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Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis

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Abstract

Fibroblast growth factors (FGFs) are a family of heparin-binding growth factors. FGFs exert their pro-angiogenic activity by interacting with various endothelial cell surface receptors, including tyrosine kinase receptors, heparan-sulfate proteoglycans, and integrins. Their activity is modulated by a variety of free and extracellular matrix-associated molecules. Also, the cross-talk among FGFs, vascular endothelial growth factors (VEGFs), and inflammatory cytokines/chemokines may play a role in the modulation of blood vessel growth in different pathological conditions, including cancer. Indeed, several experimental evidences point to a role for FGFs in tumor growth and angiogenesis. This review will focus on the relevance of the FGF/FGF receptor system in adult angiogenesis and its contribution to tumor vascularization.

Keywords: Angiogenesis; Endothelium; FGF; FGF receptors; Tumor

1. Introduction

Angiogenesis, the process of new blood vessel formation from pre-existing ones, plays a key role in various physiological and pathological conditions, including embryonic development, wound repair, inflammation, and tumor growth [1]. The local, uncontrolled release of angiogenic growth factors and/or alterations of the production of natural angiogenic inhibitors, with a consequent alteration of the angiogenic balance [2], are responsible for the uncontrolled endothelial cell proliferation that takes place during tumor neovascularization and in angiogenesis-dependent diseases [3].

Angiogenesis is a multi-step process that begins with the degradation of the basement membrane by activated endothelial cells that will migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space. Then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane [4].

Numerous inducers of angiogenesis have been identified, including the members of the vascular endothelial growth factor (VEGF) family, angiopoietins, transforming growth factor-α and -β (TGF-α and -β), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), interleukins, chemokines, and the members of the fibroblast growth factor (FGF) family.

Historically, a tumor angiogenic factor (TAF) was first isolated in 1971 from rat Walker 256 carcinoma [5]. TAF had a molecular weight of about 10 kDa and consisted of 25% RNA, 10% proteins, and 58% carbohydrates, plus a possible lipid fraction. The 1980s saw for the first time the purification to homogeneity of pro-angiogenic proteins, the breakthrough coming as a result of the observation that endothelial cell growth factors showed a marked affinity for heparin [6,7]. This led to the identification, purification, and sequencing of the two prototypic heparin-binding angiogenic growth factors FGF1 and FGF2. Since then, 22 structurally-related members of the FGF family have been identified [8]. FGFs are pleiotropic factors acting on different cell types, including

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endothelial cells, following interaction with heparan-sulfate proteoglycans (HSPGs) and tyrosine kinase FGF receptors (FGFRs). To date, more than 1200 PubMed-referenced papers related to FGFs and FGFRs in endothelial cells and during neovascularization have been published. This review will focus on the role of the FGF/FGFR system in angiogenesis.

2. Pro-angiogenic activity of FGFs

As stated above, FGFs exert their biological activities by binding to high affinity tyrosine kinase FGFRs on the surface of target cells. In vitro, endothelial cells of different origin express FGFR1 [9,10] and, under some circumstances, FGFR2 [11] whereas the expression of FGFR3 or FGFR4 has never been reported in endothelium.

Only a limited number among the 22 members of the FGF family have been investigated for their angiogenic potential in vitro and in vivo, the bulk of experimental data referring to the prototypic FGF1 and FGF2.

2.1. In vitro effects on endothelial cells

The necessity to study in detail the process of angiogenesis has led to the isolation and in vitro culture of endothelial cells [12]. A high degree of heterogeneity has been observed for endothelial cells isolated from different tissues and/or animal species. Also, significant differences exist between large-vessel and microvascular endothelium [13–15]. Nevertheless, the bulk of experimental evidence indicate that different members of the FGF family, mostly FGF1 and FGF2, can induce in vitro a complex ''pro-angiogenic phenotype'' in endothelial cells (Fig. 1) that recapitulates several aspects of the in vivo angiogenesis process, including the modulation of endothelial cell proliferation, migration, protease production, integrin and cadherin receptor expression, and intercellular gap-junction communication (summarized in [10]).

