have delayed tumor growth [193], binds to αvβ3-integrin
MMP-3	Stromelysin-1	Cartilage components, non-fibrillar collagens	Very strong fibrinolytic activity
MMP-7	Matrilysin (Pump-1)	Non-fibrillar collagen, fibronectin, laminin	Null mice have impaired intestinal tumor growth [194]
MMP-8	Neutrophil collagenase	Fibrillar collagens	Predominant in healing wounds [195]
MMP-9	Gelatinase B, type IV collagenase	Basallaminae	Knockout mice have delayed long bone growth [196]
MMP-10	Stromelysin-2	Proteoglycans, fibronectin, laminin	Implication in epithelial migration during intestinal inflammation [197]
MMP-11	Stromelysin-3	α-1 Antitrypsin	Null mice have poor response to chemical mutagenes [198]
MMP-12	Macrophage metalloelastase	Elastin, α-1 antitrypsin, myelin basic protein	Implied in generating angiostatin in vivo [199]
MMP-13	Collagenase-3	Fibrillar collagens	–
Membrane type metalloproteinases			
MT1-MMP	MMP-14	Progelatinase A	Activates MMP-2, strongest fibrinolytic activity
MT2-MMP	MMP-15	?	–
MT3-MMP	MMP-16	Progelatinase A	–
MT4-MMP	MMP-17	Probably activates growth factors	Isolated from breast cancer cDNA [200]

The importance of αvβ3 in angiogenesis has led to the development of Vitaxin, a humanized version of the LM609 antibody which has been improved using phage antibody library techniques [138]. This drug has passed phase I clinical trials where 17 patients were treated without any adverse effects. One patient had a partial regression of tumor growth and seven others had disease stabilization [139].

The almost unique expression of αv integrins on proliferating endothelial cells could be used as a target to deliver toxic drugs or diagnostic probes. A small synthetic peptide which is highly selective for αv integrins has been identified during screens with random peptide phage display libraries [140]. When this phage carrying the peptide is injected into mice bearing human tumors, an antiserum against the phage reveals exclusive homing of the phage to the endothelial walls of the tumor vasculature, mediated by the peptide [141]. Similar angiogenesis-specific peptides have been recovered after several rounds of in-vivo selection in animals bearing human tumors. Coupling a cytostatic drug to these peptides leads to concentration of the drug in the tumor vessels and stroma, thereby causing tumor regression and prolonged survival rate in a nude mice tumor model [10]. This approach has the advantage that the peptide–drug conjugate attacks both the new blood vessels and the tumor cells itself. In addition, one has the possibility to couple any other compound to the peptide which makes it a multi-purpose vehicle to deliver drugs to tumors and other angiogenesis-dependent diseases.

2.10. Matrix metalloproteinases (MMPs)

The big family of matrix metalloproteinases is another class of molecules influencing angiogenesis. These zinc-endopeptidases are either secreted or membrane-bound enzymes and degrade almost any component of the extracelullar matrix thus promoting cell motility (see Table 5). The healthy body strictly controls the expression of these potent matrix degrading proteases. Gene expression only occurs at sites where cell motility is required (e.g. wound healing) and the molecules are secreted as non-active pro-enzymes with the necessity to cleave off a fragment before initiation of proteolytic activity. Besides, there are naturally occurring non-specific protease inhibitors such as α2-macroglobulin and the group of specific tissue inhibitors of matrix metalloproteinases (TIMP) which can block the enzyme once in its active state [142]. An imbalance between MMPs and their TIMPs is responsible for the invasive phenotype of breast, colon and lung tumors [143] and a low survival rate in urothelial cancers [144]. Intense MMP-11 immunostaining in breast cancers specimens can be directly related to shorter disease-free intervals and reduced overall survival time [145] whereas MMP-1 expression predicts a low survival rate in colon cancers, independent of Duke staging results [146].

MMP-1, MMP-8 and MMP-13 preferentially degrade fibrillar collagens, whereas the gelatinases MMP-2 and MMP-9 express their proteolytic activity on basement membranes (collagen IV). The latter is thought to be a major prerequisite for the capacity of cancer cells to intravasate, since only MMP-9 expressing cells can enter blood vessels in-vivo [147]. The extracellular matrix is also a storage place for VEGFs [148], which are able to upregulate the expression of certain MMPs via the VEGFR-1 receptor present on vascular smooth muscle cells [149]. Degradation of the extracellular matrix by MMPs could then liberate even more VEGF thus establishing a positiv feedback.

MMP-2 can bind directly to αvβ3 integrin, a mechanism which helps concentrating collagenase activity to sites of endothelial cell proliferation [137]. PEX, a C-terminal hemopexin-like domain of MMP-2 can inhibit the αvβ3 interaction with MMP-2, suggesting that it may be a part of a negative feedback loop in this context [150].

An interesting finding is that MMP-7, MMP-9, [151] and MMP-12 [152] may actually block angiogenesis by converting plasminogen to angiostatin, one of the most potent angiogenesis antagonists (for review see [153]). It may be that endogenous production of angiostatin by MMPs is a part of another negative feedback loop to control MMP induced degradation of the extracellular matrix and subsequent promotion of endothelial cell motility and angiogenesis.

Important angiogenesis inhibitors in clinical trials based on MMP-blocking are Metastat, Neovastat, BMS-2752291, Mariamstat, AG3340, Bay 12-9556 and CGS 27023A (see Table 6 for drug synonyms). First Phase I clinical studies of the MMP inhibitors Marimastat and AG3340 have indicated a good tolerability of these compounds and high plasma concentrations after oral administration [154,155]. Marimastat, AG3340 and Bay 12-9556 have all entered Phase III testing for a large variety of cancers, including pancreatic and small cell lung cancer as well as breast and prostate cancers (see Tables 7 and 8). Further trials using MMPs in combination with classical chemotherapy are also underway.

Table 6
Synonyms for anti-angiogenic molecules

Drug name	Synonym(s)
TNP 470	AGM-1470, *O*-(chloroacetyl-carbamoyl)fumagillol, a fumagillin-derivate
Batimastat	BB-94
Metastat	COL-3, CMT-3
Marimastat	BB-2516
CAI	NSC 609974 (carboxyamido-triazole)
Flavopiridol	L86-8275, NSC 649890, HMR 1275
hCG (human chorionic gonadotropin)	A.P.L.
Taxol®	Paclitaxel
SU101	Leflunomide, *N*-[4-(Trifluoromethyl)-phenyl]5-methylisoxazole-4-carboxamide
Vitaxin	LM609 antibody (humanized version)
Suradista	FCE 26644 (Suramin-derivate)
Neovastat	Æ 941
ZD0101	CM101, GBS toxin

2.11. Plasminogen activator (uPA) and its inhibitor (PAI-1)

Proteases of the fibrinolytic cascade also contribute to the regulation of angiogenesis. Expression of urokinase-type plasminogen activator (uPA) by malignant cells results in an aggressive phenotype with increased tumor angiogenesis and metastatic invasion. PAI-1, the natural uPA inhibitor, is paradoxically also upregulated in human tumor samples [156]. In a clinical setting, expression of both, uPA and PAI-1 correlate with a poor prognosis of breast cancers [157].

Stable transfection of PAI-1 into a human prostate carcinoma cell line (PC-3) resulted in a less aggressive phenotype of tumors after injection into athymic mice, including decreased vascularity and metastasis [158]. The N-terminal fragment of prolactin (16K PRL) probably also mediates its anti-angiogenic effects via inhibition of FGF-2 induced expression of uPA by upregulating PAI-1 [159]. Under certain conditions, increased expression of PAI-1 could downregulate uPA activity and consequently inhibit cell motility and angiogenesis.

Results from another group add more complexity to the possible role of the uPA/PAI-1 system in angiogenesis [160]: knock-out mice lacking PAI-1 are protected against tumor vascularisation. No tumor angiogenesis occurred at the transplantation site of malignant keratinocytes whereas wildtype control animals form highly vascularisized tumors. Injection of an adenovirus inducing the production of PAI-1 in the tissue restored invasiveness and tumor angiogenesis. The authors explain the contradiction with previous findings that uncontrolled proteolytic activity, as might occur in the PAI-1 knockouts, can prevent normal evolution of endothelial cells into capillary sprouts in vitro [161]. PAI-1 possibly also exerts its angiogenic or anti-angiogenic properties depending on the time point and site of expression or the interaction with additional factors (e.g. αvβ3-integrin and vitronectin). Taken together, inhibition of uPA rather than PAI-1 activity might be a possible therapeutic target to treat human neovascularisation-dependent diseases.

3. Conclusions

The discovery that angiogenesis plays a key role in a

Table 7
Angiogenesis inhibitors in clinical trials[a]

Drug name	Mechanism	Trial	Sponsor and contact
Marimastat [154]	Synthetic MMP-inhibitor	Phase III against pancreas, non small cell lung and breast cancers	British Biotech, Annapolis, MD, USA, http://www.britishbiotech.com
AG3340 [155,201]	Synthetic MMP-inhibitor	Phase III clinical trials in combination with paclitaxel/carboplatin for non-small cell lung cancer and mitoxantrone/prednisone for hormone-refractory prostate cancer, ophtalmologic trials for age-related macular degeneration are planned	Agouron, La Jolla, CA, USA, www.agouron.com
Metastat® (CMT-3, COL-3) [202]	Synthetic MMP-inhibitor, CMT molecule = Chemically Modified Tetracycline	Phase I in solid tumors refractory to standard therapy (breast, lung, colon, prostate and brain cancers) and in patients with Kaposi's sarcoma	Collagenex, Newtown, PA, USA, http://www.collagenex.com; NCI, Bethesda, MD, USA, http://www.nci.nih.gov
BMS-2752291	Synthetic MMP-inhibitor	Phase I	Bristol-Myers Squibb, Wallingford, CT, USA, http://www.bms.com
Neovastat (Æ-941) [203]	MMP-inhibitor (MMP-2,-9,-12) and inhibition of VEGF action, protein derived from shark cartilage	Phase I/II for refractory lung, prostate and breast cancers in Canada and USA	Aeterna Laboratories, Sainte-Foy, Québec, Canada, http://www.aeterna.com
CGS 27023A [204]	Synthetic MMP-inhibitor	Phase I/II	Novartis, East Hannover, NJ, USA, http://www.novartis.com
Bay 12-9566	Synthetic MMP-inhibitor (MMP-2,-8,-9)	International phase III clinical trials in patients with solid tumors including ovarian and pancreatic cancer	Bayer, West Haven, CT, USA, http://www.bayerus.com
EMD 121974	Chemical modified cyclic peptide antagonist to αvβ3-integrin	Phase II/III against Kaposi's sarcoma and brain tumors (planned for later 1999)	Merck KgaA, Darmstadt, Germany, http://www.merck.de
Vitaxin [138]	Humanized version of LM609 antibody against αvβ3-integrin	Phase II for various cancers	Ixsys, Inc., La Jolla, CA, USA, Tel.: +619-597-4990, ext 106
RhuMAb Anti-VEGF [27]	Monoclonal antibody to VEGF	Phase II/III against lung, breast, prostate, colorectal and renal cancers	Genentech, South San Francisco, CA, USA, http://www.genentech.com
SU5416 [39]	Molecule that blocks VEGF receptor signaling	Phase I and Phase I/II for Kaposi's sarcoma and solid tumors	Sugen, Inc., Redwood City, CA, USA, http://www.sugen.com; NCI, Bethesda, MD, USA, http://www.nci.nih.gov
PTK787/ZK22584	Molecules that blocks VEGF receptor signaling	Phase I against advanced cancers, glioblastoma, Kaposi's sarcoma and von Hippel Lindau Disease	Novartis, East Hannover, NJ, USA, http://www.novartis.com
Angiozyme™ [205]	Ribozyme targets specifically VEGFR-1 mRNA	Phase I in healthy volunteers	Ribozyme Pharmaceuticals, Boulder, CO, USA, http://www.rpi.com
Purlytin (SnET2) [206,207]	Photodynamic therapy (Photopoint™) using photoreactive purpurin	Phase I/II study against metastatic cutaneous adenocarcinoma, Phase II/III against recurrent cutaneous metastatic breast cancers	Miravant Medical Technology, Santa Barbara, CA, USA, http://www.miravant.com
Suradista (Suramin derivate)	Sulphonated distamycine A derivate that blocks binding of growth factors	Phase I study in solid tumors, Phase II for recurrent primary brain tumors	Farmitalia Carlo Erba, Milan, Italy
SU101 [208]	Blocks PDGF receptor signaling	Phase III against glioblastoma, and brain cancers; Phase II combination against glioblastoma, and prostate cancer; and Phase II against ovarian cancer, and non-small cell lung cancer	Sugen, Inc., Redwood City, CA, USA, www.sugen.com
Flavopiridol [209]	Inhibition of cyclin-dependent kinases (CDK2/CDK4) and induction of apoptosis	Phase I in refractory malignancies	NCI, Bethesda, MD, USA, http://www.nci.nih.gov

Table 7 (*Continued*)

Drug name	Mechanism	Trial	Sponsor and contact
TNP-470 [210,211]	Inhibits endotheliel cell proliferation, probably through blocking of methionine aminopeptidase-2	Phase II against advanced cancer for adults with solid tumors	TAP Pharmaceuticals, Deerfield, IL, USA, http://www.tapholdings.com
Thalidomid® [7,212]	Synthetic sedative with anti-angiogenic properties, requires further species-dependent hepatic metabolisation to generate the active compound, exact mechanism unknown	Phase II against Kaposi's sarcoma, breast, prostate and primary brain cancers	Entremed, Inc, Rockville, MD, USA, http://www.entremed.com
ZD0101 (formerly CM101) [213]	Bacterial derived polysaccharide (GBS toxin) with affinity for tumor neovasculature, induces selective vascular inflammation	Phase II in a wide range of solid tumors	AstraZeneca PLC, (formerly Zeneca and Astra AB), http://www.zeneca.com
Combretastatin A-4 [214]	Tubulin-binding organic molecule derived from the stem wood of the South African tree Combretum Caffrum	Phase I studies in Europe and the US for advanced solid tumors	Oxigene, Inc., Boston, MA, USA, http://www.oxigene.com
CAI (Carboxyamido-imidazole) [215,216]	Inhibition of calcium-mediated signal transduction	Phase II against refractory or recurrent ovarian epithelial, fallopian tube, and primary peritoneal cancer; Phase I against refractory, recurrent or advanced cancers	NCI, Bethesda, MD, USA, http://www.nci.nih.gov
Squalamine [217]	Aminosterol extracted from dogfish shark liver, inhibits sodium-hydrogen exchanger	Phase I for solid tumors, Phase II to begin second quaerter 1999 in combination with cytotoxic chemotherapeutic agents in non-small cell lung cancer	Magainin Pharmaceuticals Inc., Plymouth Meeting, PA, USA, http://www.magainin.com
Taxol® [6,216,218]	Microtubule-stabilizing cytotoxic drug with anti-angiogenic activity, exact mechanism of action unknown, MMP-inhibitor?	Phase I/II in advanced refractory Kaposi's sarcoma; phase I in children with refractory cancers, various trials in combination with classical chemotherapy	Miami, FL, USA, http://www.ivax.com NCI, Bethesda, MD, USA, http://www.nci.nih.gov
SU6668	Synthetic molecule blocks VEGFR-2, PDGFR and FGFRs	Phase I for advanced cancers (breast, soft tissue sarcoma, colorectal, non-small cell lung, gastric, pancreatic, renal or prostate cancer) who have failed prior chemotherapy in the US and UK	Sugen, Inc., Redwood City, CA, USA, http://www.sugen.com
Endostatin [109]	Inhibition of endothelial cell proliferation by unknown mechanisms, (inhibition of growth factor signaling?), collagen XVIII fragment	Phase I solid tumor study to begin later in 1999	EntreMed, Rockville, MD, USA, http://www.entremed.com; University of Wisconsin, Madison, WI, USA, http://www.uwsa.edu; University of Texas, M. D. Anderson Cancer Center, Houston, TX, USA, http://www.mdanderson.org
Interleukin-12 [91,219]	Via induction of Interferon-γ and Interferon inducible protein 10 (IP-10), which then blocks FGF-2 activity	Phase I/II against Kaposi's sarcoma and solid tumors	NCI, Bethesda, MD, USA, http://www.nci.nih.gov; Genetics Institute, Cambridge, MA, USA, http://www.genetics.com
Interferon-α [8,220]	In part via inhibition of FGF-2 expression [221]	Phase II/III	Commercially available. Tel.: +1-800-4-CANCER
CT-2584 (Apra)	Mechanism of action unknown Xanthine analogue	Phase II against hormone refractive prostate cancer and soft tissue sarcoma	Cell Therapeutics, Inc., Seattle, WA, USA, http://www.cticseattle.com
IM-862	Dipeptide isolated from soluble thymus fractions, mechanism of action unknown	Phase III trial against AIDS-related Kaposi's sarcoma.	Cytran, Kirkland, WA, USA, Tel.: +425-889-5808

Table 7 (*Continued*)

Drug name	Mechanism	Trial	Sponsor and contact
BeneFin	Purified shark cartilage proteins, mechanism of action unknown	Phase II Clinical Trials for late-stage brain, breast and prostate cancer, Phase III for advanced solid tumors	NCI, Bethesda, MD, USA, http://www.nci.nih.gov; Lane Labs Inc., Allendale, NJ, USA, www.lanelabs.com

[a] Although the authors have taken care to present the most recent data of the clinical trials versions and the actual sponsors, it is possible that by the time this paper will be printed one or more clinical trials will have progressed to another phase or sponsors may have changed. Please refer to the sponsor of the drug or the NCI Cancer Trials Home Page to get the latest news on clinical trials (see also Table 8 for more information).

Table 8
Internet resources about angiogenesis and drug development

Internet address	Page description
http://cancertrials.nci.nih.gov/NCI_CANCER_TRIALS	NCI Cancer Trials
http://www.centerwatch.com	Clinical Trials Listing Service
http://www.newsfile.com	Angiogenesis Weekly
http://www.fda.gov	Food and Drug Administration Home Page
http://pharmacology.tqn.com	Pharmacology and Legal Drugs Home Page
http://www.actis.org	AIDS Clinical Trials Information Service
http://www.actis.org	The Angiogenesis Foundation

large number of severe human diseases has led to an enormous expansion of our knowledge about normal and pathological vascular development. More than thirty angiogenesis inhibitors, currently tested in clinical trials are a result of focused angiogenesis research over the last three decades. The key targets of angio-inhibitory therapies are blocking of matrix metalloproteinases (seven trials), VEGF pathways (four trials) and αvβ3-integrin action (two trials).

Other trials evaluate the anti-angiogenic potential of the signaling-inhibitors SU101 and SU6668 to block growth factors receptors for PDGF and/or FGF-2. A number of angiogenesis inhibitors act through mechanisms which are not fully understood but which might mimic endogenous angio-inhibitory systems. This includes endostatin, interleukin-12, and interferon α. Shark cartilage seems to contain powerful angiogenesis inhibitory substances, three clinical trials are based on the use of shark-derived extracts namely Neovastat, Squalamine and BeneFin.

The future will see the design of drugs based on Ties and angiopoietins because the importance of this receptor–ligand system for the development of blood vessels is as unique as are VEGFs and their receptors. To improve drugs based on natural proteins, researchers will have to identify the active anti-angiogenic regions of their candidates as it has been done for thrombospondin and PF-4. This is necessary to reduce the molecular size of future drugs (thereby decreasing the risk of immunogenecity) and to gain more insight into the structure and function of anti-angiogenic factors. Furthermore, strategies should be developed to target different angiogenic pathologies with different drugs (e.g. tumor angiogenesis versus ocular neovascularisation).

Anti-angiogenic substances may also be naturally present in parts of human nutrititon, since it has been shown very recently that a substance in green tea (epigallocatechin-3-gallate (EGCG)) significantly inhibits angiogenesis in vivo and has therefore the potential to protect against the development of angiogenesis-dependent diseases like cancer or ocular neovascular disorders [162].

Reviewers

This paper was reviewed by Dr Roy Bicknell, Molecular Oncology Laboratories, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DS, UK and Kari Alitalo, MD, PhD, Research Professor of the Finnish Academy of Sciences, Molecular/Cancer Biology Laboratory, Haartman Institute, University of Helsinki, P.O. Box 21, Haartmaninkatu 3, SF-00014 Helsinki, Finland.

Acknowledgements

The authors would like to thank Professor I. Kramer for helpful corrections. M.H. is supported by fundings from the National Academy of Medicine and ALFEDIAM.

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Biographies

Martin Hagedorn attended Medical School at the University of Cologne from 1989 to 1995. He completed his Medical thesis in the Department of Retinal and Vitreous Surgery, University Eye Clinic Cologne (Director: Professor Dr Heimann) in January 1996. From 1996 to 1998 he held a post doctoral fellowship in the laboratory of Dr Erkki Ruoslahti (Burnham Institute, La Jolla, CA, USA). Since January 1999 he has been working on a Scientific thesis in the LFCDC laboratoy, University of Bordeaux I (Director: Andreas Bikfalvi).

Andreas Bikfalvi attained his MD in Brest, France. Between 1983 and 1986 he was a Resident in Internal Medicine in Kiel, Germany. He completed a PhD at INSERM U 150 (Professor J. Caen) in Paris, France, from 1986 to 1989. He held post-doctoral fellowships at INSERM U 118 (Dr Y. Courtois) in Paris, France (1989–1990) and in the Department of Cell Biology (Professor D.B. Rifkin), NYU Medical Center, New York, USA. In 1995 he was a visiting CNRS researcher at CNRS Unit 1813, Paris (Professor D. Barritault). Since 1995 Andreas Bikfalvi has been a Professor in Cell and Molecular Biology, University Bordeaux I and Head of the Growth Factor and Cell Differentiation Laboratory (LFCDC).