2.1.1. Endothelial cell proliferation

Activation of FGFR1 or FGFR2 by angiogenic FGFs (including FGF1, FGF2, and FGF4) leads to endothelial cell proliferation [16]. Recently, also the FGF8b isoform has been shown to stimulate endothelial cell proliferation in vitro [17]. FGFR engagement involves the activation of several parallel signaling pathways as a consequence of receptor autophosphorylation followed by recruitment of Shc, FRS2, and Crk adaptor molecules (for a review see [16]). Interestingly, besides activation of the MAPK signaling pathway, a long lasting activation of protein kinase C (PKC) is required for FGF2 to exert a full mitogenic response in endothelial cells [18]. PKC down-regulation abolishes FGF2-induced endothelial cell proliferation but not urokinase-type plasminogen activator (uPA) upregulation. Also, autophosphorylation of distinct tyrosine residues in FGFR1 mediate the mitogenic and uPA-inducing activity of FGF2 [19], suggesting that the two biological responses can be dissociated at the molecular level.

2.1.2. Extracellular matrix degradation

Extracellular matrix (ECM) degradation represents an important step during the first phases of the angiogenesis process. The plasmin–plasminogen activator system and matrix metalloproteinases (MMPs) cooperate in this degradation [20]. uPA converts plasminogen into plasmin, a serine protease that degrades fibrin and other matrix proteins, and activate several MMPs, including stromelysin-1 (MMP-3), collagenase-1 (MMP-1), type IV collagenases (MMP-2 and MMP-9) [21].

FGF1, FGF2, and FGF4 upregulate uPA and MMPs production in endothelial cells. Also, FGFs modulate the expression of uPA receptor on the endothelial cell surface, thus allowing the localization of the proteolytic activity at the leading edge of the cell at the front of migration [22]. Furthermore, FGF1 and FGF2 induce the expression of the plasminogen activator inhibitor (PAI)-1 in cultured

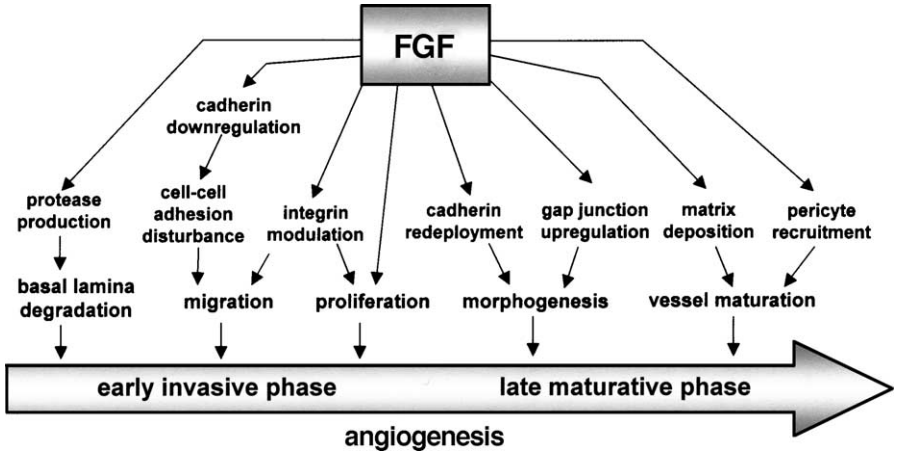

Fig. 1. Schematic representation of the events triggered by FGFs in endothelial cells that contribute to the acquisition of the angiogenic phenotype in vitro and to neovascularization in vivo.

endothelial cells leading to a fine modulation of the proteolytic balance [23–25].

Stimulation of endothelial cells by FGF2 causes also the shedding of cell surface membrane vesicles containing MMP-2 and MMP-9 together with the two MMP inhibitors TIMP-1 and TIMP-2. These vesicles stimulate capillary-like structure formation when added to endothelial cells seeded on a solubilized basement membrane preparation (Matrigel) [26].

2.1.3. Endothelial cell migration

FGF1 and FGF2 [27,28], the FGF8b isoform [17], and FGF10 [29] stimulate chemotaxis and/or chemokinesis in endothelial cells. Similar to cell proliferation, the chemotactic effect of FGF2 requires the activation of the MAPK signaling pathway [30] and is abolished by PKC down-regulation [31]. The capacity of FGFR1 to mediate chemotaxis resides in the amino acid stretch 759–773 of its cytoplasmic tail [32]. However, the biological response is independent of tyrosine phosphorylation and requires the activation of a Wortmannin-sensitive pathway [32]. Expression of a kinase-inactive c-Fes mutant interferes with FGF2-induced chemotaxis in endothelial cells [33]. Moreover, FGF2-mediated chemotaxis on fibronectin-coated substrata can be attenuated by $\alpha_v\beta_3$ integrin inhibitors [34].

The angiogenesis process can be mimicked in vitro by culturing endothelial cells on a layer of or within a 3D permissive matrix substrate [35]. Under these conditions, endothelial cells invade the substratum and organize capillary-like structures with a hollow lumen [36]. FGF2 enhances this response in type I collagen gel [37] possibly via a p38-dependent signaling pathway [38]. Endothelial cell morphogenesis can be induced by FGF2 also using three-dimensional fibrin gels [39]. Invasion of fibrin matrix can be modulated by TGF β-1 [39,40] and is mediated by CD44 [41] and integrin receptors [42].

2.1.4. Modulation of integrins, cell–cell adhesion receptors, and matrix deposition

Endothelial cell migration and proliferation are limited by lateral cell–cell adhesion and ECM interactions [43] that, in turn, are mediated by cadherin and integrin receptors. Accordingly, FGF2-mediated adhesion and migration of endothelial cells onto type I collagen depends on both integrin expression and cell density [44]. Interestingly, FGF2 regulates the expression of different integrins, including $\alpha_v\beta_3$ [45–47], and cadherins [43,48] in a complex fashion. Indeed, a brief exposure to FGF2 hampers endothelial cell–cell junctions whereas a prolonged exposure to the growth factor promotes a slow temporal redistribution of the junctional adhesion proteins, platelet/endothelial cell adhesion molecule (PECAM/CD31), vascular/endothelial cadherin, and plakoglobin. These data indicate that FGFs can promote both endothelial cell scattering, that is required during the first steps of the angiogenic process, and the formation of the cell–cell interactions required to vessel maturation [43] (Fig. 1). A similar mechanism of regulation may exist also for the interaction with the ECM that surrounds the endothelium. Indeed, FGFs initially promote the disruption of the basal lamina by inducing protease production. Lately, FGFs may induce the production of various ECM components by endothelial cells [49], contributing to the maturation of the new vessels (Fig. 1).

2.1.5. Endothelial cell morphogenesis

FGF2 [50] and FGF8b [17] can enhance endothelial cell reorganization on Matrigel. The process requires the activation of the proteolytic machinery, including type IV collagenase(s) and TIMPs production [51] as well as uPA and PAI-1 upregulation [52], $\alpha_6\beta_1$ integrin receptor engagement [53], PECAM-1 [54], and activation of the small GTPase Rac [55]. Moreover, FGF2-mediated endothelial cell morphogenesis requires signals via VEGF receptor-1 (VEGFR-1) [56], underlying the cross-talk between FGF and VEGF signaling (see below).

2.1.6. Autocrine, intracrine, paracrine mechanisms of action of FGFs in endothelial cells

FGFs can exert their effects on endothelial cells via a paracrine mode consequent to their release by tumor and stromal cells and/or by their mobilization from the ECM. On the other hand, FGF2 may also play an autocrine role in endothelial cells, as suggested by in vitro and in vivo experimental evidences (see [57] and references therein). Accordingly, FGF2 has been implicated in the pathogenesis of lesions of endothelial cell origin, including Kaposi's sarcoma [58] and hemangiomas [59]. Also, the *fgf*4 gene is over-expressed in HHV8-positive Kaposi's sarcoma [60]. To assess the biological consequences of endothelial cell activation by endogenous FGFs, we originated a stable mouse aortic endothelial cell line transfected with a human FGF2 cDNA [57]. FGF2 transfectants show an invasive and morphogenetic behavior in vitro. In vivo, they are angiogenic, cause the formation of opportunistic vascular tumors in nude mice, and induce hemangiomas in the chick embryo [61]. Accordingly, FGF2 transfection affects the expression of numerous genes implicated in the modulation of cell cycle, differentiation, cell adhesion, and stress/survival [62]. Some of these genes are similarly modulated in vitro and in vivo by administration of the recombinant growth factor [62].

A transformed morphology and an increased proliferative capacity is also observed for FGF4-transfected mouse aortic endothelial cells. However, FGF4 transfectants, although angiogenic in the chorioallantoic membrane (CAM) assay, show a limited capacity to growth under anchorage-independent conditions, to invade 3D fibrin gel, to undergo morphogenesis in vitro, and to induce hemangiomas in the chick embryo [11].