Introduction publication II

In this paper, we describe how a small portion of PF-4, the C-terminal fragment containing the amino acids 47-70, inhibits angiogenesis induced by FGF-2. We provide evidence that PF-4^{47-70} is a competitor for ^{125}I-FGF-2-binding to high and low affinity sites on endothelial cells and that it inhibits FGF-2 internalization. The peptide blocks microvascular EC proliferation in a dose-dependant manner. Anti-proliferative effects seem to be independent of cell surface proteoglycans, because proliferation of a GAG-deficient cell line (BaF3, Pro-B-lymphocytes) transfected with FGFR-1 (125) is also inhibited by the peptide. Another important event during early angiogenesis, EC motility, was tested in a monolayer-wounding assay. EC migration is impaired in the presence of PF-4^{47-70}, whereas a control peptide, PF-4^{47-70}S (C^{52} replaced by S) has no effect. Inhibition of cell migration is paralleled by down-regulation of phosphorylated MAPK kinases ERK-1/2, a phenomena that is also observed when cells are treated with the angiogenesis inhibitor angiostatin (126).

We used the rat aortic ring model to further study the effects of PF-4^{47-70} in a more complex system. This assay permits the evaluation of angiogenesis inhibitors without the addition of exogenous growth factors (127). Microvascular vessel assembly is strongly inhibited in cultures treated with PF-4^{47-70}, whereas the control peptide showed no effect at all.

To simulate a real treatment situation, the mouse sponge model was used. Angiogenesis was induced in FGF-2-soaked foam sponges implanted on the back of mice. PF-4^{47-70} or control peptide was injected intra-peritonally. A single dose of PF-4^{47-70} strongly suppressed microvessel ingrowth into the sponges as revealed by measuring erythrocyte-covered area and laminin-staining.

Circular dichroism comparison of the two peptides indicates that the substitution C^{52}S did not significantly alter the overall structure of the molecule at low resolution. However, recent data support the thesis that the substitution C^{52}S induces a defined modification in the structure of PF-4^{47-70}, which is not apparent at a low resolution (128). Taken together, the data presented in the following paper show that a small portion of PF-4 is able to counteract FGF-2 induced angiogenesis in various biochemical, in vitro and in vivo assays and that this activity is dependant on a free cysteine at position 52.

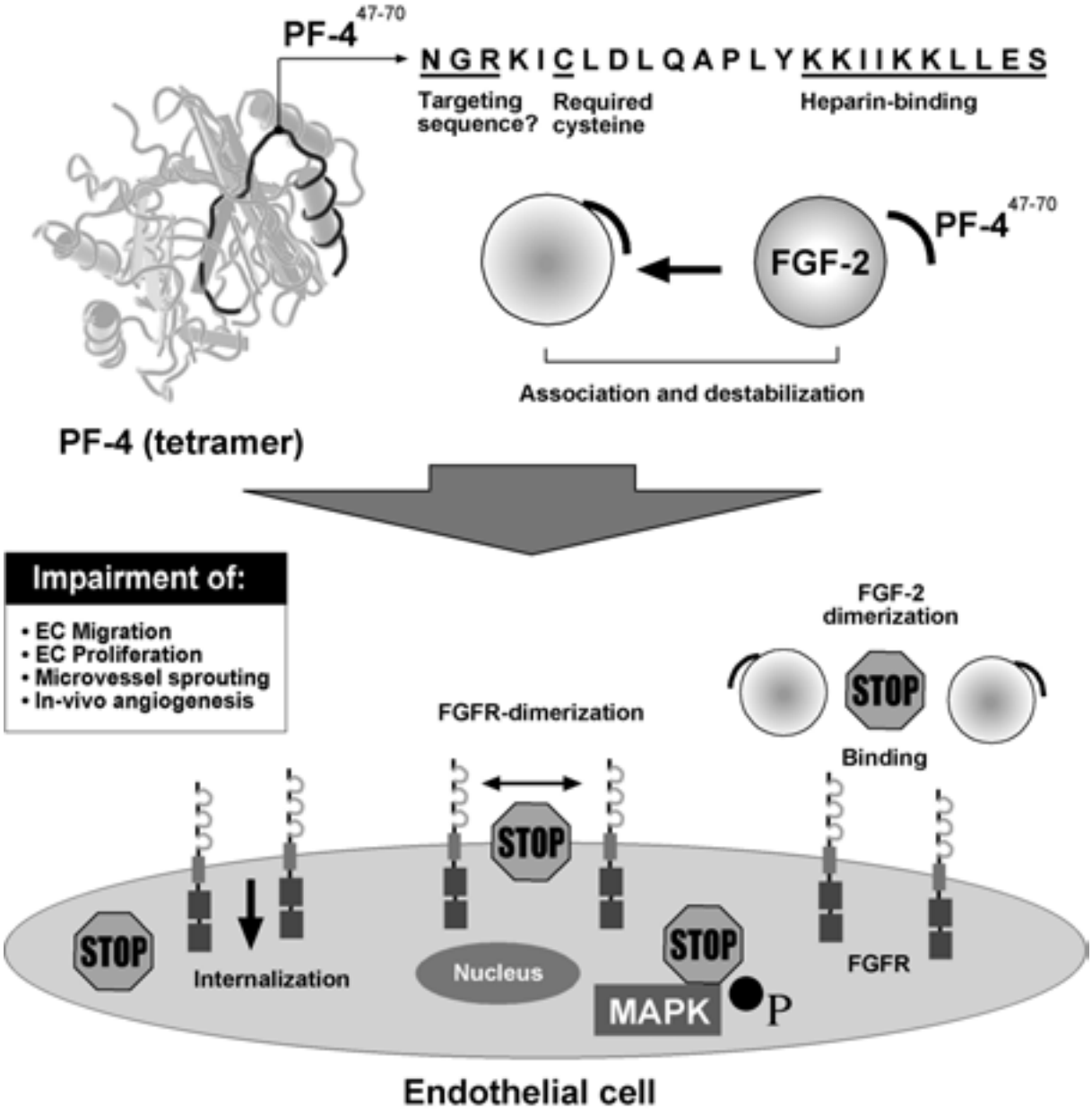

Figure 8: Mechanism of action of PF-4$^{47-70}$ on FGF-2-induced angiogenesis.

(Taken from Hagedorn et al., FASEB J. (January 5, 2001) 10.1096/fj.00–0285fje): PF-4$^{47–70}$ consists of 23 amino acids compared with the tetrameric human PF-4 (280 amino acids). It associates with FGF-2 and leads to a conformation change of the growth factor. Alteration of the secondary structure impairs FGF-2-dimerization and binding to high- and low-affinity receptors. A proper binding of the FGF-2 molecule is further necessary for receptor dimerization and internalization, and for transducing a pro-angiogenic growth signal into the cell. Blocking of FGF-2/FGFR-interaction leads to downregulation of MAPK phosphorylation, a major downstream signaling pathway. Biological consequences of these interactions are inhibition of endothelial cell proliferation, migration, microvessel assembly, and in vivo angiogenesis.

Overview: Platelet factor 4

PF-4 belongs to the CXC cytokine family that consists of small chemotactic polypeptides (<10 kDa). CXC-chemokines are, in general, pro-angiogenic when the tripeptide ELR precedes the first CXC-domain, but are anti-angiogenic, when this motif is absent (129). Exceptions are GRO-β, which contains an ELR motif, but inhibits angiogenesis in vitro and in vivo (130), and the ELR-negative stromal-derived factor 1 (SDF-1), which shows pro-angiogenic effects in vitro and in vivo (131, 132).Table 2 provides an overview of proteins of this family.

ELR^+ CHEMOKINES	ELR^- CHEMOKINES
IL-8 (Interleukin 8) **PBP** (Platelet basic protein), contains: **CTAP-III** (Connective-tissue activating peptide III), **LA-PF4** (Low-affinity platelet factor IV), **BETA-TG** (Beta-thromboglobulin) **NAP-2** (Neutrophil-activating peptide 2) **ENA-78** (Epithelial-derived neutrophil-activating peptide 78) **GCP-**2 (Granulocyte chemotactic protein 2) **Gro α, β, γ** (growth-regulated protein) **MIP2- α, β** (macrophage inflammatory protein-2) **I-TAC** (Interferon-inducible T cell chemoattractant) **9E3/CEF4** (transformation-induced protein) **Lungkine** (expressed in lungs only)	**PF-4** (Platelet Factor 4) **IP-10** (Interferon-γ induced protein) **H174** **MIG** (γ-interferon induced monokine) **SDF-1** (stromal cell-derived factor 1) **PBSF** (pre-B cell growth stimulating factor) **BRAK** (isolated from Breast and Kidney tissue)

Table 2: Members of the CXC-chemokine family.

Functions of the CXC-chemokines are mostly related to immunological processes or are unknown. There is evidence that members of this family with biological activities related to angiogenesis can be classified by the presence or absence of an ELR peptide motif at their N-terminus (129) (presence of the motif = pro-angionenic, absence = anti-angiogenic).

PF-4 can induce inflammation in vivo (133); it may form very immunogenic complexes with heparin in patients treated with heparin, leading to the disease of heparin-induced thrombocytopenia/thrombosis (HIT) (134). Administration of full-length tetrameric (ELR-negative) PF-4 restrains tumor growth and metastasis in mice models via inhibition of angiogenesis (135, 136). Survival of mice is prolonged by transducing established intracerebral glioma with an adenoviral vector encoding a secreted form of PF-4 (137). PF-4 exerts its effects most likely via interfering with FGF-2 and VEGF binding to receptors (138, 139) an activity, which is conserved in the C-terminal portion of the chemokine (140).
An important feature of PF-4, which may contribute to its anti-angiogenic effect, is the selective binding to endothelial cells in vitro and to new blood vessels in vivo. FITC-labeled PF-4 binds to proliferating endothelial cells of different origins (veins and arteries) and is quickly internalized (141). It also stains blood vessels undergoing angiogenesis in vivo as revealed by intravital microscopy (142). Another study shows that PF-4 exhibits a selective binding to regions of active angiogenesis, notably those in breast cancer tissue (143).
These data show that PF-4 is a potent inhibitor of angiogenesis. It might act as a natural antagonist of FGF-2 and VEGF induced neovascularisation.

Ex vivo systems of angiogenesis: the rat aortic ring assay

Developed in the early 90s by Nicosia and co-workers, this assay consists of the observation, that adequately cultured fragments of blood vessels give rise to endothelial outgrowths, which are able to assemble into vascular sprouts (127). The advantage of this system is that sprouts form without the addition of exogenous growth factors. It regroups angiogenic events such as basement membrane breakdown, EC proliferation, EC migration and even pericyte stabilization of microvascular sprouts (144). ECs in microvessels express typical EC markers like von Willebrand Faktor or VEGFR-2 (145) and can be stained by lectins (144).
Factors, which stimulate the growth of these structures, are thought to originate from injured ECs during preparation of rings (146). Classic angiogenic molecules such as VEGF amongst others stimulate outgrowth of microvessels when added to the serum-free cultures (147, 148). Recently, a monoclonal antibody against TIE2 with receptor-activating properties has been generated and shown to

stimulate growth of microvessels in a dose-dependent manner (149). On the other hand, anti-VEGFR-2 antibodies (148), endostatin (150) or PF-4 derived peptides inhibit this growth (publication I and III, this thesis). RGD-peptides also cause regression of microvessel formation in this assay (151). Even though not investigated by the authors, it might be that this inhibition is due to induction of apoptosis as a direct effect of RGD peptides (152).

Interestingly, ECs isolated from human embryonic aortic explants express molecules like CD34 but not CD31, suggesting a role of angiogenic precursors (angioblasts) in the participation of vessel formation in a similar type of assay (153).

Taken together, this model is very close to the in vivo situation of sprouting angiogenesis and thus very well suited to screen for angiogenesis inhibitors.

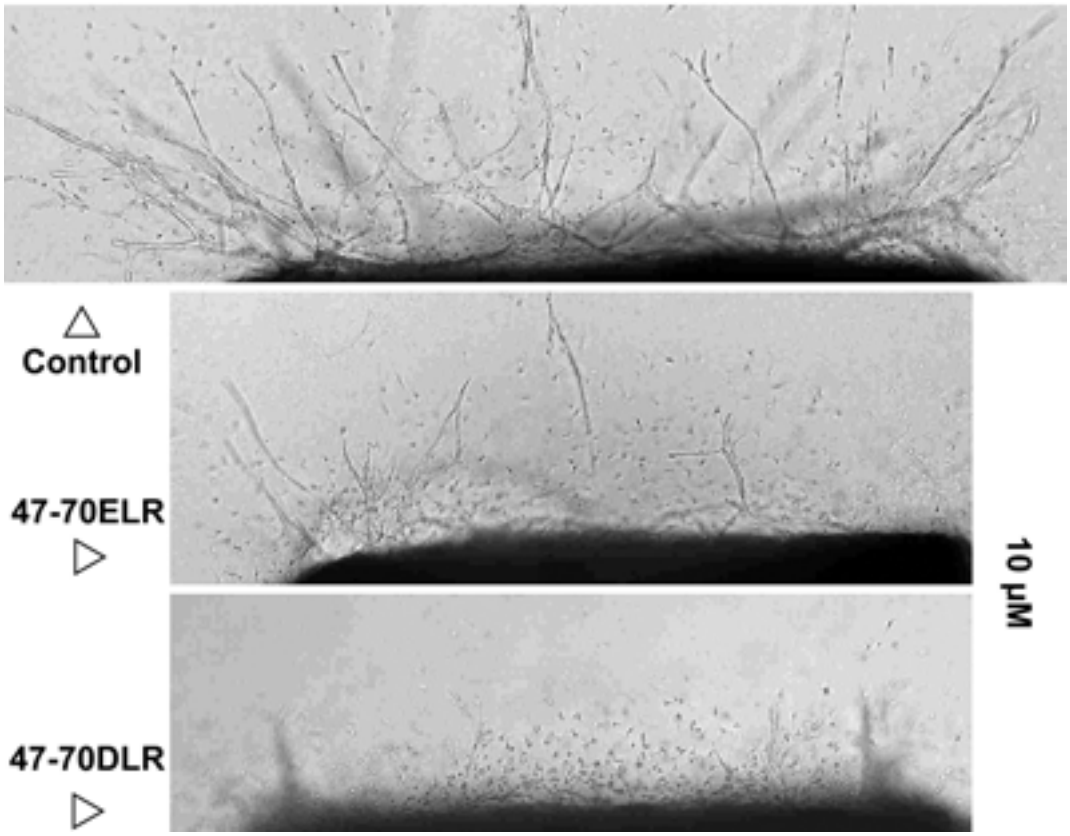

Figure 9: Microvessels radiating from the edges of aortic rings embedded in collagen gels; inhibition by PF-$^{47-70}$ELR and –DLR.

Demonstration of a typical rat aortic ring experiment, numerous vessel-like structures emerge from the edge of the aortic ring in collagen gel culture. The two lower panels demonstrate anti-angiogenic activity of two peptides derived from PF-4 (described in publication III).

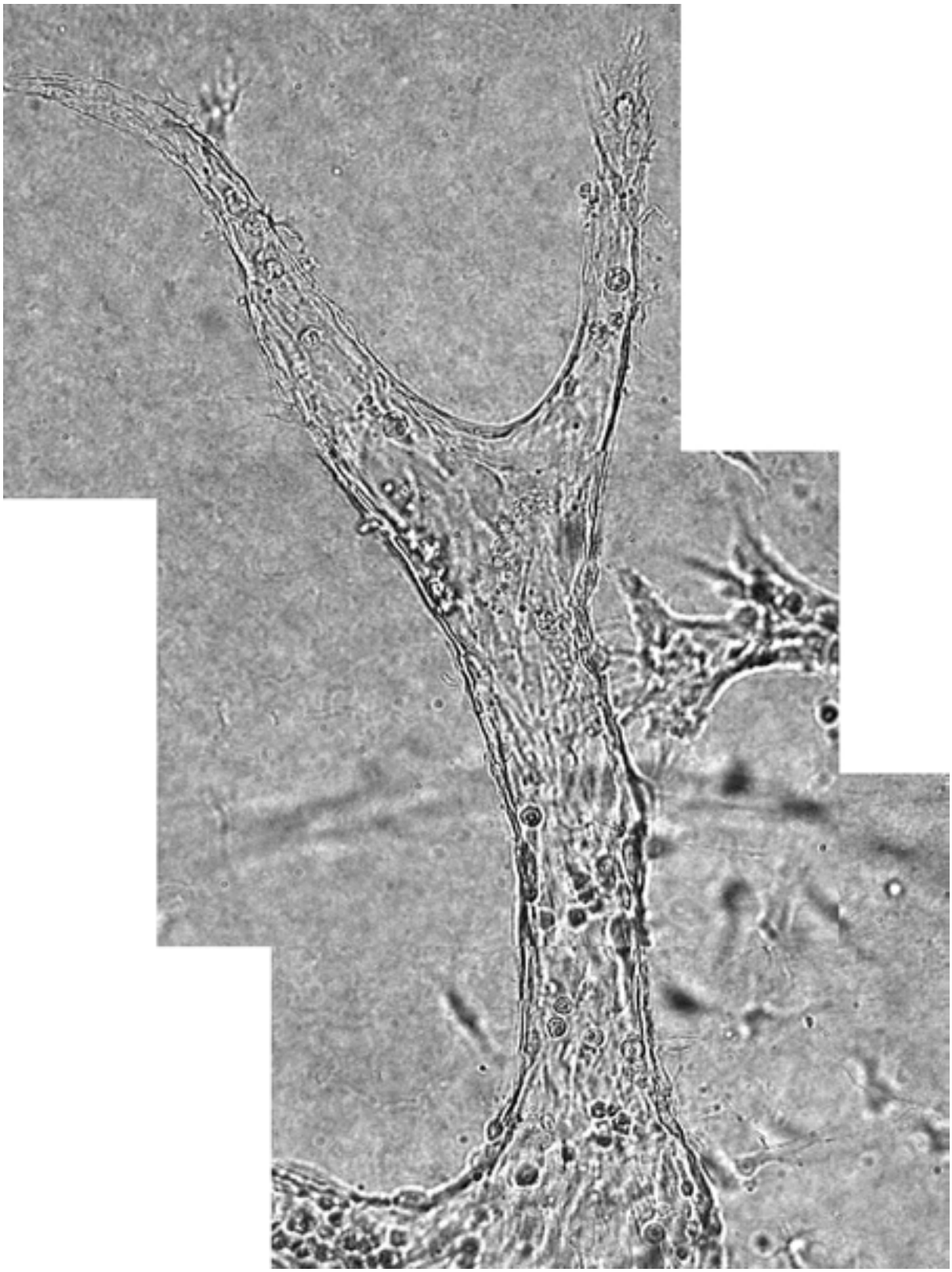

Figure 10: Reconstruction of a single vessel-like sprout from an aortic ring.

Reconstruction of a microvessel emerging from an aortic ring demonstrating the vessel-like assembly of endothelial cells after more than 7 days in culture (x100, 3 photos).

PUBLICATION II

“A short peptide domain of platelet factor 4 blocks angiogenic key events induced by FGF-2”

Hagedorn M, Zilberberg L, Lozano RM, Cuevas P, Canron X, Redondo-Horcajo M, Gimenez-Gallego G, Bikfalvi A.

Summary published in the FASEB J. 2001 Mar;15(3):550-2.
(Full version published online on January 5, 2001 as 10.1096/fj.00-0285fje)

The FASEB Journal express article 10.1096/fje.00.0285fje. Published online January 5, 2001.

A short peptide domain of platelet factor 4 blocks angiogenic key events induced by FGF-2

Martin Hagedorn*[,§], Lior Zilberberg*[,§], Rosa M. Lozano[†], Pedro Cuevas[‡], Xavier Canron*, Mariano Redondo-Horcajo[†], Guillermo Giménez-Gallego[†], and Andreas Bikfalvi*

*Growth Factor and Cell Differentiation Laboratory, University Bordeaux I, 33405 Talence, France; [†]Laboratorio de Estructura y Función de Proteínas. Centro de Investigaciones Biológicas (CSIC); Velázquez, 144, 28006 Madrid, Spain; [‡]Department of Histology, Hospital Ramón y Cajal, Madrid, Spain

[§]These authors contributed equally to this work.

Corresponding author: Professor Andreas Bikfalvi, Growth Factor and Cell Differentiation Laboratory, University Bordeaux I, 33405 Talence, France. E-Mail: a.bikfalvi@croissance.u-bordeaux.fr.

ABSTRACT

Platelet factor 4 (PF-4) is a CXC-chemokine with strong anti-angiogenic properties. We have shown previously that PF-4 inhibits angiogenesis by associating directly with fibroblast growth factor 2 (FGF-2), inhibiting its dimerization, and blocking FGF-2 binding to endothelial cells. We now have characterized a small peptide domain (PF-4^{47-70}) derived from the C-terminus of PF-4, which conserves anti-angiogenic effects of the parent protein. PF-4^{47-70} inhibited internalization of ^{125}I-FGF-2 by endothelial cells in a time-dependent manner. The peptide reduced FGF-2-stimulated cell migration to control levels in wounded monolayers of bovine capillary endothelial cells. PF-4^{47-70} also reduced FGF-2 induced phosphorylation of MAP kinases ERK-1 and ERK-2, which are essential for migration and survival of endothelial cells. In a serum-free *ex vivo* angiogenesis assay, the peptide blocked microvessel outgrowth by 89%. A single amino acid substitution within PF-4^{47-70} abolished all inhibitory activities. To simulate a real anti-angiogenic treatment situation, we administered PF-4^{47-70} systemically to mice implanted subcutaneously with FGF-2 containing gelatin sponges with the result of sparse, scattered, and immature vessel growth. The small peptide fragment derived from the angio-inhibitory CXC-chemokine PF-4 might be used as a starting point to develop anti-angiogenic designer drugs for angiogenesis-dependent pathologies such as cancer, diabetic retinopathy, and rheumatoid arthritis.