The observed differences between FGF2 and FGF4 transfectants may reflect differences in the intracellular and/

or extracellular fate of the two growth factors. The single-copy human *fgf*2 gene encodes multiple FGF2 isoforms with molecular weight ranging from 18 to 24 kDa. Both low and high molecular weight FGF2 isoforms show angiogenic activity [63]. At variance with other FGFs, FGF2 isoforms lack a leader sequence for secretion and are released in limited amounts by an alternative secretion pathway [64] or via membrane vesicle shedding [65]. Experimental evidences point to different functions of FGF2 isoforms in transfected endothelial cells [66], possibly related to differences in their subcellular localization and release. Indeed, high molecular weight FGF2 isoforms contain a nuclear localization sequence and are mostly recovered in the nucleus whereas the 18 kDa FGF2 isoform is mostly cytosolic [67]. The constitutive overexpression of high molecular weight FGF2 isoforms leads to cell immortalization whereas 18 kDa FGF2 overexpression induces a transformed phenotype [68]. In contrast, FGF4 is efficiently released and does not play an intracellular role [69]. On this basis, the biological differences observed between FGF2 and FGF4 endothelial cell transfectants may reflect differences in the intracrine and/or autocrine activities of the two growth factors. Accordingly, transfection with a secreted form of FGF1 leads to altered morphology and increased motility in endothelial cells [70].

Taken together, these data suggest that endogenous FGFs produced by endothelial cells may play important autocrine, intracrine, or paracrine roles in angiogenesis and in the pathogenesis of vascular lesions.

2.2. *In vivo effects and experimental angiogenesis assays*

FGFR1 is expressed by endothelial cells in vivo [71–73], even though a detailed analysis of FGFR expression patterns in vivo deserves further investigation. To this respect, disruption of the genes encoding for the different FGFRs in mice is not informative. Indeed, *fgfr*1 null embryos are developmentally retarded and dye during gastrulation, the early embryonic lethality occurring prior to a stage in which the role of FGFR1 in blood vessel development can be evaluated [74,75]. However, adenovirus-mediated expression of dominant-negative FGFR1 results in a significant impairment of blood vessel development and maintenance in mouse embryos cultured in vitro [76]. *Fgfr*2 mutation results in a later embryonic lethality characterized by the lack of a functional placenta and limb buds [77]. *Fgfr*3-deficient mice are normal during gestation and exhibit bone alterations during postnatal development [78]. Finally, *Fgfr*4-null animals are developmentally normal [79].

In keeping with the expression of FGFR1 on endothelial cells in vivo, the angiogenic activity of recombinant FGF1 and FGF2 proteins has been demonstrated in various experimental models, including the chick embryo CAM [80], the avascular rabbit [81] or mouse [82] cornea, and subcutaneous Matrigel injection [83]. Similarly, the delivery of either one of the two growth factors via retroviral, adenoviral, lentiviral, and adeno-associated viral vectors or via implantation of different FGF-overexpressing cell transfectants result in a potent angiogenic response in various experimental animal models (see below).

The CAM assay is a well-established assay for studying the effects of growth factors on blood vessel growth [80]. As compared to the application on the CAM of a single bolus of recombinant FGF2, cell implants overexpressing the growth factor allows the continuous delivery of FGF2 produced by a limited number of cells, thus mimicking more closely the initial stages of tumor angiogenesis [84]. Indeed, the release of 1.0 pg FGF2 per day from viable cells triggers an angiogenic response quantitatively similar to that elicited by 1.0 μg of the recombinant molecule [84]. Also, neutralizing anti-FGF2 antibodies prevent CAM neovascularization, supporting the key role of endogenous FGF2 in the development of vascular system in avian embryo [85]. Accordingly, FGFRs are expressed in the CAM until E10, when the angiogenic process is switched off [86].

In contrast with the potent angiogenic response elicited by exogenous FGF2 in different in vitro and in vivo models, the role of endogenous FGF2 in angiogenesis remains uncertain. Indeed, *fgf*2 knockout mice are morphologically normal [87] and do not show differences in neovascularization following injury [88] or hypoxia [89]. Conversely, transgenic overexpression of FGF2 does not result in spontaneous or inherent vascular defects, even though an amplified angiogenic response can be observed after wounding or s.c. implantation of a Matrigel plug [90]. The apparently normal vascularization in $fgf2^{-/-}$ mice as well as in double $fgf2^{-/-}/fgf1^{-/-}$ mice may reflect the wide redundancy in the FGF family [91] and the contribution to angiogenesis of several other angiogenic growth factors, including VEGF (see below).

Angiogenic activity has been shown also for other members of the FGF family. FGF3/int-2 oncogene expressing human epithelial mammary cells or their conditioned culture medium exert a potent angiogenic response in the CAM assay. The same conditioned medium triggers angiogenesis also in the mesentery of i.p. injected rats [92]. Analysis of mammary glands from *fgf*4 transgenic mice confirmed preliminary in vitro data about the angiogenic properties of FGF4 mediated through VEGF-A upregulation [93]. The pro-angiogenic activity of FGF4 is confirmed by the angiogenic effect exerted by FGF4-encoding adenovirus in a rabbit hind limb ischemia model [94] and by FGF4-transfected endothelial cells in the CAM assay [11]. Intracoronary gene transfer of FGF5 increases blood flow and contractile function in ischemic heart possibly related to an increased vascularization [95]. Subnanomolar concentrations of FGF7/KGF induce neovascolarization in the avascular rat cornea [29]. FGF8b elicits an angiogenic response in the CAM assay [17] significantly enhanced by heparin co-administration (Presta, unpublished observations). Transient expression of FGF9 in transgenic mice results in alterations of retinal pigment

epithelium possible related to alterations of the choroidal vasculature [96]. Finally, FGF10, which is structurally related to FGF7, elicits a pro-migratory effect on capillary endothelial cells, suggesting a possible pro-angiogenic activity in vivo [29].

2.3. FGF/VEGF cross-talk

For many years FGF1 and FGF2 occupied a central stage in the angiogenesis field. Then, the VEGF family of angiogenic growth factors came to the limelight after the discovery of their pivotal role in vasculogenesis and angiogenesis during embryonic development and under numerous physiologic and pathologic conditions in adults [97]. The VEGF family comprises six members (VEGF-A denoting the originally identified VEGF) that differently interact with three cell surface tyrosine kinase VEGFRs. To date, VEGF-A/VEGFR-2 interaction appears to play a major role in blood vessel angiogenesis whereas VEGF-C and -D are mainly involved in lymphangiogenesis by interacting with VEGFR-3 expressed on lymphatic endothelium [97].

An intimate cross-talk exists among FGF2 and the different members of the VEGF family during angiogenesis, lymphangiogenesis, and vasculogenesis. Several experimental evidences point to the possibility that FGF2 induces neovascularization indirectly by activation of the VEGF/VEGFR system. Indeed: (i) VEGFR-2 antagonists inhibit both VEGF and FGF2-induced angiogenesis in vitro and in vivo [98]; (ii) expression of dominant-negative FGFR1 or FGFR2 in glioma cells results in a decrease in tumor vascularization paralleled by VEGF down-regulation [99]; (iii) both endogenous and exogenous FGF2 modulate VEGF expression in endothelial cells [82]; (iv) in the mouse cornea, the quiescent endothelium of vessels of the limbus express both VEGF mRNA and protein only after FGF2 treatment. In the same model, systemic administration of anti-VEGF-A neutralizing antibodies dramatically reduces FGF2-induced vascularization [82]; (v) VEGFR-1-blocking antibodies or the expression of a dominant-negative VEGFR-1 result in a significant reduction of FGF2-induced cell extensions and capillary morphogenesis [56]; (vi) FGF2 upregulates the expression of both FGFRs and VEGFRs in endothelial cells [100].

On the other hand, endothelial cell tube formation stimulated by VEGF in murine embryonic explants depends on endogenous FGF2 [101]. Also, FGF2 and VEGF may exert a synergistic effect in different angiogenesis models [102–104] even though this may not be the case when the two factors are applied onto the chick embryo CAM [105].

Recently, we analyzed the vascularization of xenografts originating from different clones of the same human tumor cell line but differing for the expression of VEGF and/or FGF2 [106]. The two growth factors exert a synergistic effect on tumor blood vessel density. However, FGF2 and VEGF exert a different impact on blood vessel maturation and functionality (see below). Accordingly, the study of the transcriptional changes occurring in cultured endothelial cells revealed that, together with a cluster of angiogenesis-related genes that were similarly modulated by FGF2 and VEGF, the two growth factors affected the expression of distinct subsets of transcripts [107,108]. Accordingly, FGF2, but not VEGF, induces the upregulation of telomerase activity in endothelial cells, thus preventing the early onset of senescence [109]. Distinct patterns of vascular morphology upon FGF2 or VEGF stimulation are described also in the quail embryo CAM assay [86]. Finally, increased endothelial fenestration is observed in the blood vessels of the chick embryo CAM stimulated by VEGF-overexpressing cells, but not by FGF2-overexpressing cells, despite the quantitatively similar angiogenic response elicited by the two transfectants [84].