Key words: CXC-chemokines ● fibroblast growth factor biology ● peptide angiogenesis inhibitor

Angiogenesis is the growth of new blood vessels out of established vessels. A large number of growth factors, extracellular matrix proteins, proteolytic enzymes, and chemokines act in concert to control new vessel growth tightly under physiological conditions (for reviews see Refs. 1, 2). During malignant tumor growth, the balance is biased so that pro-

angiogenic molecules like vascular endothelial growth factor (VEGF), FGF-2, and metalloproteinases dominate over endogenous inhibitors. This condition results in higher vascularized tumors, tumor dissemination, and poorer survival rates of patients (3, 4). Angiogenic molecules are also important mediators of disease progression in advanced stages of diabetic retinopathy and age-related macular degeneration, retinopathy of prematurity, rheumatoid arthritis, and several dermatological disorders (5, 6).

Numerous endogenous angiogenesis inhibitors have been identified, many of which are generated by proteolytic cleavage from proteins originating from the hemostatic system, the extracellular matrix, or basement membranes. These include angiostatin (kringle 1-4 of plasminogen) (7), endostatin (part of collagen XVIII) (8), canstatin (9) (fragment of the α2 chain of collagen IV), troponin I (from cartilage) (10), the cytoplasmic domain of tissue factor (11), PEX (part of MMP-2) (12), and aaAT (anti-angiogenic antithrombin) (13).

Anti-angiogenic proteins, which are not cleavage products from larger molecules, are thrombospondin (14) and platelet factor 4 (PF-4) (15). Human PF-4 is a tetramer of four identical 70-amino-acid polypeptide chains. It has potent antitumor efficacy and binds to regions of active angiogenesis in human breast carcinoma xenografted to nude mice, which suggests a physiological role in the regulation of new blood vessel growth (16, 17).

PF-4 belongs to the CXC-chemokine group of proteins, which have attracted a lot of interest in the past decade because of their implication in a wide variety of biological events such as neutrophil activation, HIV infection, and angiogenesis. The presence of an ELR-peptide motif preceding the CXC domain at the N-terminus in this protein family generally determines pro-angiogenic behavior (18). The ELR^- chemokines PF-4 and interferon–gamma-inducible protein (IP-10) are angiostatic (18, 19), whereas ELR^+ chemokines such as interleukin-8 (IL-8) are angiogenic (20). The ELR-motif is required for specific interaction with chemokine cell surface receptors CXCR1 and CXCR2 (formerly IL-8RA and IL-8RB). Grafting this sequence into PF-4 converts it into a potent neutrophil-activating protein (21). CXC-chemokines also play an important role in vasculogenesis because mice defective for CXCR4, the receptor for stroma-derived factor 1, have a lethal defective organization of the small intestine vessel network (22).

No specific cell surface receptor has been identified yet for PF-4, which specifically transduces an anti-angiogenic signal. It is believed that the inhibitory properties of PF-4 are a result of interference with angiogenic growth factors. We have previously shown that human PF-4 can complex with FGF-2 and inhibit endogenous and heparin-induced FGF-2 dimerization (23). PF-4 also blocks FGF-2 internalization and binding to its receptor. These data indicate that impairing the physiological interactions of FGF-2 with its receptors is one important way by which PF-4 achieves anti-angiogenic activity. It has also been reported that PF-4 hinders $VEGF_{165}$ binding to VEGFR-2 and blocks $VEGF_{121}$-induced endothelial cell proliferation (24). PF-4 also interferes with cell cycle proteins and inhibits proliferation of endothelial cells induced by epidermal growth factor by blocking down-regulation of the cyclin-dependent kinase inhibitor p21(Cip1/WAF1) (25). Previous experiments showed that the anti-angiogenic activity of PF-4 was conserved within peptides derived from the C-terminal region, which inhibited FGF-2 and VEGF-mediated angiogenesis *in vitro* by impairing ligand-receptor interactions, whereas central or N-terminal peptides were inactive (26).

On the basis of these observations, we evaluated the anti-angiogenic potential of C-terminal amino acids 47-70 (PF-4^{47-70}) of human PF-4. By using a variety of different angiogenesis assays *in vitro*, *ex vivo*, and *in vivo*, we show that PF-4^{47-70} fully retains the inhibitory features of the whole molecule by counteracting FGF-2-induced angiogenesis and that suppressing FGF-2 function is sufficient to nearly completely suppress new vessel growth. The anti-angiogenic effect depends on the presence of a cysteine at position 52 (C^{52}), because its substitution by serine greatly affects inhibitory activity. This finding is the first well-characterized anti-angiogenic peptide derived from a CXC-chemokine. Thus, PF-4^{47-70} may be substantially beneficial for the treatment of angiogensis-dependent diseases like cancer, diabetic retinopathy and rheumatoid arthritis.

MATERIAL AND METHODS

Peptide synthesis and growth factors

C-terminal peptides of human PF-4 ^{47}NGRKICLDLQAPLYKKIIKKLLESS^{70} (PF-4^{47-70}) and ^{47}NGRKISLDLQAPLYKKIIKKLLESS^{70} (PF-4^{47-70}S) were synthesized by using standard solid-phase methodology and were purified by high-performance liquid chromatography (HPLC) by using a C18 column and a 0%–80% linear acetonitrile gradient in 0.1% trifluoroacetic acid. Lyophilized peptides were dissolved in sterile $_{dd}H_2O$ and stored at –20°C prior to use. Recombinant human FGF-2 used in this study was kindly provided by Dr. Hervé Prats (INSERM U 397, Toulouse, France).

Endothelial cells

Bovine capillary endothelial cells (BCE cells) were supplied by Dr. Daniel B. Rifkin (NYU Medical Center, New York). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum, 1% glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin (all from GibcoBRL Life Technologies, Cergy Pontoise, France) at 37°C in a 5% CO_2 atmosphere. All experiments were done between passage P14 and P18. Adrenal cortex capillary endothelial cells (ACE cells) were donated by Dr. Jean-Jacques Feige (CENG, Grenoble, France) and were grown in the same media as the BCE cells. ACE cells were used at passage 15.

Blocking of ^{125}I-FGF-2 binding to high- and low-affinity receptors

FGF-2 was labelled with ^{125}I-Na by using Iodogen (Pierce, Rockford, Ill.) as coupling agent, according to the manufacturer's indications and according to Moscatelli (27). The specific activity of ^{125}I-FGF-2 was 50,000 cpm/ng. FGF-2 binding experiments to high- and low-affinity sites were performed essentially as described by Moscatelli (27). Cells were seeded at 2.5×10^5 per cm^2 and cultured in complete medium in 3.5 cm diameter dishes for two days. Cells were washed twice with ice-cold phosphate buffer saline (PBS) and incubated in the presence of 10 ng/ml ^{125}I-FGF-2 in DMEM and peptides PF-4^{47-70} or PF-4^{47-70}S plus 20 mM Hepes (pH 7.4) and 0.15% gelatin for 2 h at 4°C. Cells were washed again three times with ice-cold PBS, and ^{125}I-FGF-2 was dissociated from its cellular low-affinity binding sites by two 20-s washes with ice-cold 20 mM Hepes (pH 7.4), 2 M NaCl and from its high-affinity sites by two 20 s washes with ice-cold 20 mM NaAc (pH 4.0), 2 M NaCl. Bound ^{125}I-FGF-2 was quantified by using a Kontron

MR250 γ-counter (Saint-Quentin-Yvelines, France). Nonspecific binding was determined by incubating separate dishes with ^{125}I-FGF-2 and a 100-fold excess of unlabeled ligand. Specific binding was determined by subtraction nonspecific binding from total binding.

FGF-2 internalization assay

^{125}I-FGF-2 internalization experiments were performed as described by Roghani and Moscatelli (28). Briefly, BCE cells were incubated with 10 ng/ml ^{125}I-FGF-2 with or without 20 μM PF-4$^{47-70}$ at 37°C in a 5% CO_2 atmosphere (5×10^5 cells per 3.5 cm dish). The specific activity of ^{125}I-FGF-2 was 127,000 cpm/ng. At indicated timepoints, cells were washed three times with PBS and cell-surface bound material was extracted by washing the cells for 20 s twice with 2M NaCl in 20 mM Hepes buffer (pH 7.4) and twice with 2M NaCl in 20 mM acetic acid (pH 4). The amount of internalized radioactivity was determined by solubilizing the cells with extraction buffer containing 10% glycerol, 2% SDS, 1.6 mM EDTA in 125 mM Tris-HCl (pH 6.8), and subsequent gamma counting.

Proliferation assay using a FGFR-1 transfected cell line (BaF3/FR1c-11)

To further confirm that PF-4$^{47-70}$ acts *in vitro* via blocking of FGF-2 interaction with tyrosine kinase receptors, we performed proliferation assays by using the BaF3 cell line transfected with FGFR-1 (BaF3/FR1c-11) provided by D. M. Ornitz (St. Louis University, St. Louis, Mo.). Cells were cultured in RPMI 1640 media supplemented with 10% neonatal bovine serum, L-glutamine, penicillin-streptomycin/β-mercaptoethanol and conditioned media form AX63 plasmocytoma cells transfected with plasmid BMGNeo mIL3 as a source for interleukin-3 (29). During the proliferation assay, only serum-free medium containing only 60 ng/ml FGF-2 was used. Proliferation assays were performed as described previously by Ornitz et al., 1996 (30) except that cell proliferation was assessed by using a XTT colorimetric assay (Roche, Cell Proliferation Kit II) and that no heparin had been added to the culture medium. Peptides were added and cells were incubated for 48 h. XTT was added 6 h before the end of this period, absorbance was measured at $A_{492} - A_{690}$ nm. The experiments were conducted in quadruplicates.

Migration assay

Migration of BCE cells was performed by using a method described by Sato and Rifkin (31), with modifications. Briefly, 1.5×10^5 BCE cells were seeded out in 35-mm culture dishes and were allowed to grow to confluence. Then complete medium was replaced with serum-free DMEM containing 1% glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin and incubation was continued overnight. One linear scar per dish was drawn in the cell monolayer with a sterile pipette tip, and each scar was divided into several fields by scratching lines under the bottom of the dish for further analysis of individual scar fields during light microscopy. A set of digital photos was taken of each scar, and the border between cell and dish surface after induction of the scar was marked by a line on the computer by using the Biocom VisionL@b 2000 software (Les Ulis, France). The dishes were washed carefully to remove detached cells and were further incubated for 18 h in fresh serum-free medium containing 0.1% BSA and peptides PF-4$^{47-70}$ or PF-4$^{47-70}$S at indicated concentrations. After that, we took a second set of photos, which matched the exact fields of the first set. Photos were superimposed on the

computer screen, and nuclei of endothelial cells migrated across the line drawn at the border of the scar in the first photo set were counted. Statistical analysis was performed by using the Student's *t*-test.

ERK-1 (p44) / ERK-2 (p42) phophorylation Western blot

Antibodies for phospho-specific MAPK p42/p44 and MAPK p42/p44 were obtained from New England Biolabs (Ozyme, France). To detect primary antibodies in Western blots, we used horseradish peroxidase-conjugated mouse and rabbit IgG (Dako SA, Trappes, France)and detected immunoreactivity by enhanced chemoluminescence. Bovine capillary endothelial cells (BCE) were cultivated as described above, and subconfluent cultures were serum-deprived for 24 h. Peptides PF-4^{47-70} or PF-4^{47-70}S were then added for 5 min in the presence of 10 ng/ml FGF-2. Cells were carefully scraped off the dish and lysed for 20 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 (all reagents from Bio-Rad, Ivry-sur-Seine, France). The cytoplasmic extracts were resolved by SDS-PAGE on 12% gels under reducing conditions and electrotransferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Orsay, France) by using a semi-dry blotting system (Bio-Rad, Ivry-sur-Seine, France). The blocked membranes were then incubated with primary antibodies, washed, and incubated with secondary antibodies coupled to peroxidase. Blots were visualised by using the ECL*plus* Western blot detection system (Amersham Pharmacia Biotech, Orsay, France).

Rat aortic ring assays

Rat aortic ring cultures were prepared according to a method developed by Nicosia et al. (32), with modifications. Female Sprague Dawley rats, about 200 g, (Harlan, Gannat, France) were deeply anaesthetised with a mixture of ketamine (150 mg/kg), (Merial, Lyon, France) and xylazine (15 mg/kg), (Bayer Pharma, Puteax, France). Aortas were removed by using sterile microsurgical instruments and put directly on ice in MCDB131 medium supplemented with L-glutamine, bicarbonate, and Pen-Strep (all cell culture media and supplements were from GibcoBRL Life Technologies, Cergy-Pontoise, France). Further preparation of aortic rings was performed under a dissection microscope in a sterile tissue culture hood. After cleaning the vessel from surrounding periadventitial tissue, about 15–20 rings each ~1-mm long were cut per aorta and washed 5 times in 15 ml ice-cold serum-free MCDB131. A collagen solution of pH 7.4 was prepared on ice by mixing rat-tail collagen I solution (2 mg/ml, Serva Electrophoresis, Heidelberg, Germany) with bicarbonate, NaOH, 10x MEM, and Pen-Strep. To gel the collagen, 24-well chambers were coated with 300 µl collagen solution and incubated in a tissue culture incubator at 37°C in a 5% CO_2 atmosphere for 10 min. Rings were then placed with sterile forceps in the center of each well so that the lumen of the aorta was orientated parallel to the bottom of the dish. Another 300 µl of collagen solution was added on top of each ring, and cultures were incubated for another 10 min; 1 ml of serum-free MCDB131 was added to each well and replaced every two days. Rings were treated with different concentrations of PF-4^{47-70} every two days; controls received no peptide or the mutated peptide PF-4^{47-70}S. After 5–6 days, we took photographs by using a Sony digital camera connected to a Nikon inverted microscope and microvessel length and number were measured by using the Biocom VisionL@b 2000

software (Les Ulis, France). Statistical analyses of the differences in vessel length and number compared with untreated controls were analyzed by using the Student's *t*-test.

Mouse sponge angiogenesis assays

Sterile gelatin sponges, 10 mm^3 (Curaspon Dental, Clinimed Holding, Zwanenburg, Netherlands), containing 200 µl of 10 ng/ml FGF-2 in PBS (*n*=20) or PBS alone (*n*=10, negative background control) were implanted subcutaneously in the back of C57/BI/6 mice (Charles River, Spain), and wounds were closed with sutures. Twenty-four hours after surgery, mice with sponges containing PBS alone (*n*=10) or PBS plus FGF-2 (*n*=10) were treated with a single intraperitoneal injection of 16 µM $PF4^{47-70}$ in PBS. All procedures in these experiments were performed under sterile conditions and in agreement with the animal welfare guidelines of the National Institutes of Health and the European Union. After 7 days, animals were killed and the gelatin sponges were removed, fixed in 10% neutral buffered formalin solution, and embedded in paraffin. Sponges were sectioned at 6 µm, and slides were stained with hematoxylin and eosin for histological examination. Ingrowth of neovessels was quantified by measuring surface area with erythrocyte content. Counts were performed in four predetermined visual fields by using an eyepiece graticule at 10-fold magnification. At this magnification, the graticule covered an area of 0.11 mm^2. The average area occupied by erythrocytes in all the fields scanned by two observers was calculated. Statistical analyses were performed by using the Student's *t*-test.

For immunhistochemical analysis, the same experiment as described above was performed, except that one additional control group (*n*=10 animals) received sponges incubated with 45 µg/ml of $PF4^{47-70}$ in PBS. Gelatin sponges were fixed in 4% paraformaldehyde in PBS for 24 h and embedded in paraffin. Sections were mounted on aminopropytriethoxysilane-coated slides. Paraffin sections were deparaffinized and rehydrated prior to incubation with 0.1% trypsin (Sigma Chemical, St. Louis, Mo.) to unmask antigen before incubation with primary antibody. Vessels were detected by incubating the sections with a polyclonal anti-laminin antibody (Sigma Chemical). The primary antibody was used at a 1:25 dilution at 4ºC overnight. After rinsing, slides were incubated by a biotin-linked anti-rabbit secondary antibody (1:200) (Vector Laboratories, Burlingame, Calif.). The antigen was visualized with the Vector Laboratories ABC inmunoperoxidase kit, with 0.05% diaminobenzidine in 0.005% H_2O_2. Sections were counterstained with hematoxylin. Parallel sections served as negative control for the primary antibody and were processed identically except that no primary antibody was added. Three sections from all specimens, each 300-µm apart from one another, were used to count immunostained vessels. Microvessel density was confined to the periphery of sponges by counting all vessels under 40-fold magnification with the use of an eyepiece screen. Total microvessel density per treatment group was calculated by adding the numbers of all sections. Values were reported as the mean ± SE, and the difference between the groups was found to be statistically significant as evaluated by the Student's *t*-test (***p*<0.001).

Comparison of PF-4^{47-70} and PF-4^{47-70}S by circular dichroism spectroscopy

Circular dichroism (CD) experiments were carried out in a Jasco J-720 spectropolarimeter fitted with a thermostated cell holder and interfaced with a Neslab RTE-110 water bath. Isothermal wavelength spectra were acquired at a scan speed of 50 nm/min. The response time was 2 s.

RESULTS

PF-4$^{47-70}$ inhibits FGF-2 binding to high- and low-affinity receptors, FGF-2 internalization, and FGF-2-dependent cell proliferation

We first investigated the ability of the PF-4 peptides to interfere with FGF-2 binding to its tyrosine kinase receptors (FGFRs) or low-affinity receptors (heparan sulfate proteoglycans, HSPGs), because both receptor types are required for normal FGF-2 function. We found previously that recombinant PF-4 inhibited binding of ^{125}I-FGF-2 to both classes of receptors (23). Peptide PF-4$^{47-70}$, but not PF-4$^{47-70}$S, blocked binding of ^{125}I-FGF-2 to high- and low-affinity receptors on BCE cells. Concentrations necessary for half-maximal inhibition (IC_{50}) were calculated as ~2 μM for high- affinity receptors and ~4 μM for HSPGs-type receptors. Inhibition was complete between 10 and 20 μM (Fig. 1A). In this range, PF-4$^{47-70}$ also inhibited internalization of ^{125}I-FGF-2 in BCE cells, with the first effect after 1–2 h and reaching a plateau after 12 h. The rate of internalization in the presence of PF-4$^{47-70}$ decreased by 4.6-fold compared with untreated controls (Fig. 1B).

We also checked the effects of PF-4$^{47-70}$ and PF-4$^{47-70}$S on endothelial cell proliferation. The number of adrenocortical endothelial cells (ACE) or BCE cells stimulated with 10 ng/ml FGF-2 and 1% serum was counted after 6 days in culture. At 10 μM, the anti-proliferative effects of PF-4$^{47-70}$ became evident; at 20 μM, cell proliferation was suppressed below levels of 1% serum treated cells (negative control). PF-4$^{47-70}$S had no significant effect on proliferation (data not shown).

To assess the specificity of the interaction of the peptide with FGF-2 *in vitro*, we used the FGFR-1 transfected cell line BaF3/FR1c-11, which does not express HSPGs on its surface (30). FGF-2-induced proliferation was suppressed strongly in these cells between 10 and 15 μM PF-4$^{47-70}$ with an IC_{50} of 3.6 μM. PF-4$^{47-70}$S showed a greatly reduced inhibitory effect at these concentrations (Fig. 1C). These results suggest that PF-4$^{47-70}$ inhibits FGF-2-induced cell proliferation by a mechanism independent of cell-surface HSPGs.

Endothelial cell migration is blocked in presence of PF-4$^{47-70}$

ERK activation by growth factor receptors or integrins has been linked recently to a migratory program in COS-7 and FG carcinoma cell lines (33), and blocking of the ERK pathway with the specific inhibitor PD 98059 leads to a decrease of cell motility in a wounding assay and on vitronectin (34, 35). When we induced a single scar in BCE cell monolayers with a pipette tip, very few BCE cells migrated into the wounded scar area after 18 h of serum deprivation (Fig. 2A). Cells from the positive control, which received 10 ng/ml FGF-2, migrated towards the scar field in serum-free media (Fig. 2B).

During the 18 h period, the cell number was not increased by FGF-2 compared with unstimulated cultures, as determined by cell counting with a Coulter counter (data not shown). The mutated peptide control PF-4$^{47-70}$S did not counterbalance the stimulatory effects of FGF-2 (Fig. 3C), whereas PF-4$^{47-70}$ at the same dose (20 μM) nearly completely blocked cell migration (Fig. 2D). In some experiments, PF-4$^{47-70}$S did enhance FGF-2 induced endothelial cell migration (20%–50%), but no inhibitory effects were observed. The inhibitory effects of PF-4$^{47-70}$ on FGF-2 (10

ng/ml)-stimulated BCE cell migration are dose-dependent. Inhibition started around 10 µM and reached a plateau at 20 µM, where migration was suppressed to control levels (Fig. 2E).

PF-4^{47-70} inhibits the activation of mitogen-activated kinases ERK-1 (p44) and ERK-2 (p42)

Binding of FGF-2 or VEGF to its high-affinity receptors induces rapid phosphorylation of ERK-2 *in vivo* (35) and endogenous angiogenesis inhibitors like the 16-kDa fragment of prolactin or angiostatin can block FGF-2 induced phosphorylation of ERK isoforms p42/p44 *in vitro* (36, 37). We therefore investigated whether this important signalling event in the angiogenic cascade was affected by PF-4^{47-70}. BCE cells stimulated by 10 ng/ml FGF-2 for 5 min showed a strong increase in phosphorylation of ERK-isoforms. If co-incubated with 20 µM of peptide PF-4^{47-70}, ERK activation decreased markably, whereas PF-4^{47-70}S did not influence ERK phosphorylation. Similar data have been obtained in several independent experiments, and one representative Western blot is shown (Fig. 2F).