Thus, FGF2 may require the activation of the VEGF/VEGFR system for promoting angiogenesis. Conversely, VEGF may require FGF2 for exerting its angiogenic potential under defined experimental conditions. Nevertheless, the two growth factors retain distinct biological properties exerting different biological effects on endothelial cells during angiogenesis.

Lymphatic system drains extravasated fluid, proteins, and immune cells, and transport them back to the venous circulation via the collecting lymphatic vessels and the thoracic duct. In tumors the development of the lymphatic network may play a critical role in facilitating the metastatic spread of malignant cells. Recent data demonstrate that a FGF/VEGF cross-talk may occur also during lymphangiogenesis. FGF2 pellets implanted in the mouse cornea trigger both angiogenesis and lymphangiogenesis, lymphatic vessels being more sensitive than blood vessels to FGF2 [110]. However, the lymphangiogenic activity of FGF2 is mediated by endogenous VEGF-C and VEGF-D upregulation, leading to VEGFR-3 activation [111]. Interestingly, no endothelial fenestration was observed in FGF2, VEGF-A, or VEGF-C-induced lymphatic vessels [112].

The VEGF/VEGFR system is essential for the development of embryonic vasculature [113]. The situation is much less well-defined for the FGF/FGFR system. As stated above, the phenotype of *fgfr* knockout mice is scarcely informative even though adenovirus-drive dominant-negative FGFR1 expression leads to severe vascular alterations in mouse embryos [76]. Also, FGF2 promotes the proliferation and differentiation of VEGFR-2^+ hemangioblast precursors from the mesoderm [114]. In embryoid bodies, embryonic stem cells can differentiate into a variety of cell lineages, including endothelial cells [115]. In this model, both VEGF and FGF2 lead to improved angioblast survival but only VEGF supports the formation of primitive endothelial tubes [116]. Also, in embryoid bodies in which VEGF/VEGFR function is impaired, FGF2 stimulates the formation of endothelial cell clusters that fail to develop into primitive vessels. In contrast, VEGF induces the formation of a characteristic vascular plexus also in *fgfr*$1^{-/-}$ embryoid bodies [117].

3. FGF interaction with endothelial cell surface, extracellular matrix, and free molecules

As stated above, FGFs interact with signaling FGFRs expressed on the endothelial cell surface. However, various other binding partners can affect the biological activity and angiogenic potential of FGFs (Fig. 2). These molecules can interact with FGFs in the extracellular environment, thus modulating their bioavailability, stability, local concentration, interaction with endothelial receptors, and intracellular fate. The complexity of this network of interactions is manifold: (i) FGF-binding molecules, heterogeneous in nature, are present in the blood stream or in body fluids as free molecules or associated to ECM; (ii) under different conditions, certain FGF-binding molecules may be present as free, ECM-associated, or cell membrane-associated molecules, possibly exerting different effects on FGF activity (see below); (iii) endothelial cell receptors distinct from FGFRs may activate signal transduction pathways complementary to those activated by FGFRs; (iv) some FGF-binding molecules can also bind FGFRs, leading to possible agonist/antagonist effects.

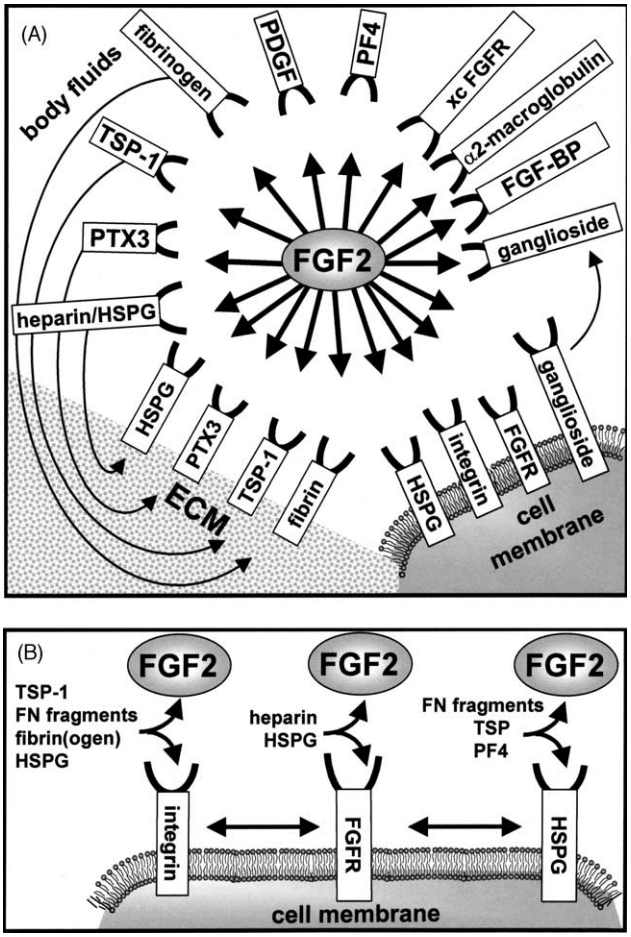

Fig. 2. FGF2-binding molecules. (A) Proteins, polysaccharides, and lipids present as free molecules in body fluids, associated to ECM, or anchored to endothelial cell membrane bind FGF2. Some of these molecules can change their status from an immobilized to a free form (arrows) exerting opposite effects on the biological activity of FGFs. (B) Some FGF binders are also able to interact with FGF-binding sites/receptors present on the surface of endothelial cells, possibly exerting agonist/antagonist effects (see text for further details).

FGF2 is present in blood at concentrations equal to 0.6 pM in physiological conditions and up to 6.0 pM under different pathological conditions [see [118] and references therein] whereas its binding partners are present at concentrations that are up to 1,000,000 times higher (Table 1). Thus, FGFs should exist mainly as immobilized molecules bound to the ECM and/or cell surface or as circulating complexes. The relative concentrations of the various FGF-binders may change greatly during different physio-pathological conditions, shifting the binding of FGFs from one ligand to another with repercussion on their bioavailability, endothelial cell interactions, and biological activities. Since the bulk of experimental data refer to the interaction of FGF2 with a number of extracellular molecules other than FGFRs, we will focus on FGF2 and its binding partners, even though many of the interactions described below may apply also to other members of the FGF family and, possibly, to other cytokines.

3.1. Cell surface and ECM components

3.1.1. Heparin and HSPGs

FGFs are heparin-binding proteins. Heparin is a negatively charged glycosaminoglycan released in the blood stream during inflammation. However, FGFs are more likely to interact with the heparan sulfate glycosaminoglycan chains attached to HSPG core proteins. HSPGs are expressed on the surface of almost all the cell types, including the endothelium, where they can be found as membrane-associated receptors, as ECM components, or released as free molecules [119]. HSPGs modulate angiogenesis by interacting with pro and negative regulators [120]. In particular, heparin/HSPGs bind FGF1, FGF2, FGF4, FGF7, and FGF8, modulating their biological activities in vitro and in vivo [121]. The interaction of heparin/HSPGs with FGFs occurs with low affinity and is mediated by the negatively charged sulfated groups of the saccharidic chain [122] that bind to basic amino acid motifs [123].

The alternative binding of FGFs to heparin, or to free, ECM-associated, or cell-surface HSPGs results in a fine control of the bioavailability and endothelial cell interaction of these growth factors (reviewed in [121]). In general, free heparin/HSPGs sequester FGFs in the extracellular environment and act as FGF antagonists. On the contrary, cell-associated HSPGs can directly activate a signal transduction pathway in response to FGF2 [124], promote FGF2 internalization [125,126], and are required for a correct presentation of FGFs to FGFRs, leading to the formation of productive HSPGs/FGF/FGFR ternary complexes [121]. Finally, HSPGs of the ECM act as a reservoir for FGF2 that reaches higher local concentrations and sustains the long-

Table 1
FGF2-binding molecules

Endothelial cell surface receptors	FGF2 affinity (K_d)	Reference	Number per cell	Reference
FGFR	20.0 pM	[125]	~10–20,000	[125]
$\alpha_v\beta_3$ integrin	6.5 nM[b]		~1×10^{6}[c]	[141]
HSPGs[a]	413.0 nM	[125]	~0.5–1.0 $\times 10^{6}$[d]	[125]
Gangliosides	3.0 nM	[148]	N.D.	