Microvessel assembly in aortic ring cultures embedded in collagen gels is hindered in presence of PF-4^{47-70}

To investigate microvessel assembly, thus the first step towards formation of a blood vessel after endothelial cell migration and proliferation, the rat aortic ring angiogenesis assay was used (32). When living aortic ring cultures were treated every 2 days with 20 µM of PF-4^{47-70}, a profound effect on spontaneous microvascular sprouting was observed. In control cultures where no peptide had been added, numerous vessels developed after 4–6 days. The vessels grew into the collagen matrix originating from the cut edges of the ring, which was placed parallel with its lumen to the bottom of the culture dish (Figs. 3A, C). When rings were placed with the lumen parallel to the walls of the dish, no reproducible, quantifiable growth of vessels could be observed (data not shown). In cultures treated with 20 µM peptide PF-4^{47-70} vessels, formation was strongly inhibited, whereas cellular (fibroblastic and/or endothelial) outgrowth was less affected (Figs. 3B, D). If vessels developed, they were much thinner and shorter than vessels in control cultures. Branching microvessels with lumina became clearly visible in untreated cultures, whereas PF-4^{47-70}-treated rings are surrounded mainly by cells that did not assemble into microvessels (Fig. 3D). PF-4^{47-70} reduced the mean vessel length by 89% and the vessel number by 86% (four rings per group in three independent experiments, Fig. 3E). The inhibiton of angiogenesis at 20 µM PF-4^{47-70} measured by the length and number of microvessels was statistically significant (Student's *t*-test, $p<0.001$). Inhibitory effects of PF-4^{47-70} were dose-dependent, with a narrow therapeutic range between 10 and 20 µM. Adding 20 µM of mutant peptide PF-4^{47-70}S to cultures did not inhibit formation of vessels, and they resembled those of the untreated controls (Fig. 3F). Dose-response results were repeated twice with similar findings.

Systemic treatment with PF-4^{47-70} in mice reduced vessel ingrowth in FGF-2-containing subcutaneous sponges

To simulate a more realistic treatment situation, we injected a single dose of PF-4^{47-70} (16 µM) in PBS or PBS alone into mice with subcutaneous implants of FGF-2-containing gelatin sponges. One week later, animals were killed, and sponges were examined histologically or by immunhistochemistry with an anti-laminin antiserum. No new capillaries were found in sponges incubated with PBS alone or in that with peptide PF4$^{47-70}$ in PBS (4A+B). In contrast, sponges

with 10 ng/ml FGF-2 developed neovessels; neutrophils and macrophages were present (4C+E). When mice received a single intraperitoneal injection of $PF4^{47-70}$ 24 h after implantation of FGF-2 sponges, vessel number decreased markedly, vessels were scattered, were of immature nature, and had smaller diameters, whereas slightly more inflammatory cells were present.

The angiogenic response was quantified by calculating the areas that contained erythrocytes or by counting laminin-positive vessels. Statistical analyses were done by using the Student's *t*-test, and the difference between PF-4^{47-70}-treated and control groups was considered significant (**$p<0.001$). The histogram further illustrates that $PF4^{47-70}$ treatment blocked angiogenesis compared with control groups by 86% when assessed by calculating areas with erythrocytes and by 81% when laminin was used as a marker for angiogenic vessel growth in an independant experiment (Fig. 4G).

Low-resolution structural characterization o PF-4^{47-70} and PF-4^{47-70}S

As shown in Figure 5, the far-UV circular dichroism spectrum of PF-4^{47-70}S (dotted line) is quite similar to that PF-4^{47-70} (solid line). These sorts of spectra are typical for polypeptides in which the α-helix is the only defined element of secondary structure. Estimation of the α-helix contents of those structures, on the basis of the molar ellipticity of the spectrum at 222 nm (38), yields values of approximately 28% for PF-4^{47-70}S and 36% for PF-4^{47-70}. The effect of TFE addition on the circular dichroism spectra of PF-4^{47-70}S undoubtedly confirms its propensity to fold almost exclusively in α-helix conformation. According to the molar ellipticity of the spectrum at 222 nm, the α-helix content increased to almost 100% with the addition of TFE (30% v/v). As demonstrated in Figure 5, this finding is also the case for PF-4^{47-70}. These results suggest that the substitution $C^{52}S$ does not modify the secondary structure of PF-4^{47-70} peptides at low resolution.

DISCUSSION

The anti-angiogenic activity of recombinant human PF-4 was first described in 1990 by using the chick allantoic membrane (CAM) assay (15). PF-4 was described further to be an effective inhibitor of metastatic melanoma growth if administered systemically (16). Furthermore, adenoviral vector-mediated delivery of a secreted form of PF-4 to established intracerebral glioma leads to hypovascular tumors and increased survival rates in nude mice (39).

As anti-angiogenic therapy may require long-term treatment with protein drugs, it is useful to identify small active regions of endogenous inhibitors to reduce production costs and immunogenicity and eventually to increase bioavailability. We recently screened a series of overlapping peptides of PF-4 for their anti-angiogenic potential and identified the C-terminal domain 47-70 (PF-4^{47-70}) as the major inhibitory region, which interferes with the activity of the angiogenic growth factors FGF-2 and VEGF by blocking several ligand-receptor interactions (26). We have now characterized the potency of PF-4^{47-70} as an inhibitor of FGF-2-induced angiogenesis in various *in vitro*, *ex vivo*, and *in vivo* angiogenesis models.

The inhibitory effects observed with PF-4^{47-70} are based on the defined interaction of the peptide with FGF-2 and its receptors. ^{125}I-FGF-2-binding to high- and low-affinity receptors on the surface of bovine capillary endothelial cells is reduced strongly in the presence of PF-4^{47-70}, but not by the $C^{52}S$ mutant PF-4^{47-70}S. Blocking the high-affinity FGF receptors alone by the

synthetic compound PD 166866 leads to inhibition of microvessel outgrowth from cultured artery fragments of human placenta (40). Low-affinity sites (HSPGs) modulate binding of FGF-2 to high-affinity receptors and influence the availability of the growth factor on the cell surface (for reviews see Ref. 41). RG-13577, a synthetic molecule that mimics heparan sulfate proteoglycans biological properties, can FGF-2 angiogenic effects *in vitro* and in the *ex vivo* rat aortic ring angiogenesis assay (42). These experiments show that selected compounds sufficiently interfer with high- or low-affinity FGF-receptors to block important events of the angiogenic cascade but that PF-4^{47-70} impairs both the high- and low-affinity receptor systems.

After binding, the FGF/FGFR complex is internalized and intracellular signaling mechanisms are activated. We investigated the effect of PF-4^{47-70} on these post-receptor events. PF-4^{47-70} strongly inhibited the rate of ^{125}I-FGF-2 internalization in BCE cells in a time-dependent manner. This effect of the peptide is likely to contribute to its inhibition of endothelial cell proliferation, because it has been shown that internalization and nuclear translocation of FGF-2 enhances endothelial cell proliferation (43, 44). Proliferation of endothelial cells is a key event during the initial phase of new blood vessel growth, and FGF-2 is a well-known and potent endothelial mitogen. Treatment of ACE cells with micromolar concentrations of PF-4^{47-70} strongly inhibited FGF-2 induced cell proliferation (26), whereas mutant PF-4^{47-70}S did not show any inhibitory effects (data not shown). Proliferation of BaF3/FR1c-11 cells is also inhibited by PF-4^{47-70} but not by PF-4^{47-70}S. These findings suggest that cell surface HSPGs are not essential for the inhibitory action of PF-4^{47-70} and that anti-proliferative effects are due to a specific interaction of PF-4^{47-70} with the FGF/FGFR system.

In BCE cells, binding of FGF-2 to its high-affinity receptors FGFR-1/2 initiates activation of the Ras/mitogen-activated protein (MAP) kinase pathway, with subsequent phosphorylation of extracellular signal-regulated kinases ERK-1/2 (45). VEGF also activates the phosphorylation of ERK-1/2 in a time- and concentration- dependent manner, thus preventing apoptosis of endothelial cells under serum-free conditions (46). The endogenous angiogenesis inhibitor angiostatin diminishes ERK activation in human dermal microvascular endothelial cells stimulated by FGF-2 and VEGF, and this effect might contribute to the inhibition of collagen gel invasion by these cells (37). The efficacy of an angiogenesis inhibitor should therefore ideally be reflected by an inhibition of ERK-1/2 phosphorylation. When FGF-2-stimulated BCE cells were treated with various concentrations of PF-4^{47-70}, a strong inhibition of ERK-1/2 phosphorylation occured at 20 µM. The related mutant peptide PF-4^{47-70}S did not show any effect on ERK-1/2 phosphorylation.

We next checked whether endothelial cell motility, an important pre-requisite for angiogenesis, was affected by PF-4^{47-70}. When BCE cell monolayers were injured with a pipette tip, PF-4^{47-70} completely abolished FGF-2-induced cell migration in serum-free media in a dose-dependent manner, whereas the control peptide PF-4^{47-70}S did not inhibit cell migration mediated by FGF-2. It is likely that inhibition of wound repair-associated migration of endothelial cells by PF-4^{47-70} is a result of impaired ERK phosphorylation. This finding would be in accordance with the results of Pintucci et al., who found that migration of endothelial cells after wounding of endothelial cell monolayers requires ERK-1/2 phosphorylation, because PD 98059 a specific blocker of ERK, inhibits cell migration after injury (34). Accordingly, Klemke et al. recently have proposed a model by which both growth receptors and integrins can activate directly the RAS/MEK/ERK pathway leading to phosphorylation of myosin light chain kinase (MLCK) and myosin light

chains (MLC) with activation of myosin and actin resulting in cell migration (33). It must be noted at this point, however, that these experiments were done by using non-endothelial cells, namely cancer cells (FG carcinoma) and fibroblasts (COS-7).

Further assembly of endothelial cells into a functional vessel requires chord organization and lumen formation, a process known as sprouting (47). The aortic ring assay is a serum-free *ex vivo* model, which mimics this important step of the angiogenic cascade. Microvessel growth in this assay is promoted by endogenous release of FGF-2 and VEGF from injured aortic endothelial and smooth muscle cells (48, 49). PF-4^{47-70} inhibits almost all the microvessel outgrowth in this assay, but affects cellular outgrowth around rings less efficiently. Miao et al. have found similar results by treating ring cultures with a synthetic heparin/heparansulfate compound (RG-13577) that mimics activity of normal heparin and impairs interaction of FGF-2 with one of its high-affinity receptors (42). RG-13577 caused a profound reduction in the number of microvessels, whereas cellular outgrowth around the rings was not affected. These data suggest that blocking FGF-2 function is essential for microvessel assembly but is not sufficient to inhibit completely stroma cell proliferation in this assay. It is possible, that other growth factors such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF), which are not blocked by RG-13577 or PF-4^{47-70}, may be present in small amounts in the injured aortic vessel wall and promote cellular outgrowth around the rings. Recently, murine endostatin has been evaluated by using this method and was found to block vessel sprouting around concentrations of 500 µg/ml. Peptide PF-4^{47-70} reaches maximal anti-angiogenic activity at 55 µg/ml (20 µM), thus at 9 times lower concentrations. The therapeutic range for PF-4^{47-70} is relatively small in this assay, between doses from 10 µM (inhibition starts) to 20 µM (nearly complete inhibition). One explanation for this effect might be that a large percentage of FGF-2 must be inactivated by the peptide before an anti-angiogenic effect can be seen, an alternative explanation may be that other pro-angiogenic molecules can compensate a decrease of FGF-2 activity for a certain time.

The optimal lead drug for anti-angiogenic therapy should be a substance that exerts its anti-angiogenic effects at the remote site of vascular vessel growth if delivered systemically. Using the mouse sponge assay of FGF-2 induced angiogenesis, we showed that a single injection of PF-4^{47-70} (16 µM/mouse) is sufficient to suppress the angiogenic response in the FGF-2 containing sponge compared with mice treated with PBS alone by 81% or 86%, depending on the quantification method used. There were slightly more inflammatory cells present in sponges from animals treated with PF-4^{47-70} (Fig. 4F). One reason for this result might be a possible pro-inflammatory motif in PF-4^{47-70}, because it has been shown that a peptide termed C-41 (derived from the C-terminus of PF-4) has neutrophil-recruiting activity *in vivo* (50).

It is tempting to speculate that the N-terminal NGR sequence of PF-4^{47-70} enhances biological effects *in vivo* by targeting the peptide to sites of active angiogenesis. This speculation would be in accordance with the work of Borgstrom and co-workers, who found that recombinant PF-4 accumulates at sites of active angiogenesis (17). Further support for this hypothesis comes from Arap et al., who identified the NGR peptide motif by phage display technologies as a neovascular homing domain, which enhances the *in vivo* anti-tumor toxicity of doxorubicine if coupled to it (51). Work is underway in our laboratory to investigate these interactions.

The C-terminal region contains a lysine-rich heparin-binding domain (KKIIKK) and originally has been described as responsible for the anti-angiogenic activity of PF-4 (15). But changing the

KKIIKK motif to QEIIQE yields a PF-4 mutant devoid of heparin-binding properties, which fully retains anti-angiogenic activity (52). This finding suggests that this heparin-binding motif is not sufficient for anti-angiogenic effects of PF-4. NMR analysis has revealed an additional, even stronger, heparin-binding region in PF-4, consisting of a group of arginines (R 20, 22, 49), which can interact tightly with a heparin-derived dodecasaccharide (53). PF-4^{47-70} contains only one of those arginines, which may not be sufficient for a strong interaction with heparin. Furthermore, previous results have shown that peptides 47–58 (containing R49) and 58–70 (containing the KKIIKK motif) of PF-4 did not have anti-angiogenic effects *in vitro* (26).

Recently, we have revealed the structure of PF-4^{47-70} and have studied its interaction with FGF-2 by using several biophysical methods (Lozano et al., unpublished results). PF-4^{47-70} in aqueous solution adopts a structure that contains two helical regions approximately between residues 50–53 and 57–67, separated by a spacer of three amino acids. Gel filtration and equilibrium sedimentation studies have shown that PF-4^{47-70} associates with FGF-2 at a stochiometric ratio of 1:1. Shorter sequences of PF-4^{47-70} did not show this effect. That PF-4^{47-70}S is devoid of any anti-angiogenic activity indicates that a free cysteine (C^{52}) must be present for angiogenesis inhibition. Circular dichroism data presented in Figure 5 indicate that the substitution C^{52}S did not significantly alter the overall structure of the molecule at a low resolution. One possible explanation for the loss of activity of the mutant might be that PF-4^{47-70} exerts its anti-angiogenic effects by dimerization with itself or target molecules through disulfide bonding. However, preliminary data support the thesis that the substitution C^{52}S induces a defined modification in the structure of PF-4^{47-70}, which may not be apparent at a low resolution. Further studies are underway to solve this issue.

Other angiogenesis inhibitors also act by impairing FGF-2 function. Thrombospondin (TSP) and its 140-kDa fragment inhibit binding of FGF-2 to endothelial cells at nanomolar concentrations and block endothelial cell proliferation, but not cell motility. The inhibition of binding of FGF-2 to low-affinity sites by TSP is more prominent than it is for tyrosine kinase receptors (54). The anti-angiogenic activity could be mimicked by a small C-terminal peptide derivative, peptide 4N1K. It exerts similar effects as PF-4^{47-70}; like inhibition of FGF-2 stimulated *in vitro* tube formation of a murine brain capillary endothelial cell line and it also blocked neovascularization *in vivo* in the mouse cornea assay (55). Binding of TSP-1 to its receptor CD36 and activation of p59fyn, caspase-3-like proteases and p38 mitogen-activated protein kinases may also contribute to the anti-angiogenic properties of the molecule (56). The well-known angiogenesis inhibitor, suramin, blocks FGF-2-binding to BCE cells, to high- and low-affinity receptors and inhibits BCE cell proliferation and migration, but at >5 (proliferation) to 10-fold (migration) higher doses as PF-4^{47-70} (57). These observations suggest that blocking the FGF-2 pathway is an effective way to suppress angiogenesis *in vitro* and *in vivo*. It may not be ruled out, however, that down-regulation of VEGF as a result of decreased FGF-2 action contributes to the anti-angiogenic effects of PF-4^{47-70}, because it has been shown that VEGF expression can depend on FGF-2 release via an autocrine pathway (58).

Taken together, our results show that the fragment PF-4^{47-70} regroups most of the important features of potent angiogenesis inhibitors. These features include inhibition of FGF-2 binding to high- and low-affinity receptors, internalization of FGF-2, downstream ERK-signalling, endothelial cell proliferation, migration, *ex vivo* vessel assembly in collagen gels, and *in vivo* angiogenesis. It therefore acts as an inhibitor on some of the main biological events of the

angiogenic cascade. Further studies should investigate its effects on vessel maturation and arteriogenesis. Because of the small size of the peptide and its ability to affect many aspects of the angiogenic cascade, PF-4^{47-70} is a very promising candidate for further development as an anti-angiogenic drug for treatment of cancer and other angiogenesis-dependent diseases.

ACKNOWLEDGMENTS

We would like to thank Rebecca Ralph (Department of Neurosciences, University of California, San Diego) for critical reading of the manuscript; Concha Muela and Argentina Fernandez (Department of Histology, Hospital Ramón y Cajal) for technical help with the sponge assay; and M. Zazo for excellent technical assistance (Laboratorio Estructura Proteinas (LESP), Centro Investigaciones Biologicas (CIB), Madrid, Spain). This work was supported by grants from Retina France, La Ligue de Recherche contre le Cancer (comité de la Gironde) and the Ministère de la Recherche (MSR, JoBxI) to Andreas Bikfalvi, and by the Dirección General de Investigación Científica y Técnica and Fundación Futuro. Martin Hagedorn is a recipient of a fellowship from the Berthe Fouassier Foundation, and R. M. Lozano was a recipient of a Contrato de Reincorporación from the Spanish Ministerio de Educación y Ciencia.

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Received June 26, 2000; revised October 16, 2000.

Fig. 1

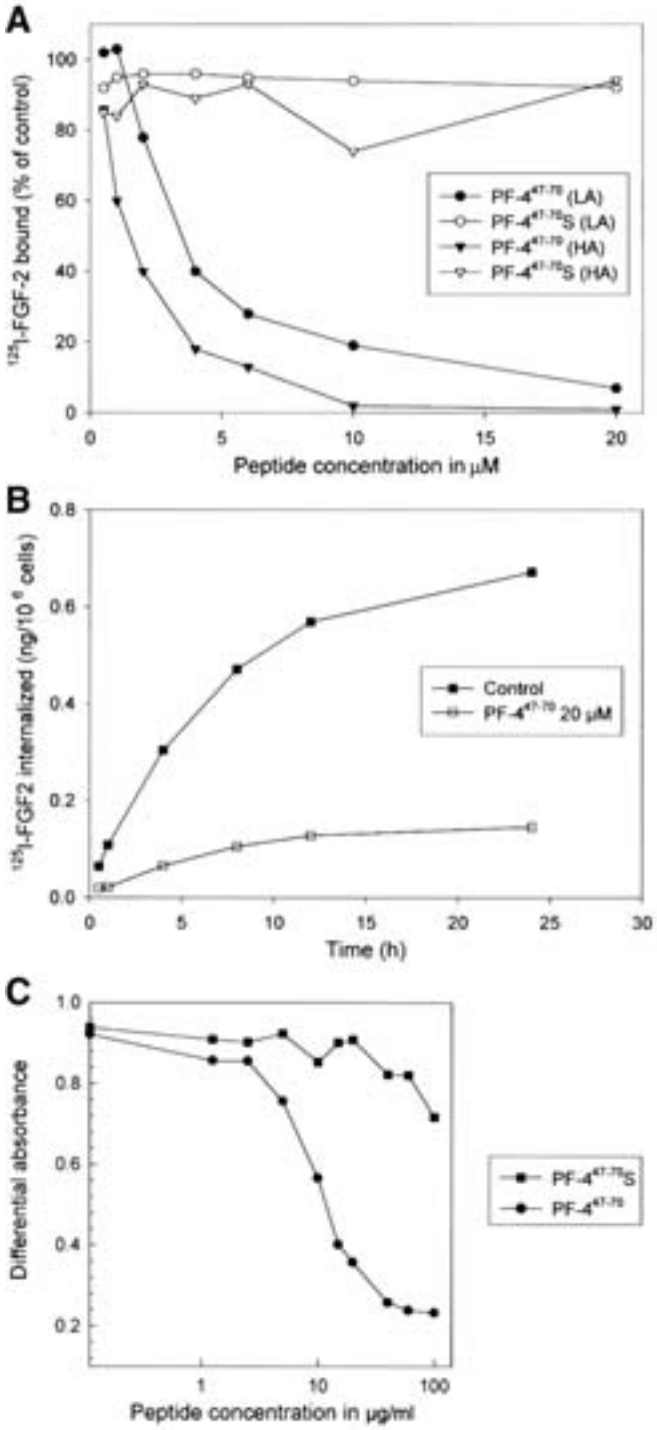

Figure 1. Inhibition of FGF-2 binding to low-affinity (LA) and high-affinity (HA) receptors, FGF-2 internalization and proliferation by PF-4$^{47-70}$. BCE cells are incubated with 10 ng/ml ^{125}I-FGF-2 and peptides at indicated concentrations. Peptide PF-4$^{47-70}$ but not PF-4$^{47-70}$S blocked binding of ^{125}I-FGF-2 to HA and LA receptors on BCE cells with half-maximal inhibition (IC_{50}) at ~2μM for HA receptors and ~4 μM for HSPGs receptors. Total inhibition was achieved at 20 μM**(A)**. At this concentration, PF-4$^{47-70}$ also inhibited internalization of ^{125}I-FGF-2 in BCE cells 4.6-fold **(B)**. Effects of PF-4$^{47-70}$ and PF-4$^{47-70}$S on the proliferation of a cell line transfected with FGFR-1 (BaF3/FR1c-11) in the presence of FGF-2 measured by the XTT colorimetric assay at an OD at A_{492}-A_{690} **(C)**. Cells were stimulated at a mitogen concentration of 60 ng/ml, which elicits a cell-proliferation response 85% of the maximum stimulation obtained in FGF-2-concentration proliferation-dependency assays of BaF3/FR1c-11 cells in the absence of heparin.

Fig. 2

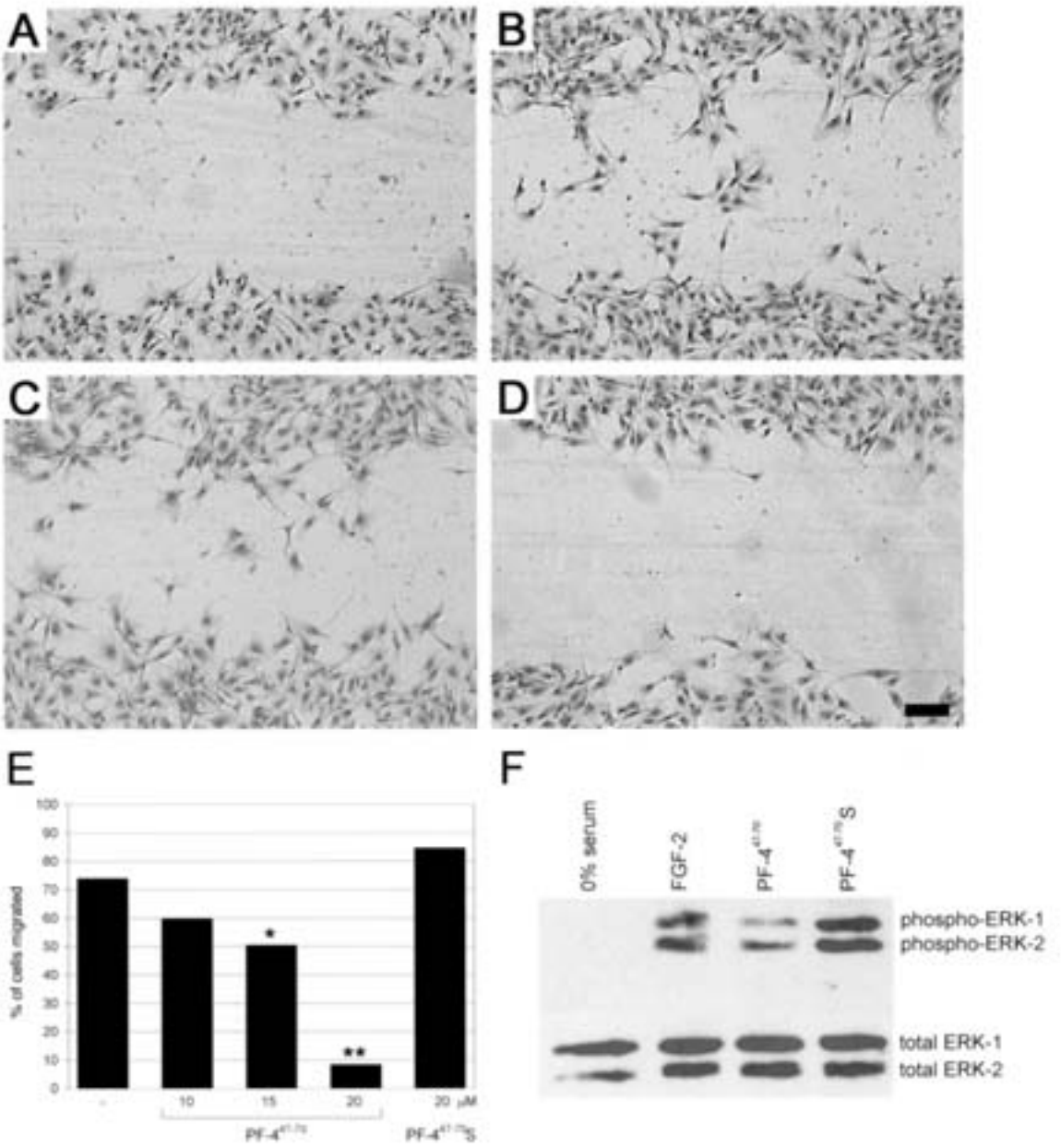

Figure 2. Photographs of a representative endothelial cell-migration experiment. After serum deprivation for 20 h, no BCE cells migrated into the scar drawn in the cell monolayer **(A)**. In the positive control (10 ng/ml FGF-2), numerous cells migrate into the denuded scar area **(B)**. PF-$4^{47\text{-}70}$S cannot counteract the stimulatory effects of FGF-2 **(C)**, whereas PF-$4^{47\text{-}70}$ at the same dose nearly completely blocks migration **(D)**. Effects with the dose of 20 µM PF-$4^{47\text{-}70}$ have been reproduced in five independent experiments. Black bar equals 130 µm on all photos. Inhibition of FGF-2-induced migration by PF-$4^{47\text{-}70}$ in a dose-dependent manner is demonstrated in **(E)**, with first effects starting around 10 µM and reaching a plateau already at 20 µM, where migration reaches control levels. Data are expressed in percentage of the mean number of migrated cells of seven fields over background cell migration of negative controls (without FGF-2). Dose-dependency experiments were performed in duplicates with similar results; data of one experiment are shown. Statistical analysis showed a significant difference in the migration response of untreated versus treated cells at 15 and 20 µM PF-$4^{47\text{-}70}$ (Student's *t*-test; *$p<0.05$; **$p<0.001$). PF-$4^{47\text{-}70}$, but not PF-$4^{47\text{-}70}$S, diminishes phosphorylation of MAPK ERK-1 and ERK-2 in BCE cells. Cells were stimulated with 10 ng/ml FGF-2 for 5 min and co-incubated with 20 µM of peptide **(F)**. The lower panel shows stripped probes incubated with total anti-MAPK p44/42 antibody, which demonstrates that same amounts of proteins were loaded on the gel.

Fig. 3

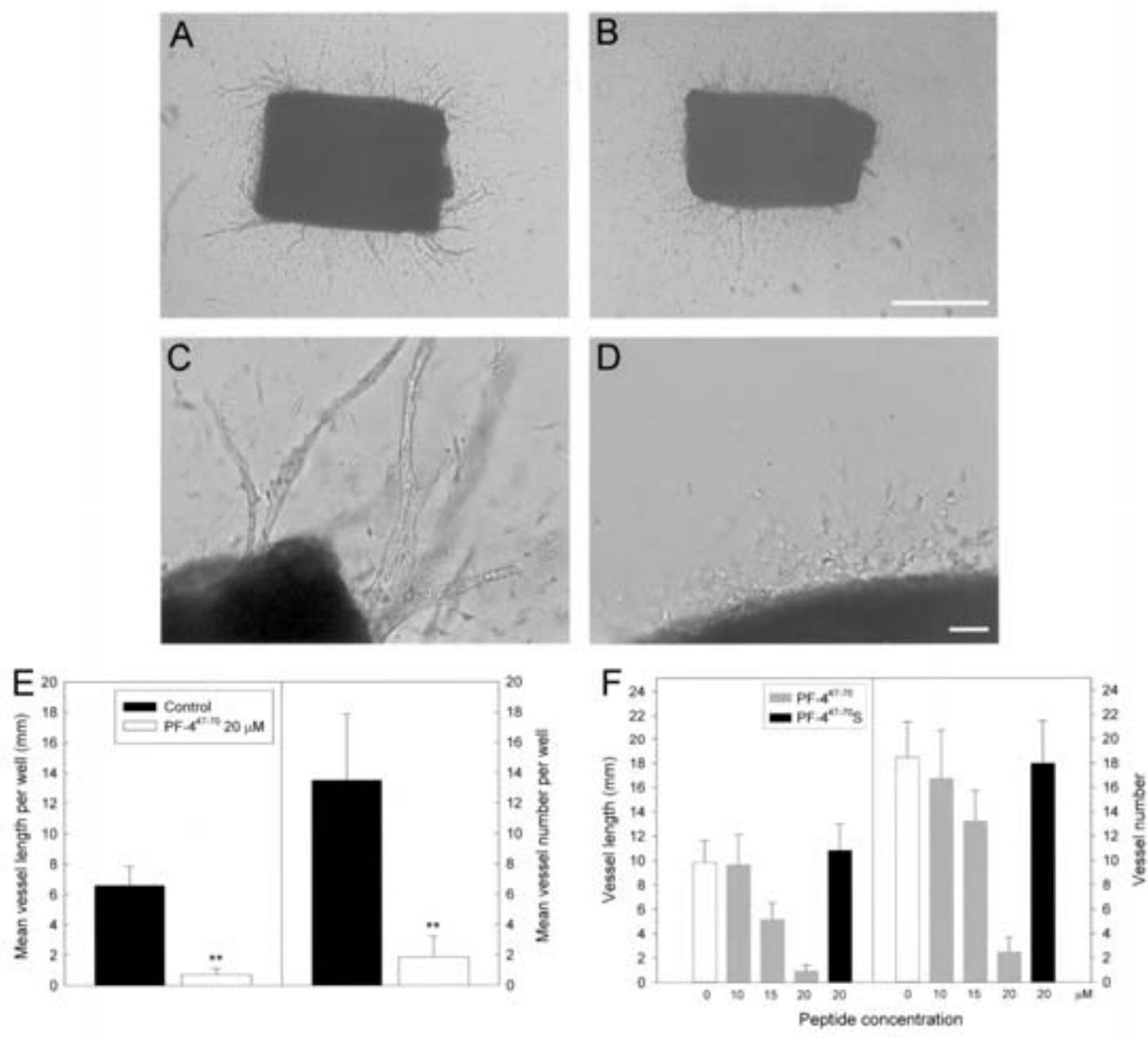

Figure 3. Inhibitory effects of PF-4$^{47-70}$ on spontaneous microvascular sprouting in the serum-free rat aortic ring model. Photographs from control **(A, C)** and PF-4$^{47-70}$-treated **(B, D)** living aortic ring cultures. In control cultures, numerous vessels are visible after 4-6 days. In ring cultures treated with 20 µM peptide PF-4$^{47-70}$, vessel formation is strongly inhibited, whereas cellular (fibroblastic) outgrowth is less effected **(B, D)**. If vessels developed, they were much thinner and shorter than vessels in control cultures **(B)**. At higher magnification, branching microvessels with formed lumina become clearly visible in untreated cultures, whereas PF-4$^{47-70}$ treated rings are surrounded mainly by stroma cells, which do not assemble to vascular sprouts. Magnification ×40 (A, B; bar equals 1 mm) and ×200 (C, D; bar equals 130 µm). As demonstrated in **(E)**, PF-4$^{47-70}$ reduces vessel length by 89% and vessel number by 86%. Each bar represents mean values and standard errors from four rings per group from three independent experiments. Difference of vessel length and number was analyzed by using the Student's *t*-test and was considered significant (**, $p<0.001$) **(E)**. The inhibitory effects of PF-4$^{47-70}$ are dose-dependent, with a narrow treatment window between 10 and 20 µM per culture **(F)**. A control peptide, where the cysteine in position 52 had been replaced by a serine (PF-4$^{47-70}$S), showed no effect. The experiment was repeated twice with similar results.

Fig. 4

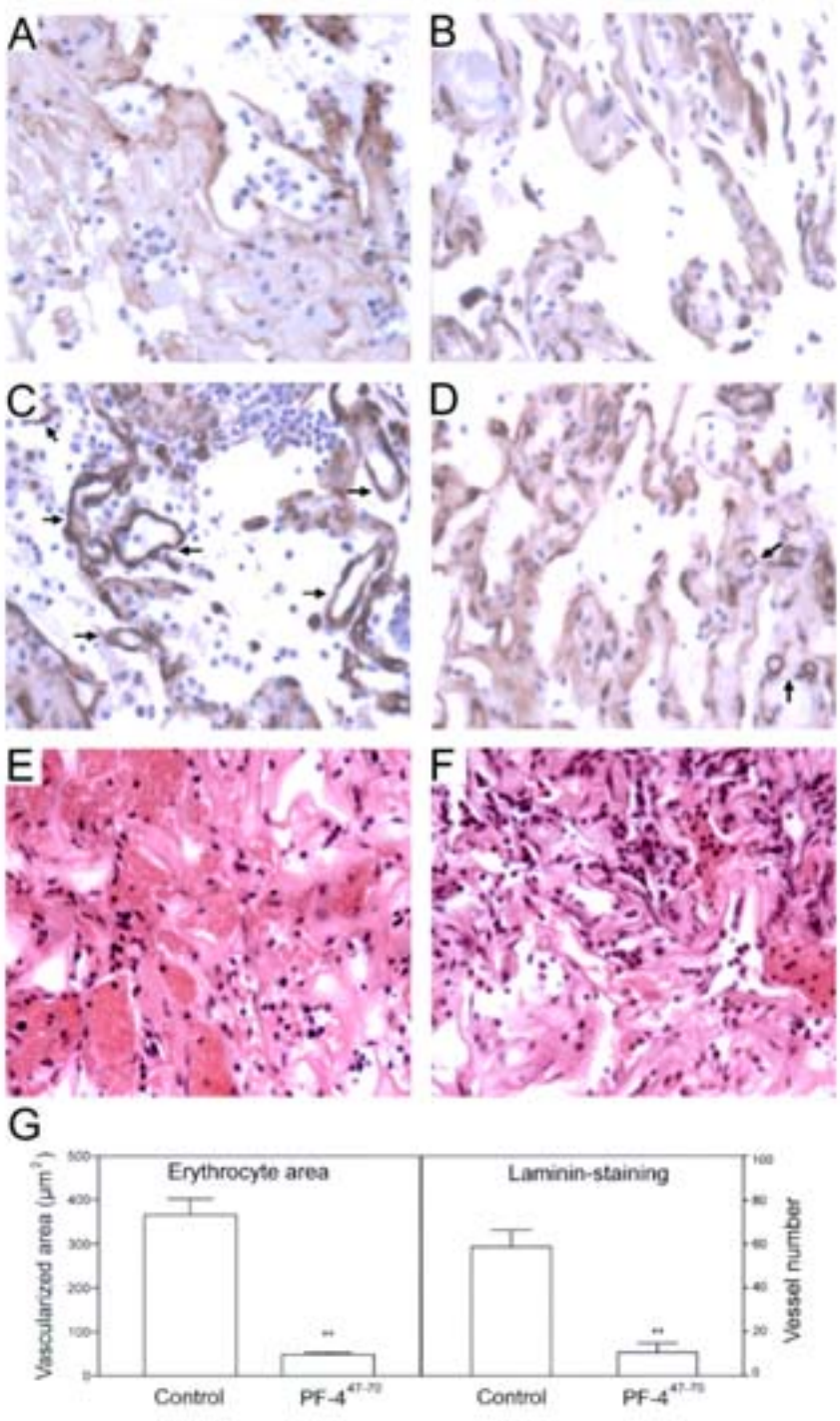

Figure 4. Inhibition of *in vivo* angiogenesis by PF4$^{47-70}$ in the mice gelatin sponge assay. Photomicrographs (original magnification x40) of 6-µm-thick sections of sponges pre-incubated with 200 µl of PBS alone **(A)**, 45 µg/ml of PF4$^{47-70}$ in PBS **(B)**, 10 ng/ml FGF-2 in PBS plus an injection of PBS 24 h after surgery **(C+E)** or 10 ng/ml FGF-2 plus one injection of 16 µM PF4$^{47-70}$ **(D+F)**. Note the absence of neovessels in sponges embedded with PBS or PF4$^{47-70}$ alone (A+B). Arrows indicate neovessels, counterstaining by Hematoxylin and Eosin **(E+F)** or Hematoxylin **(A-D)**. Quantification of the angiogenic response in sponges containing FGF-2 from animals treated intraperitoneally with PF4$^{47-70}$ or vehicle (PBS) was done either by calculating the area covered by erythrocytes or by counting new vessels identified by immunohistochemistry by using a polyclonal anti-laminin antibody **(G)**. The data are presented as mean ± SE, and differences were considered significant (Student's *t*-test, **p<0.001).

Fig. 5

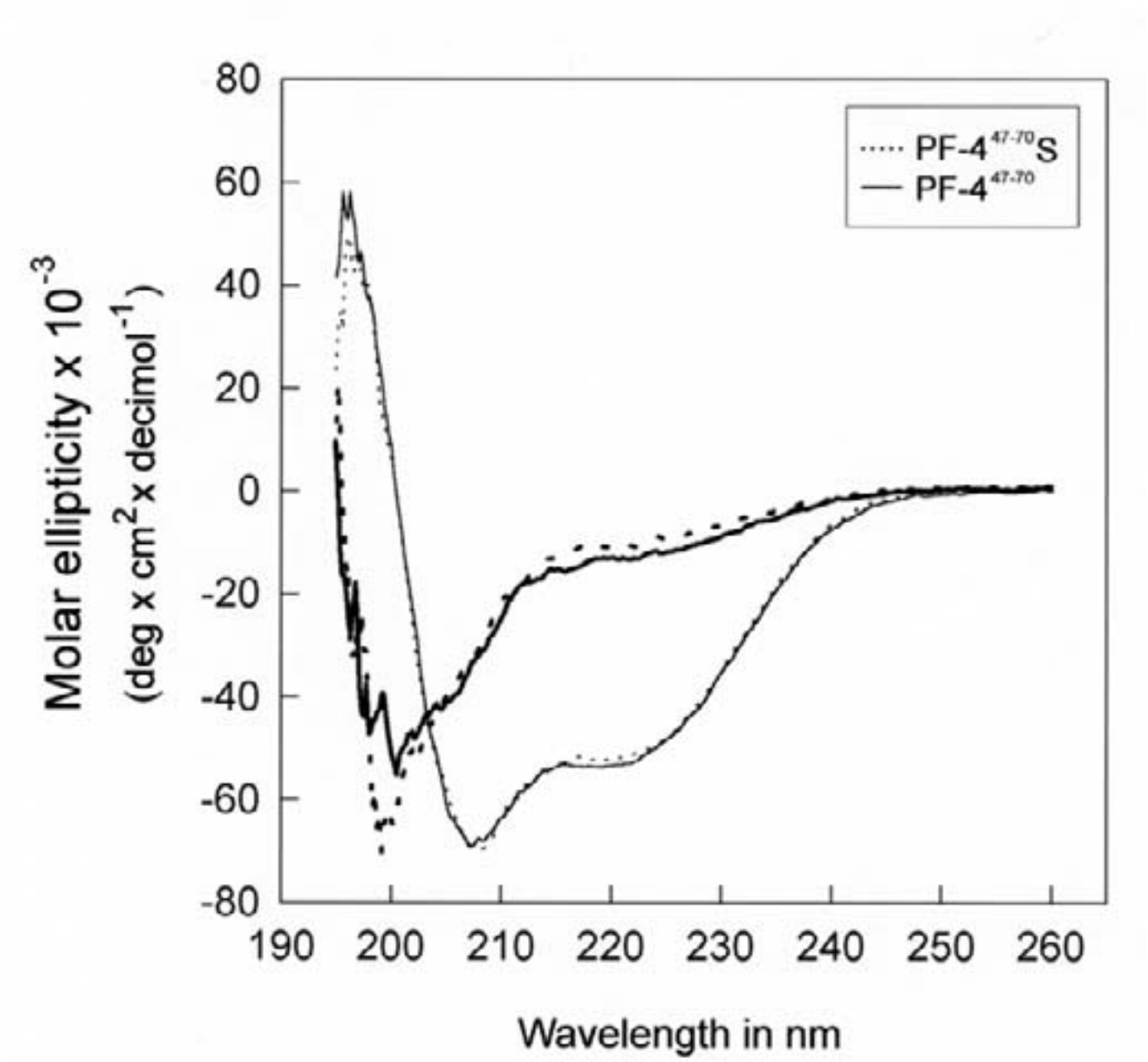

Figure 5. Molar ellipticity of PF4$^{47\text{-}70}$ (solid line) and PF4$^{47\text{-}70}$S (dotted line) solutions at 298 K. Protein (72 μM) was in 10 mM Na-phosphate, NaCl 80 mM buffered at pH 7.2. A considerable increase to almost 100% in the α-helix content was observed when 30% v/v TFE was added.

Introduction publication III

As we have shown in the previous publication, anti-angiogenic activity of a fragment derived from the CXC-chemokine PF-4 can be used to suppress angiogenesis in vivo. The PF-4^{47-70} peptide contains a DLQ motif, previously described as essential for inhibition of myeloid progenitor proliferation of full-length PF-4 (154). When we replaced this motif by the ELR tripeptide, present in pro-angiogenic chemokines like IL-8 or NAP-2, we expected to produce a molecule with pro-angiogenic activity. The advantage would have been to have a system consisting of three peptides, very similar in structure, with opposing effects on angiogenesis. In contrast, anti-angiogenic activity of PF-4^{47-70}ELR was enhanced compared to PF-4^{47-70}. An even stronger effect was observed with peptide PF-4^{47-70}DLR (Q56 to R), which had initially been designed as a control for peptide PF-4^{47-70}ELR, because the ELR to DLR mutation in the N-terminus of IL-8 greatly diminishes its function (154).

We show that FGF-2 as well as VEGF-induced angiogenesis can be blocked by these peptides using various in vitro and in vivo assays. Binding of iodinated FGF-2 and VEGF to ECs is strongly inhibited in the presence of both peptides. EC proliferation induced by FGF-2 or VEGF is also suppressed by the peptides but the growth of two glioma cell lines, U87 and C6 is not affected. Both, PF-4^{47-70}ELR and –DLR, can block FGF-2 or VEGF-mediated EC migration at lower doses as the original peptide. In the serum-free rat aortic ring assay, only PF-4^{47-70}DLR showed stronger anti-angiogenic activity than PF-4^{47-70}, but capillary angiogenesis induced by $VEGF_{165}$ on the day 13 CAM was strongly inhibited by both peptides at a dose, where PF-4^{47-70} was inactive. Finally we show that the growth of established intracerebrellar glioma in nude mice is greatly reduced by PF-4^{47-70}DLR compared to the same dose of PF-4^{47-70}.

Modified C-terminal peptides of PF-4 are potent inhibitors of VEGF- and FGF-2-induced angiogenesis in vitro and in vivo. They might be especially useful in the treatment of tumors expressing high levels of these growth factors, like glioblastoma.

In vivo systems to evaluate angiogenesis inhibitors: The chick chorio-allantoic membrane assay

The development of anti-angiogenic drugs requires effective and realistic screening techniques to test for biological activity. The CAM assay is one of the oldest and most widely used animal models to study embryology and more recently, angiogenesis. The physiological function of the CAM includes gas exchange, calcium adsorption for bone development and absorption of waste products.

The allantois forms around day 3.5 (stage 18, Hamilton & Hamburger) as an evagination from the ventral wall of endodermal hind gut, and protrudes in the extraembryonic coelem. During day 6 and 7, it fuses with the adjacent mesodermal layer of the chorion to form the chorio-allantoic membrane (155). From day 5 to 6, the CAM surface expands 20-fold (156). During day 5 to 7, sprouting angiogenesis prevails, thereafter the capillary meshwork is remolded by intussusceptive growth and around day 13 the CAM reaches a developmental state where small further changes occur in vessel morphology (16). Accordingly, cell proliferation studies using radioactive thymidine incorporation or BrdU labeling have shown that the CAM endothelium displays a high mitotic rate until day 10 which decreases several-fold at day 11 and thereafter (157, 158). This demonstrates endogenous angiogenesis and high susceptibility of endothelial cells during early CAM development. Additionally, non-specific carrier effects often render interpretation of effects of anti- or pro-angiogenic molecules difficult before day 13 (159).

We therefore chose to test the modified PF-4 peptides using a modified version of the previously described day-13 CAM assay so that we repeatedly could stimulate and/or inhibit angiogenesis and reduce unspecific carrier effects. For that purpose, Thermanox plastic rings were produced and placed on the CAM. Because of the high adhesiveness of the CAM surface, liquid placed in the center of these rings remains in place and is absorbed by the CAM within one hour. This assay allowed us for the first time to demonstrate by bio-microscopy and semi-thin sectioning of the CAM area covered by the ring, the efficacy of an angiogenesis inhibitor on VEGF-induced capillary growth (see figure 5, publication III).

PUBLICATION III

Domain swapping in a C-terminal fragment of platelet factor 4 generates potent angiogenesis inhibitors

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

-Accepted for publication by “Cancer Research”, October 04th, 2002-

Title: Domain swapping in a C-terminal fragment of platelet factor 4 generates potent angiogenesis inhibitors

Authors: Martin Hagedorn,* Lior Zilberberg,* Jörg Wilting,[†] Xavier Canron,* Giorgio Carrabba,[‡] Carlo Giussani,[‡] Mauro Pluderi,[‡] Lorenzo Bello,[‡] and Andreas Bikfalvi*

Institutions: * INSERM EMI 0113 'Molecular Mechanisms of Angiogenesis', Université de Bordeaux I, Avenue des Facultés, 33405 Talence, France; [‡] Neurosurgery**,** Department of Neurosurgery, Department of Neurological Sciences, University of Milano, Ospedale Maggiore Policlinico, IRCCS, 20122 Milan, Italy; [†] Universitätskinderklinik Göttingen, Abteilung Pädiatrie I, Robert-Koch-Strasse 40, 37570 Göttingen, Germany

Running title: Angiogenesis inhibition by modified PF-4 peptides

Key words: Vascular endothelial growth factor, fibroblast growth factor, CAM assay, intracerebral glioma model, anti-angiogenic peptides

Footnotes:

This work was supported from grants of the 'Ligue Nationale Contre le Cancer', the INSERM, the 'Ministère de la Recherche' (all to A. B.); and of 'Ricerca Finalizzata, Ospedale Maggiore di Milano', and 'Fondazione Monzino', Milano, Italy (to L. B.). M. H. was a fellowship of the 'Féderation de la Recherche Médicale' (F.R.M.) and the 'Féderation des Aveugles et Handicapés visuels de France' (F.A.F.). L. Z. was supported by the 'Association pour la recherche contre le cancer' (ARC). We are grateful to Mrs. S. Antoni and Mr. G. Frank (Anatomisches Insitut I, Universitaet Freiburg, Germany) for their excellent technical assistance and to Dr. Françoise Dellu-Hagedorn (U.M.R 5541, Neuropsychobiologie des désadaptations, Bordeaux, France) for help with the statistical analysis.

Animal studies (intracranial glioma model) have been approved by the ethical committee of the University of Milano, Italy**.**

Abbreviations used in this paper: VEGF(R), vascular endothelial growth factor (receptor); FGF(R), fibroblast growth factor (receptor); PF-4, platelet factor 4; CAM, chorio-allantoic membrane; GAG, glycosaminoglycan; BCE, bovine capillary endothelial; BAE, bovine aortic endothelial; ACE, adrenal cortex capillary endothelial; EC, endothelial cell.

Reprint requests to: Professor Andreas BIKFALVI, INSERM EMI 0113 « Mécanismes Moléculaires de l'Angiogenèse », Université Bordeaux I, Avenue des Facultés, 33 405 Talence, FRANCE. Fax: (33) 5 56 84 87 05, E-mail: a.bikfalvi@croissance.u-bordeaux.fr

Abstract

A few peptide residues in structurally important locations often determine biological functions of proteins implicated in the regulation of angiogenesis. We have recently shown that the short C-terminal segment PF-4^{47-70} derived from platelet factor 4 (PF-4) is the smallest sequence that conserves potent anti-angiogenic activity *in vitro* and *in vivo*. Here we show that modified C-terminal PF-4 peptides containing the sequence ELR (or related DLR), a critical domain present in pro-angiogenic chemokines, surprisingly elicit several times greater anti-angiogenic potential than the original peptide. The modified peptides inhibit binding of iodinated vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2) to endothelial cell (EC) receptors, EC proliferation, migration and microvessel assembly in the rat aortic ring model at lower doses than PF-4^{47-70}. On the differentiated chick chorio-allantoic membrane (CAM), topical application of 40 µg of modified peptides potently reduces capillary angiogenesis induced by $VEGF_{165}$, a dose were peptide PF-4^{47-70} was inactive. Established intracranial glioma in nude mice decreased significantly in size when treated locally with a total dose of 250 µg of peptide PF-4^{47-70}DLR (n=10) compared to the same dose of the original PF-4^{47-70} peptide (n=10) or controls (n=30). Tailored PF-4 peptides represent a new class of anti-angiogenic agents with a defined mode of action and a strong *in vivo* activity.

Introduction

VEGFs and FGFs are amongst the most important angiogenic factors. FGF-2 binds to tyrosine kinase receptors on endothelial cells in a heparan-sulfate-dependant manner (1). FGF-2 and its receptors play a critical role during normal development and maintenance of the embryonic vasculature (2, 3); monoclonal antibodies against FGF-2 inhibit tumor growth in nude mice through blocking of angiogenesis (4). Impairing FGFR-1 signaling by pyrimidine derivates or dominant negative receptors leads to angiogenesis inhibition *in vitro* and *in vivo* (3, 5, 6). A peptide derived from PF-4 interacts with FGF-2 in a defined manner (7) and counteracts its biological activity *in vitro* and *in vivo* (8, 9). FGF-2 also induces production of VEGF via an autocrine feedback loop (10) and blocking antibodies against FGF-2 can counteract angiogenic effects of VEGF *in vitro* (11).

In growing tumors and the ischemic retina, hypoxia is the main regulator of VEGF expression through induction of transcription factor HIF-1α (12). Five VEGF isoforms are thus generated by alternative splicing that exhibit different biochemical properties; variant $VEGF_{165}$ represents the most abundant form and is one of the strongest angiogenic growth factors (13, 14). The biological response of VEGF is mediated by two endothelial cell-specific tyrosine kinase 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) as well as soluble dominant-negative receptors (21).

PF-4 belongs to the CXC-chemokines, which, in general, are pro-angiogenic when the tripeptide ELR precedes the first CXC-domain, but are anti-angiogenic, when this motif is absent (22). Exceptions are growth-related protein beta (GRO-beta), which contains an ELR motif, but inhibits angiogenesis *in vitro* and *in vivo* (23), and the ELR-negative stromal-derived factor 1 (SDF-1), which shows pro-angiogenic 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 C-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 N-terminal DLQ domain of PF-4 with the corresponding ELR motif of the angiogenic CXC-chemokines IL-8 or neutrophil activating protein 2 (NAP-2) confers CXCR-2-dependent neutrophil activation of these proteins to PF-4 (31, 32). On the other hand, mutation of ELR to DLQ or DLR 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-4^{47-70}, recently described as the smallest anti-angiogenic portion of PF-4 (8) by inserting ELR or DLR mutations. The modified peptides surprisingly inhibited binding of iodinated VEGF or FGF-2 to cellular receptors at several times lower concentrations as the unmodified peptide and abrogated VEGF or FGF-2-induced EC proliferation but not glioma cell growth. Using a newly developed anti-angiogenesis assay on the differentiated CAM, where capillary angiogenesis is induced in the stroma by VEGF, we show anti-angiogenic 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 anti-angiogenic peptide can determine to a great extend biological activity *in vitro* and *in vivo.* Modified PF-4 peptides represent good candidates for the development of peptide-based anti-angiogenic drugs for cancers with high expression of VEGF and FGF-2.

Material and Methods

Synthetic peptides

C-terminal PF-4 peptides (PF-4^{47-70}: NGRKICLDLQAPLYKKIIKKLLES; PF-4^{47-70}ELR: NGRKICLELRAPLYKKIIKKLLES; PF-4^{47-70}DLR: NGRKICL-DLRAPLYKKIIKKLLES; PF-4^{47-70}S: NGRKISLDLQAPLYKKIIKKLLES) were purchased from ThermoHybaid, Ulm, Germany or were donated by Rhône-Poulenc Rorer, Paris, France. All peptides were purified by HPLC to more than 95% and calculated molecular weight was confirmed by mass spectroscopy.

Growth factors

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 (34, 35). Human $VEGF_{165}$-encoding baculovirus was a kind gift of Dr. Jean Plouët (Institut de Pharmacologie et de Biologie Stucturale, UMR 5089, Toulouse, France).

Cells

Bovine capillary endothelial (BCE) cells were a kind gift of Dr. Daniel B. Rifkin (NYU Medical Center, New York, USA). Bovine aortic endothelial (BAE) cells were from Dr. Georg Breier (Department of Molecular Biology, Max-Planck-Institut fur physiologische und klinische Forschung, Bad Nauheim, Germany). Adrenal cortex capillary endothelial (ACE) cells were donated by Dr. Jean-Jacques Feige (INSERM EPI 0105, CEA-Grenoble, France). All endothelial cells were grown at 37°C, 5% CO_2 in DMEM, 10% new born calf serum (NBCS), 1% glutamine and antibiotics (GibcoBRL Life Technologies, Cergy Pontoise, France) and were used up to passage 25. BCE and ACE cells were grown in the presence of 2 ng/ml FGF-2. C6 rat glioma cells (a kind gift from Dr. Paul Canioni, CNRS UMR 5536, Université Bordeaux II, Bordeaux, France) were grown in DMEM, 10% fetal bovine serum (FBS) and antibiotics. U87 glioma cells (ATCC) were grown in MEM alpha medium, 10% FBS plus antibiotics. HI5 insect cells were a kind gift of Dr. Jean Plouët and were cultured without CO_2 at 28ºC in IPL41 insect cell medium (GibcoBRL Life Technologies, Cergy Pontoise, France) containing 10% FBS, L-glutamine and antibiotics.

Binding assays

$VEGF_{165}$ and FGF-2 were labeled with ^{125}I-Na using Iodogen (Pierce, Rockford, IL, USA) as coupling agent according to the manufacture's instructions and (36). ACE cells were seeded at 2.5 x 10^5 density in gelatin-coated 6-well plates and cultured in complete medium for two days. Cells were washed twice with ice cold PBS and incubated with 10 ng/ml ^{125}I-FGF-2 and peptides at indicated concentrations in binding medium (DMEM, 20 mM Hepes, pH 7.4; 0.15% gelatine) for 2 h on a shaker at 4°C. ^{125}I-FGF-2 from high and low affinity binding sites was recovered and quantified as described earlier (8). ^{125}I-VEGF-binding was evaluated essentially in the same manner, except that cells were detached (2% triton, 10% glycerol, 1 mg/ml BSA) prior to gamma counting. Each condition was tested in duplicates. Data are expressed as percentage of total radioactivity and IC_{50} for every peptide was calculated from smooth curve fits.

Proliferation assays

ACE cells were seeded in 6-well culture plates overnight in 10% NBCS at 5000-7500 cells per well. Medium was changed to 1% NBCS, 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 $VEGF_{165}$-induced cell proliferation (10 ng/ml), BAE cells were used in conditions similar to ACE cells. IC_{50}'s 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, www.lim.cz). The dishes were washed and fresh serum-free medium containing 0.1% BSA, 10 ng/ml FGF-2 (BCE migration) or 10 ng/ml of

$VEGF_{165}$ (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 endothelial cells 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, aortic rings from male or female Sprague Dawley rats, between 200 and 500 g, (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 day 0, 2 and 4 and photos were taken on day 7; controls received no peptide or peptide PF-$4^{47\text{-}70}$S. Microvessel length and number were measured using digital image analysis software (Lucia G, www.lim.cz). 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, USA).

CAM anti-angiogenesis assay

Fertilized chicken eggs (*Gallus gallus*) (E.A.R.L. Morizeau, Dangers, France) were incubated at 37ºC 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 Thermanox® coverslips) were put on the CAM. 3 µg of $VEGF_{165}$ was pre-mixed 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 minutes at room temperature and the area containing the ring was cut out for further 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 $VEGF_{165}$-induced angiogenesis from 0 to 2 (0 = none, 1 = medium, 2 = high). Means of the obtained scores were analyzed by the Kruskal-Wallis one-way analysis of variance and pair wise comparisons of the different treatment groups (CRUNCH software corporation, Oakland, CA, USA). For histological studies, samples were fixed in 3% glutaraldehyde and 2% formaldehyde in 0.12 M sodium cacodylate buffer, post-fixed 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 (x200 magnification) were taken using a Leica DMR microscope.

Intracranial glioma model

Groups of 10 six weeks old nude mice (Charles Rivers Italia, Monza, Italy) were implanted intracranially with 50.000 human U87 glioma cells using an open window technique (38). After 15 days, animals were implanted with 2004 Alzet osmotic minipumps (ALZET, Cupertino, CA, USA). 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 mg, 0.5 mg or 1 mg of the peptides 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 ($width^2$ x length/2) (38). Statistical analysis of tumor volumes was performed with a two-way ANOVA (peptide x

dose) followed by analyses of simple main effects to compare the effects of the 3 peptides at each dose. Pairwise comparisons between peptides at each dose were performed by the Newman-Keuls post-test. Immunhistochemistry was carried out using the Vectastain® Elite kit (Vector Laboratories, CA, USA). Anti-CD31 antibody (Pharmingen USA) was used at 1:100 dilution. Signal was visualized with DAB chromogen and sections were counterstained with hematoxylin. Microvessel counts and density were scored as previously reported (39). Apoptotic cells were detected with the ApopTag™ plus kit (Intergen, MS, USA) and quantified as described earlier (39).

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-FGF-2 and ^{125}I-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. Half-maximal concentrations (IC_{50}) necessary to inhibit ^{125}I-$VEGF_{165}$ binding to its receptors were 0.4 µM for PF-4$^{47-70}$DLR, 0.56 µM for PF-4$^{47-70}$ELR and 1.58 µM for PF-4$^{47-70}$ (figure 1 A). IC_{50} for inhibition of FGF-2 binding to low or high (in brackets) affinity sites were 0.75 µM (0.46 µM) for PF-4$^{47-70}$DLR, 1.51 µM (0.53 µM) for PF-4$^{47-70}$ELR and 3.47 µM (2.06 µM) for peptide PF-4$^{47-70}$ (figure 1 B, 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 VEGF165 (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 µM) (figure 2 A), followed by PF-4$^{47-70}$ (2.15 µM) and PF-4$^{47-70}$ELR (2.91 µM). 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 µM and 2.34 µM in comparison to 11.17 µM for PF-4$_{47-70}$ (figure 2 B). In contrast to ECs, proliferation of two glioma cell lines, human U87 and rat C6 cells, was not inhibited by normal or modified PF-4 peptides at all doses (figure 2 C).

Inhibition of micro- and macrovascular EC migration. Without stimulation, some random background migration occurred (figure 3 A). BAE cells migrated into the denuded scar area when stimulated with $VEGF_{165}$ (figure 3 B) and, in a very similar way, when FGF-2 was added to BCE cells (not shown). We tested the modified peptides at a concentration, where PF-4$^{47-70}$ was inactive or much less active (5 µM on BAE cells, 10 µM on BCE cells). PF-4$^{47-70}$ ELR strongly inhibited EC migration (figure 3; E, G); PF-4$^{47-70}$DLR induced complete inhibition of VEGF-induced migration (figure 3; F, G), PF-4$^{47-70}$S showed no effect (figure 3 C) and PF-4$^{47-70}$ only a small one (figure 3; D, G). One representative BAE migration experiment is shown; modified peptides showed a similar increase in activity compared to PF-4$^{47-70}$ in FGF-2-induced BCE 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 (figure 4 A). PF-4$^{47-70}$S at the highest concentration tested in this assay did not show any effects on vessel length or number (figure 4; E, F). A strong reduction of the mean microvessel length was observed only for peptide PF-4$^{47-70}$DLR at 10 µM compared to the same dose of PF-4$^{47-70}$ ($p<0.05$) or PF-4$^{47-70}$ELR ($p<0.002$; Newman-Keul post-hoc test after ANOVA analysis of all groups: $F(7.79) = 11.23$; $p<0.001$) (figure 4; D, E, F).

Statistical analysis of peptide effects on microvessel number showed that groups differed significantly from each other (One-way ANOVA: $F(7,79) = 28.37$; $p<0.001$). 40% inhibition was observed in cultures treated with 5 µM PF-4$^{47-70}$ and 63.5% at 10 µM compared to PF-4$^{47-70}$S. Mean vessel number was reduced by 33% at 5 µM (40% at 10 µM) in cultures treated with PF-4$^{47-70}$ELR compared to control peptide PF-4$^{47-70}$S. 50% inhibition was reached at 5 µM and 90% inhibition was achieved at 10 µM in cultures treated with PF-4$^{47-70}$DLR (figure 4 F). Thus, in this assay, only peptide PF-4$^{47-70}$DLR showed a clear increase in inhibitory activity in comparison to PF-4$^{47-70}$. Post-hoc pairwise comparison between PF-4$^{47-70}$DLR and PF-4$^{47-70}$ or PF-4$^{47-70}$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. 3 µg of recombinant human $VEGF_{165}$ applied in the center of a plastic ring on the surface of the CAM induced strong capillary formation (figure 5, B). The effect of VEGF is also present around the site of application, due to diffusion. Water alone had no effect (figure 5, A). When pre-mixed with the growth factor and deposed on the CAM, a clear anti-angiogenic effect of peptide PF-4$^{47-70}$ELR and PF-4$^{47-70}$DLR was visible inside the ring (figure 5; C, D). PF-4$^{47-70}$ELR also causes a hyperplasia of the chorionic epithelium, comparable to hyperosmolar effects (insert of figure 5, C).

Control peptide PF-4$^{47-70}$S did not inhibit VEGF-induced capillary growth in the CAM stroma (figure 5, E). At the low dose tested, PF-4$^{47-70}$ failed to stop capillary growth in this assay (figure 5, F) and did not differ from the control peptide ($p<0.05$; after analysis of differences between all treatment groups, Kruskal-Wallis test: $H(4) = 28.27$, $p<0.0001$). The modified peptides PF-4$^{47-70}$ELR and PF-4$^{47-70}$DLR both showed an equally strong anti-angiogenic effect at the dose tested compared to PF-4$^{47-70}$S (** = $p<0.002$ for both peptides) (figure 5, G). In semi-thin sections of control CAMs treated with sterile water, the capillary layer stays within

the chorionic epithelium (arrow, insert in A); larger conduit vessels are present in the stroma, filled with erythrocytes. $VEGF_{165}$ induces a strong *de novo* growth of capillaries, which are present throughout the stroma (arrows, insert in B). Control peptide PF-4^{47-70}S does not neutralize the capillary angiogenesis induced by $VEGF_{165}$ (insert in E). PF-4^{47-70} at the low dose tested (2 x 20 mg) also does not inhibit capillary angiogenesis (insert in D). PF-4^{47-70}ELR (insert in C) as well as PF-4^{47-70}DLR (insert in D) markedly decreases angiogenesis nearly to control levels.

Inhibition of intracerebral tumor growth. Animals treated with control peptide PF-4^{47-70}S, with PBS, or those without pumps developed large tumors (figure 6 A, upper panel). Animals receiving different doses of peptide PF-4^{47-70}DLR or PF-4^{47-70} showed a strong reduction of tumor volumes (figure 6 A, lower panel). Immunohistochemical analysis with CD31 antibody revealed that inhibition of tumor growth in animals treated with PF-4^{47-70}DLR or PF-4^{47-70} is associated with a decreased vessel density (figure 6 B) and an increased number of apoptotic cells (figure 6 C). Statistical analysis of tumor volumes of animals treated with the equal dose of active peptides (PF-4^{47-70} and PF-4^{47-70}DLR) revealed a significant difference in anti-tumor efficacy and showed a clear advantage of peptide PF-4^{47-70}DLR, especially at the lowest dose tested, 0.25 mg (**$p<0.005$).

Discussion

PF-4$^{47-70}$ is the smallest anti-angiogenic 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 by the ELR tripeptide, present in pro-angiogenic chemokines like IL-8 or NAP-2, we expected a peptide with pro-angiogenic activity. In contrast, anti-angiogenic activity was enhanced compared to PF-4$^{47-70}$ in a number of assays. An even stronger effect was observed with PF-4$^{47-70}$DLR (Q56 to R), which had initially been designed as a control for PF-4$^{47-70}$ELR, because the ELR to DLR mutation in the N-terminus of IL-8 greatly diminishes its function (33). FGF-2 and VEGF$_{165}$ binding to tyrosine kinase receptors on ECs is strongly suppressed by PF-4$^{47-70}$ELR and -DLR. Endostatin also inhibits VEGF$_{165}$ binding to VEGFR-2, but the effect seems to depend greatly on pre-incubation of endostatin with cells prior to addition of iodinated VEGF$_{165}$ (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 VEGF$_{165}$ or FGF-2 in the low micromolar range. Comparable results have been obtained with a designed peptide inhibitor termed “Anginex”, a 33-mer beta-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 where several times higher than those of PF-4$^{47-70}$ELR or –DLR, and only FGF-2-induced angiogenesis has been studied. C-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-4$^{47-70}$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-4$^{47-70}$ELR did not very well inhibit microvessel sprouting in this assay. Since the ELR motif is crucial for receptor activation of the pro-angiogenic chemokine IL-8 (44), it is possible that PF-4$^{47-70}$ELR stimulates chemokine receptors on residual leukocytes and ECs (45) present in the aortic wall, which may partially overcome 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$^{47-70}$DLR shows maximal activity between 14 and 28 µg/ml (5-10 µM), thus at

18 to 35 times lower concentrations. This strong activity is probably due to the fact that the peptide interferes with both, VEGF and FGF-2, by inhibition of binding to their receptors.

We tested effects of C-terminal PF-4 peptides for their ability to interfere with $VEGF_{165}$-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 day 13 CAM induced by human recombinant $VEGF_{165}$ is strongly reduced by PF-4^{47-70}DLR and –ELR. In some eggs, PF-4^{47-70} and PF-4^{47-70}S also showed some minor activity, but this may result from inter-individual variations, the high sensitivity of the assay system or the difficulty to exactly quantify differences between low inhibitory effects. Metastatin, 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 alphaVbeta3-integrin showed strong anti-angiogenic effects in the day 10 CAM at 300 µg; again, the angiogenic response was induced by VEGF or FGF-2 at doses less than 1 µg (49). These comparisons indicate that modified C-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 recently been shown 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). 250 µg of peptide PF-4^{47-70}DLR were already sufficient to reduce tumor growth to one half compared to PF-4^{47-70}-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 anti-angiogenic molecule PEX; alone or in combination with low-dose chemotherapy. When PEX was included in the treatment, animals survived significantly longer and showed no measurable side effects (38, 39).

Binding of FGF-2 or $VEGF_{165}$ to tyrosine kinase receptors is facilitated by heparan sulfate proteoglycans on the cell surface and is essential for their pro-angiogenic activities (55). It is possible that the peptides, which contain the C-terminal heparin-binding motif of PF-4 consisting of two double lysine cluster (56), operate in part via inhibition of this interaction. Three positively-charged arginines within full-length PF-4 (R20, R22, R49) contribute to its

particularly high heparin-binding capacity (57). This might explain the improved anti-angiogenic activity, since both PF-4$^{47-70}$ELR and -DLR contain an additional arginine (R56) together with the internal R49. Similar observations have been made with peptides mimicking the surface of endostatin: an increase of arginine residues enhances their anti-angiogenic effects probably due to an increased affinity for heparin (58).

It has been shown recently that unmodified PF-4$^{47-70}$ associates directly with FGF-2, independently of GAG-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 due to an increase in affinity for FGF-2 and perhaps, VEGF too. Taken together, modified C-terminal peptides of PF-4 are potent inhibitors of VEGF- and FGF-2-induced angiogenesis *in vitro* and *in vivo*. They might be especially useful in the treatment of tumors expressing high levels of these growth factors; like glioblastoma.

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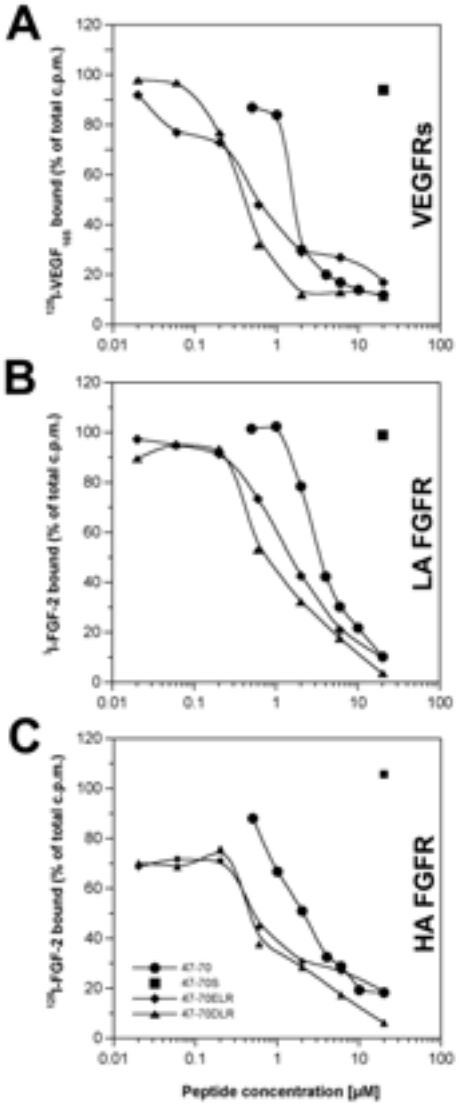
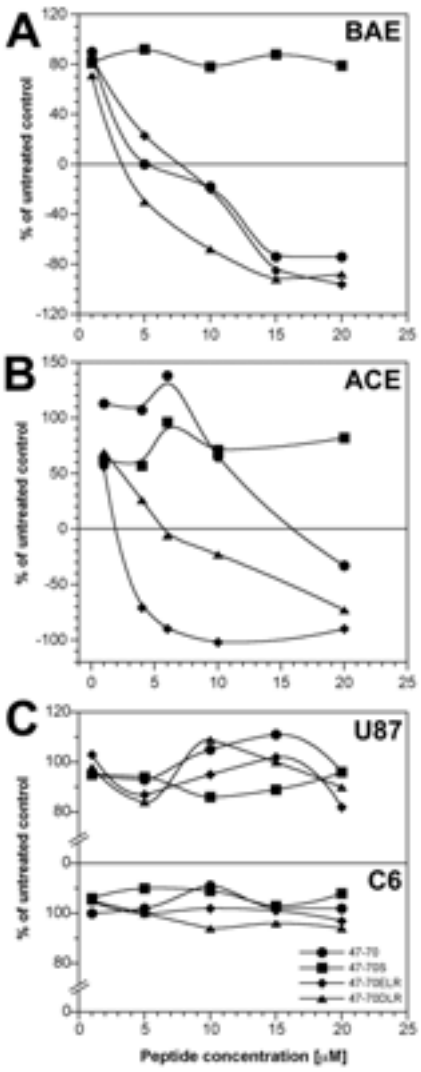
Figure 1



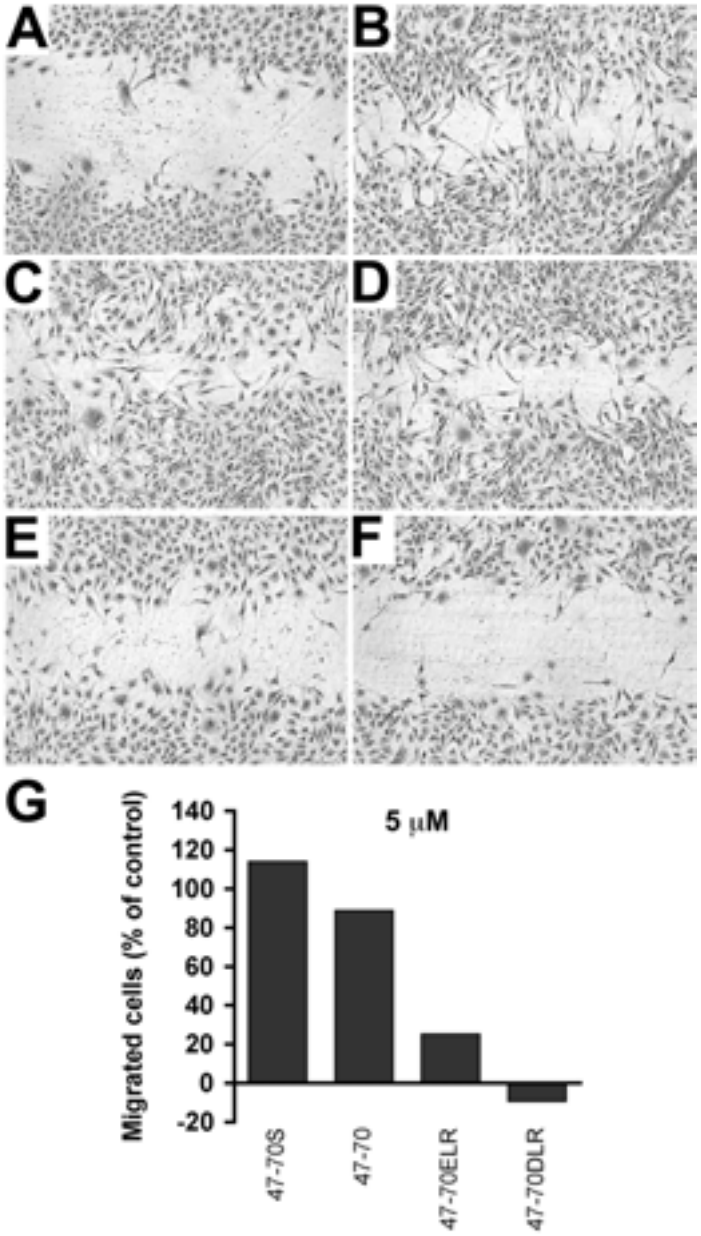
ACE cells were incubated with 10 ng/ml of ^{125}I-VEGF$_{165}$ (A) or ^{125}I-FGF-2 (B, C) and peptides at indicated concentrations. Control peptide PF-4$^{47\text{-}70}$S at the highest dose tested (20 μM) had no effect. Half-maximal inhibitory concentrations (IC_{50}) were calculated from smooth curve fits. Peptide PF-4$^{47\text{-}70}$ELR inhibited ^{125}I-VEGF binding to VEGFRs at approximately 3 times lower concentrations and PF-4$^{47\text{-}70}$DLR at four times lower concentrations than peptide PF-4$^{47\text{-}70}$ (A). FGF-2 binding to low affinity receptors (LA FGFR) was affected in a similar way: PF-4$^{47\text{-}70}$ELR-induced inhibition of binding was 2 times stronger and PF-4$^{47\text{-}70}$DLR-induced inhibition nearly 5 times stronger than that of PF-4$^{47\text{-}70}$ (B). FGF-2 binding to high affinity receptors (HA FGFR) was inhibited at approximately 4 times lower concentrations by peptides PF-4$^{47\text{-}70}$ELR and PF-4$^{47\text{-}70}$DLR than by peptide PF-4$^{47\text{-}70}$ (C).

Figure 2



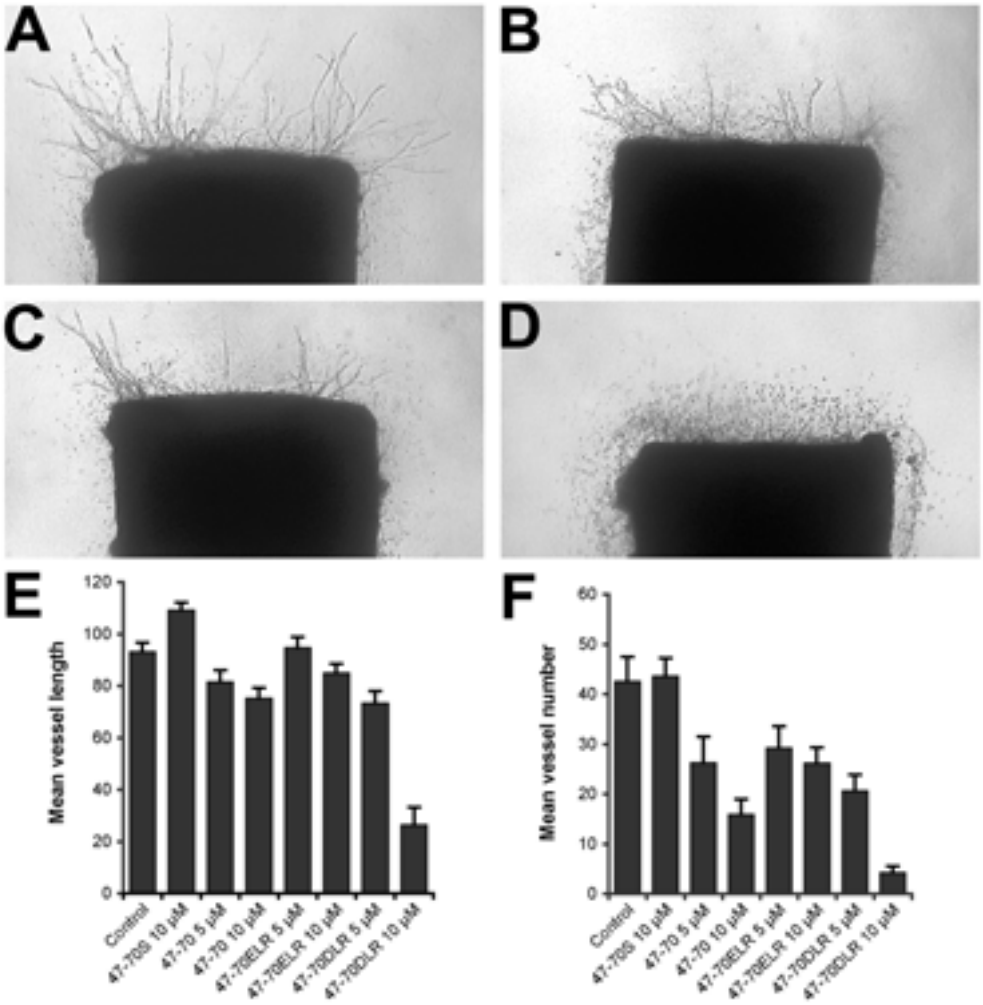
Proliferation was stimulated with $VEGF_{165}$ using BAE cells (A) or FGF-2 and ACE cells (B). PF-$4^{47\text{-}70}$, PF-$4^{47\text{-}70}$ELR and PF-$4^{47\text{-}70}$DLR inhibited cell proliferation in a concentration-dependent manner, peptide PF-$4^{47\text{-}70}$S did not show any effects at all concentrations tested. Horizontal line at 0 indicates conditions were 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 following order of activity (highest to lowest): PF-$4^{47\text{-}70}$DLR, PF-$4^{47\text{-}70}$, PF-$4^{47\text{-}70}$ELR for VEGF-induced proliferation and PF-$4^{47\text{-}70}$DLR, PF-$4^{47\text{-}70}$ELR, PF-$4^{47\text{-}70}$ for FGF-2-induced proliferation. In contrast, serum-induced U87 or C6 glioma cell proliferation was not affected by the peptides (C).

Figure 3



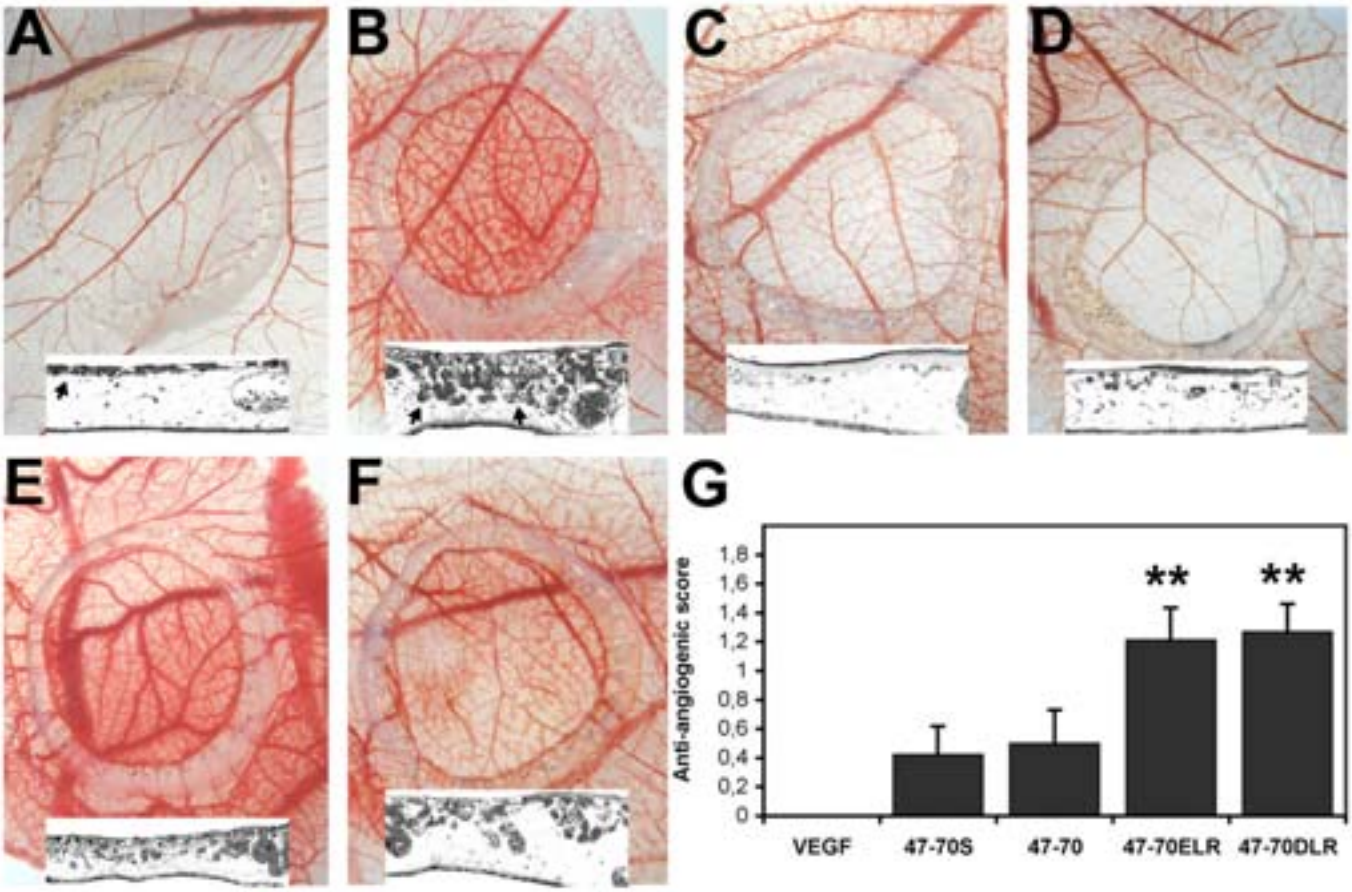
Endothelial cell migration was stimulated with 10 ng/ml $VEGF_{165}$ on BAE cells. During the 18 h period in serum-free medium, some random cell migration occurs (A). $VEGF_{165}$ induces cell migration into the denuded area of the scar (B). At 5 µM, control peptide PF-$4^{47\text{-}70}$S (C, G) had no effects. PF-$4^{47\text{-}70}$ (D, G) does only slightly antagonize pro-migratory effects of $VEGF_{165}$. PF-$4^{47\text{-}70}$ELR (E, G) had profound effects on $VEGF_{165}$-induced migration; PF-$4^{47\text{-}70}$DLR (F, G) suppressed it below control levels.

Figure 4



Rat aortic rings were incubated in MCDB131 medium without serum and growth factors (A) or treated with PF-4-derived peptides at 10 µM (B-D) or at indicated doses (E, F). Peptide PF-4$^{47\text{-}70}$S did not interfere with microvessel growth, whereas peptides PF-4$^{47\text{-}70}$ (B) and its derivates PF-4$^{47\text{-}70}$ELR (C) and PF-4$^{47\text{-}70}$DLR (D) impaired this process; with different efficacy at the same dose (E, F). Mean vessel length (E) is strongly reduced under treatment with 10 µM PF-4$^{47\text{-}70}$DLR compared to the same dose of PF-4$^{47\text{-}70}$ ($p<0.05$) or PF-4$^{47\text{-}70}$ELR ($p<0.002$). When comparing mean vessel number, PF-4$^{47\text{-}70}$ showed a dose-dependent inhibition, whereas PF-4$^{47\text{-}70}$ELR displayed only a moderate, not dose-dependent effect. PF-4$^{47\text{-}70}$DLR strongly reduced mean vessel number in a dose-dependent manner (PF-4$^{47\text{-}70}$DLR vs. PF-4$^{47\text{-}70}$ or PF-4$^{47\text{-}70}$ELR: $p<0.001$). Data for statistical analysis were pooled from three independent experiments and are plotted as mean ±SEM. Photos

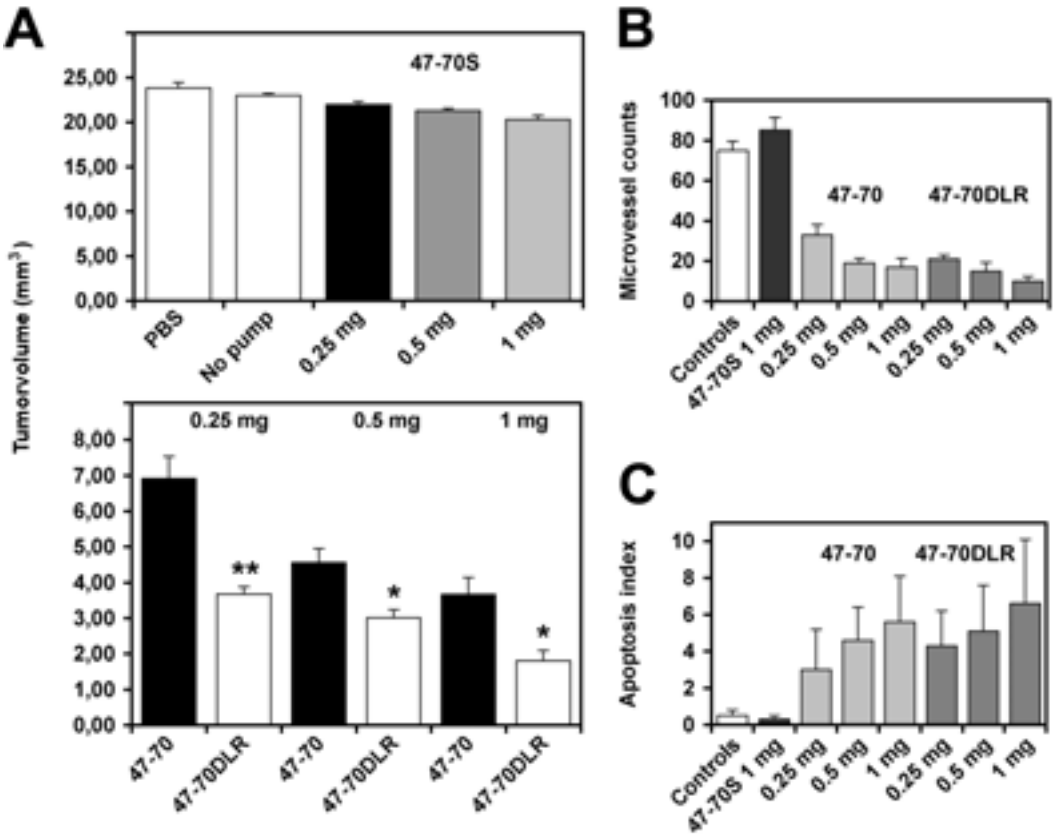
Figure 5



Sterile water and plastic rings alone did not interfere with CAM vasculature (n = 8) (A). A mixture of $VEGF_{165}$ and water (n = 17) (B) or $VEGF_{165}$ and 20 µg of peptides PF-$4^{47\text{-}70}$S (n = 13) (E), PF-$4^{47\text{-}70}$ (n = 12) (F), PF-$4^{47\text{-}70}$ELR (n = 14) (C) and PF-$4^{47\text{-}70}$DLR (n = 13) (D) was placed in the center of the rings. $VEGF_{165}$ induces brush-like capillary formation surrounding pre-capillary arterioles (B). Control peptide PF-$4^{47\text{-}70}$S (E) and peptide PF-$4^{47\text{-}70}$ (F) were not able to counterbalance growth of new capillaries. Both modified peptides PF-$4^{47\text{-}70}$ELR (C) and –DLR (D) had a strong anti-angiogenic effect in the CAM; statistical analysis proved the difference compared to PF-$4^{47\text{-}70}$S to be significant (**$p<0.002$) (G).

Semi-thin sections of controls show that the capillary layer stays within the chorionic epithelium (arrow, insert in A); larger conduit vessels are present in the stroma, filled with erythrocytes. $VEGF_{165}$ induces a strong *de novo* growth of capillaries, which are present throughout the stroma (arrows, insert in B). Control peptide PF-$4^{47\text{-}70}$S does not neutralize the capillary angiogenesis induced by $VEGF_{165}$ (insert in E). PF-$4^{47\text{-}70}$ at the low dose tested (2 x 20 µg) also does not inhibit capillary angiogenesis (insert in F). PF-$4^{47\text{-}70}$ELR and -DLR both markedly decreases angiogenesis nearly to control levels (inserts in C, D).

Figure 6



Locally administered peptide PF-4$^{47\text{-}70}$ or PF-4$^{47\text{-}70}$DLR strongly reduced tumor volumes of established intracerebral tumors in nude mice at all three doses (A, lower panel). PF-4$^{47\text{-}70}$DLR displayed a greater anti-tumor activity than PF-4$^{47\text{-}70}$ 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 Newmann-Keuls post-test: 0.25 mg: **$p<0.005$; 0.5 mg: *$p<0.02$; 1 mg: *$p<0.03$). 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; $t = 30$, $df = 18$; NS) (A, upper panel). Control peptide PF-4$^{47\text{-}70}$S 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-4$^{47\text{-}70}$ or PF-4$^{47\text{-}70}$DLR (B, C).

Final conclusions: Biological activities of modified PF-4 peptides

The work presented here has shown that the small fragment PF-4^{47-70}, composed of 23 amino acids, can conserve an important biological activity of its parent protein: inhibition of angiogenesis. Full-length PF-4 is a monomer of 70 amino acids, which is thought to assemble into a tetramer under physiological conditions. PF-4^{47-70} shows defined activities against FGF-2-induced angiogenic events: inhibition of receptor binding, EC proliferation and migration and MAPK kinase phosphorylation. It also blocks assembly of microvessels in the rat aortic ring assay and in vivo blood vessel growth in a mouse model of FGF-2-induced angiogenesis. Modifying the model peptide PF-4^{47-70} resulted in one peptide with loss of activity (PF-4^{47-70}S) and two peptides with enhanced anti-angiogenic activity: PF-4^{47-70}ELR and –DLR.

FGF-2 and $VEGF_{165}$ binding to their tyrosine kinase receptors on ECs is strongly suppressed by the modified peptides PF-4^{47-70}ELR and -DLR in the low micromolar range. This effect is most likely the reason for their strong anti-angiogenic activities. Disturbing VEGF and FGF-2 functions at the receptor level leads to impairment of crucial angiogenic events such as EC proliferation and migration. Another peptide containing PF-4 sequences has recently described. “Anginex”, a 33-mer β-sheet-forming peptide containing sequences from PF-4, IL-8 and bactericidal-permeability increasing protein (160). However, Anginex doses necessary for inhibition of EC migration where several times higher than those of PF-4^{47-70}ELR or –DLR, and only FGF-2-induced angiogenesis has been studied.

Assembly of EC into microvessels in serum-free cultures of aortic rings was differently affected by the peptides. Peptide PF-4^{47-70}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-4^{47-70}ELR did not very well inhibit microvessel sprouting in this model. It is possible that the ELR motif which is crucial for receptor activation of the pro-angiogenic chemokine IL-8 (161), is presented in PF-4^{47-70}ELR in a way that chemokine receptors on residual leukocytes and ECs

(162) are activated, partially overcome the direct inhibitory effects on endogenous VEGF and FGF-2. Endostatin completely inhibits vascular sprouting in the aortic ring assay at 500 µg/ml (150), whereas PF-4^{47-70}DLR shows maximal activity between 14 and 28 µg/ml (5-10 µM), thus at 18 to 35 times lower concentrations. This strong activity is probably due to the fact that the peptide interferes with both VEGF and FGF-2 by inhibition of binding to their receptors.

We tested effects of C-terminal PF-4 peptides for their ability to interfere with $VEGF_{165}$-induced angiogenesis in the differentiated CAM (day 13); to our knowledge the first attempt to study angiogenesis inhibitors in this modified type of assay. The typical brush-like formation of capillaries into the stroma of the day 13 CAM is strongly reduced by co-application of PF-4^{47-70}DLR and –ELR with human recombinant $VEGF_{165}$. In some eggs, PF-4^{47-70} and PF-4^{47-70}S also showed some minor anti-angiogenic activity, but this may result from inter-individual variations, the high sensitivity of the assay system or the difficulty to exactly quantify differences between low inhibitory effects. The doses necessary to achieve considerable VEGF-inhibition were relatively low with PF-4^{47-70}DLR and –ELR; CAMs received a total of 40 µg of peptide accompanied by 6 µg of the growth factor. Metastatin, a recently described angiogenesis inhibitor, was active in the day 10 CAM assay at a two times higher dosage, and additionally, angiogenesis was induced with VEGF at a 300-fold lower dose compared to our assay (163). Cyclic peptide antagonists for $\alpha v\beta 3$-integrin showed strong anti-angiogenic effects in the day 10 CAM at 300 µg; again, the angiogenic response was induced by VEGF or FGF-2 at doses less than 1 µg (21). These comparisons suggest that modified C-terminal PF-4 peptides display a very high affinity for VEGF *in vivo*, and that their binding leads to a functionally relevant inactivation of the angiogenic factor.

Encouraged by these results, the C-terminal PF-4 peptides were tested for their ability to block tumor angiogenesis [1]. We did not test the PF-4^{47-70}ELR peptide in this assay because of the somewhat uncertain results in the rat aortic ring assay. Glioma growth is strongly promoted via up-regulation of angiogensis by VEGF and FGF-2; especially the U87 cell line we used in our model produces high levels of these growth factors in culture (164). 250 µg of peptide PF-4^{47-70}DLR were already sufficient to reduce tumor growth to one half compared to PF-4^{47-70}-

[1] Lorenzo Bello and colleagues at the University of Milano, Italy carried out these experiments.

treated animals. This effect is paralleled by a reduction of blood vessels within the tumor and elevated EC and/or tumor cell apoptosis. Increased apoptosis in tumors is commonly observed after treatment with angiogenesis inhibitors (111, 165, 166). Similar results have been obtained previously by systemic treatment using the anti-angiogenic molecule PEX; alone or in combination with low-dose chemotherapy. Animals bearing U87 glioma survived significantly longer and there were no measurable side effects (167, 168).

The reason why C-terminal PF-4 peptides are such potent angiogenesis inhibitors *in vivo* and why their activity is enhanced by the replacement of a few amino acids is perhaps related to arginine residues. Three positively-charged arginine residues within full-length PF-4 (R20, R22, R49) contribute to its particularly high heparin-binding capacity (169). This may in general explain the augmented anti-angiogenic activity, since both, PF-4^{47-70}ELR and -DLR contain an additional arginine (at position 56) in company with the internal R49, leading to an increased net charge of the peptides. This could lead to an augmented capacity to derange interactions of FGF-2 and VEGF with heparin. Binding of FGF-2 or $VEGF_{165}$ to tyrosine kinase receptors is facilitated by heparin sulfate proteoglycans on the cell surface and is essential for their pro-angiogenic activities (for review see (105)). Similar observations have been made with peptides mimicking the surface of endostatin: an increase of arginine residues enhances their anti-angiogenic effects probably due to an increased affinity for heparin (170).

These findings might be of importance for the future design of anti-angiogenic drugs. If one considers treatment of patients with protein angiogenesis inhibitors, some important issues are associated with this kind of pharmacological approach: 1) production of recombinant human proteins is time consuming and expensive, 2) some structural features of more complex proteins important for activity (e.g. glycolysation) is dependent on the production system used (yeast, bacteria, fungi, plants or insect cells) and can raise immunogeneity problems, and 3) purification can be very complicated and contaminations have to be eliminated. In contrast, short synthetic peptides with a strong activity like PF-4^{47-70}DLR could be produced at large scale without most of the above-mentioned drawbacks.

Taken together, modified C-terminal peptides of PF-4 represent a new class of potent angiogenesis inhibitors due to dual inhibition of VEGF and FGF-2 pathways and might be especially useful in the treatment of tumors expressing high levels of these growth factors.

Perspectives – Anti-angiogenesis assay in the CAM and SNA-1 lectin staining

During the characterization of the different PF-4-derived peptides we have come across a general problem in biological sciences related to the development of treatment strategies: finding an appropriate model for screening of in vivo effects. Because VEGF plays an exclusive role in tumor angiogenesis, it is of primary importance to show activity of an angiogenesis inhibitor against that growth factor. Therefore we developed a new anti-angiogenesis model on the differentiated chick CAM based on the earlier work of Wilting et al. (171, 172), which shows that a strong capillary angiogenesis can be induced by application of recombinant VEGF. Bio-microscopy and semi thin section analysis of treated CAMs showed that both modified peptides have strong anti-VEGF activity at a dose at which the unmodified peptide is inactive.

One important drawback of the chicken CAM system is the relative difficulty of visualizing the entire vascular network. The growth of new blood vessels on the CAM can be analyzed by bio-microscopy, confocal laser scanning microscopy, corrosion casts or classical histology (semi thin and ultra thin sectioning) as well as electron microscopy and in-situ hybridization. All these methods are relatively labor-intense and not well suited to do screening studies. In addition, few or no EC specific markers are available for the chicken CAM, whereas the quail vasculature can be identified using the QH1 antibody (173). In addition, receptor homologues to VEGFR-2 (Quek-1) and VEGFR-3 (Quek-2) have been cloned and characterized in the quail embryo (174, 175). Proteins known to be expressed in the chick endothelium which have served as markers for endothelium include the TGF-beta receptor II (176), endoglin (177) and integrin $\alpha v\beta 3$ (21), but so far no specific antibody or other marker has been developed for identification of the entire chick vascular endothelium.

Different lectins have selectivity towards either chick or quail tissues and difference in binding were sometimes dependent on developmental stage of the embryo (178). We have now shown that *sambucus nigra* lectin (SNA-1) can be used to stain the whole vascular network of the chick chorio-allantoic membrane at different developmental stages with high selectivity, no other structures except the vascular walls were stained (see figure 11). Newly formed capillaries induced by $VEGF_{165}$ can be visualized equally well. This lectin can be used in combination with other cellular markers to study vascular morphology during development, for example, to elucidate the contribution of mural cells (eg. pericytes and vascular smooth muscle cells) during physiological and VEGF-induced angiogenesis (179).

Combining the anti-angiogenesis model of VEGF-induced capillary growth on the differentiated day 13 CAM with the newly discovered staining method described above, will provide a powerful tool to study effects of molecules, which interfere with vascular growth and morphogenesis.

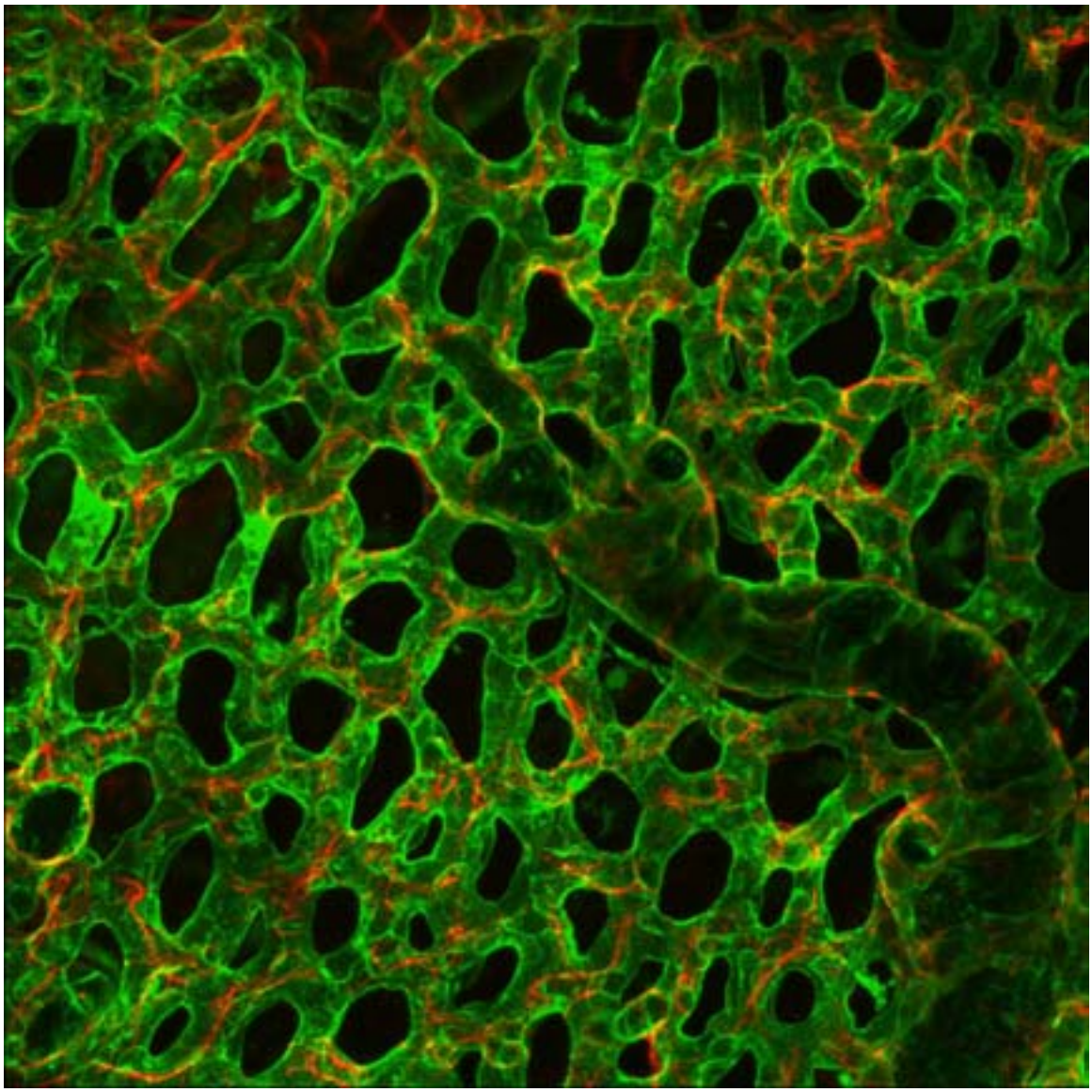


Figure 11: Sambucus nigra lectin and desmin staining of the capillary bed of the day 17 CAM.

Three-dimensional reconstructed confocal microscopy image (magnification x600) of the day 17 CAM capillary bed. The whole mount tissue was stained with FITC-coupled sambucus nigra lectin (green) and a mouse-anti human desmin antibody revealed by a secondary antibody coupled to Alexa Fluor® 488 (red). Note that the entire capillary bed is stained and capillaries are associated with pericytes, to a varying degree. From the right bottom to the center, a pre-capillary vessel merges into the capillary layer. (Image generated by Dr Benoît ROUSSEAU using Bitplane AG IMARIS® software).

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RESUME

L'angiogenèse joue un rôle important dans de nombreuses pathologies telles que le cancer, la rétinopathie diabétique, l'arthrite rhumatoïde et certaines maladies de la peau.

Elle est définie par la croissance de nouveaux vaisseaux sanguins à partir de vaisseaux préexistants. L'angiogenèse est contrôlée par un équilibre entre des facteurs stimulants et inhibiteurs endogènes. Les stimulateurs les mieux connus sont le facteur de croissance de l'endothélium vasculaire (VEGF) et le facteur de croissance des fibroblastes 2 (FGF-2). Parmi les inhibiteurs, on trouve l'endostatine, l'angiostatine, certaines interleukines, la thrombospondine et le facteur plaquettaire 4.

Nous avons caractérisé dans la région C-terminale du facteur plaquettaire 4, la séquence peptidique essentielle à son activité. Ce peptide de 23 acides aminés (PF-4^{47-70}) montre des effets anti-angiogéniques dans de multiples tests d'angiogenèse *in vitro* et *in vivo*. La liaison du VEGF et du FGF-2 iodés aux récepteurs présents sur des cellules endothéliales est fortement inhibée par ce peptide. Le changement d'un acide aminé dans sa séquence supprime son activité ($C^{52}S$ = PF-4^{47-70}S) alors que l'introduction d'un autre la potentialise ($Q^{56}R$ = PF-4^{47-70}DLR et $D^{54}E/Q^{56}R$ = PF-4^{47-70}ELR). Ces peptides modifiés montrent une activité supérieure au peptide PF-4^{47-70} dans des tests de prolifération et de migration des cellules endothéliales. La prolifération des cellules tumorales de type gliome n'est pas affectée. Dans un modèle de bourgeonnement angiogénique sur des anneaux d'aorte de rats, seul le peptide PF-4^{47-70}DLR montre une plus forte action anti-angiogénique.

Pour confirmer ces résultats *in vivo*, nous avons développé un nouveau test d'angiogenèse sur la membrane chorio-allantoïdienne chez le poulet. La croissance des capillaires est induite par le VEGF déposé dans un anneau de plastique à la surface de la membrane et les peptides à tester sont appliqués avec le VEGF dans cet anneau. Les résultats obtenus ont montré que les deux peptides modifiés bloquent presque à 100% l'angiogenèse induite par le VEGF, à une dose où les deux autres peptides ne montrent pas d'effet.

Des souris « nudes » avec des gliomes intracérébraux, traitées avec le peptide PF-4^{47-70}DLR présentent des tumeurs deux fois plus petites que celles qui sont traitées avec le peptide non modifié PF-4^{47-70}. Des souris contrôles (traitées avec le peptide PF-4^{47-70}S ou avec du PBS) ont des tumeurs 4 à 5 fois plus grandes.

Ces travaux ont identifié et caractérisé des peptides provenant du PF-4 comme étant des candidats possibles pour le développement de futures drogues à action anti-angiogénique. La double inhibition de FGF-2 et du VEGF est très probablement la raison de leur forte activité *in vivo*.

Des patients avec des maladies dépendantes de l'angiogenèse pourraient éventuellement profiter d'un traitement par ces peptides ou par des dérivés synthétiques (peptidomimétiques) qui pourraient être produits par l'industrie pharmaceutique.