Free molecules	FGF2 affinity (K_d)	Reference	Blood concentration	Reference
FGF-binding protein	10 nM	[153]	N.D.	
Free gangliosides	6.0 μM	[147]	10 μM[e]	[249]
Heparin	42.0 nM	[139]	N.D.	
TSP[a]	30.0 nM	[150,151]	0.26–15.0 nM[f]	[250]
PTX3[a]	10.0 nM	[166]	0.16–0.36 nM[g]	[251]
Fibrin(ogen)[a]	1.3–260.0 nM[h]	[252]	7.0 μM	[118]
α_2 macroglobulin	62.0 nM	[162]	5.0 μM	[162]
xcFGFR1[a]	5.0–10.0 nM	[158]	N.D.	
PDGF	23.0 nM	[168]	74.0–204.0 nM[i]	[253]
PF4	37.0 nM	[170]	5.0–8.7 nM[j]	[254]

Affinity of different FGF2-binding partners, their number per cell or their blood concentration are indicated. Please note that FGF2 concentration in blood may range between 0.6 and 6.0 pM (see text). N.D.: not determined.

[a] These molecules can also be found associated to ECM.
[b] Our unpublished data.
[c] Value calculated by using radiolabeled fibronectin.
[d] These values refer to the number of FGF2-binding sites, as a single HSPG receptor can bind multiple FGF2 molecules.
[e] Total serum sialic acid content in healthy subjects.
[f] Values measured in the absence or in the presence of platelet activation.
[g] Values measured in the absence or in the presence of acute myocardial infarction.
[h] The two values are representative of the biphasic nature of the binding.
[i] Values measured in healthy and tumor-bearing individuals.
[j] Values measured in health and coronary disease.

term stimulation of endothelial cells [127]. A schematic representation of the effects exerted by the heparin/HSPGs system on the biology of FGFs is shown in Fig. 3.

The binding of FGFs to different HSPGs may have different biological consequences. This is the case for syndecan, betaglycan, and perlecan, all able to bind FGF2 but with different effects. For instance, syndecan inhibits the mitogenic activity of FGF2 whereas perlecan promotes FGF2-induced cell proliferation and angiogenesis (reviewed in [128]). It is interesting to note that modifications of HSPGs composition can regulate the sensitivity of the cell to different FGFs [129] and that FGFs themselves can modulate HSPG synthesis [130].

Also, FGF2 regulates the synthesis of HSPGs as well as the production of protease/glycosidase that digest the core protein/saccharidic chains of HSPGs inducing the mobilization of free HSPGs/HS chains [131]. ECM degradation can lead to mobilization of entrapped FGF2 with consequent activation of an angiogenic response [132].

The capacity of various angiogenic factors, including FGFs, to bind heparin/HS indicates that molecules able to interfere with this interaction may act as angiogenesis inhibitors. The ability of low molecular weight heparin fragments to reduce the angiogenic activity of FGF2 and VEGF support this hypothesis. On this basis, several heparin-like anionic molecules and heparin derivatives have been developed as possible candidate drugs (reviewed in [128]).

3.1.2. Integrin receptors

Integrins are transmembrane, adhesion receptor heterodimers comprised of α and β subunits. The combination of different subunits originates distinct integrins that mediate cell adhesion to a variety of adhesive proteins of the ECM [133]. Integrins regulates also the response of endothelial cells to soluble growth factors, including FGF2 [134], but the molecular mechanism(s) of this regulation are not fully elucidated. $\alpha_v\beta_3$ integrin is expressed on endothelial cells where it plays a central role in neovascularization. For this reason it has been considered as a target for the development of anti-cancer therapies [135].

Similar to classical adhesive proteins, FGF2 binds $\alpha_v\beta_3$ [136]. Consequently, immobilized FGF2 promotes endothelial cell adhesion and spreading, leading to uPA upregulation, cell migration, proliferation, and morphogenesis [137]. $\alpha_v\beta_3$/FGF2 interaction and endothelial cell adhesion to immobilized FGF2 lead to the assembly of focal adhesion plaques containing $\alpha_v\beta_3$ and FGFR1, whose presence is an absolute requirement for the activation of $ERK_{1/2}$ and cell proliferation [137]. Accordingly, a direct $\alpha_v\beta_3$/FGFR1 interaction is required for a full response to FGF2 [138].

Endothelial cell adhesion and activation by immobilized FGF2 may have relevance in vivo. Indeed, as stated above, FGF2 accumulates as an immobilized protein in the ECM, mainly by binding to HSPGs. Relevant to this point, heparin-bound FGF2 retains it cell-adhesive capacity [139]. Also,

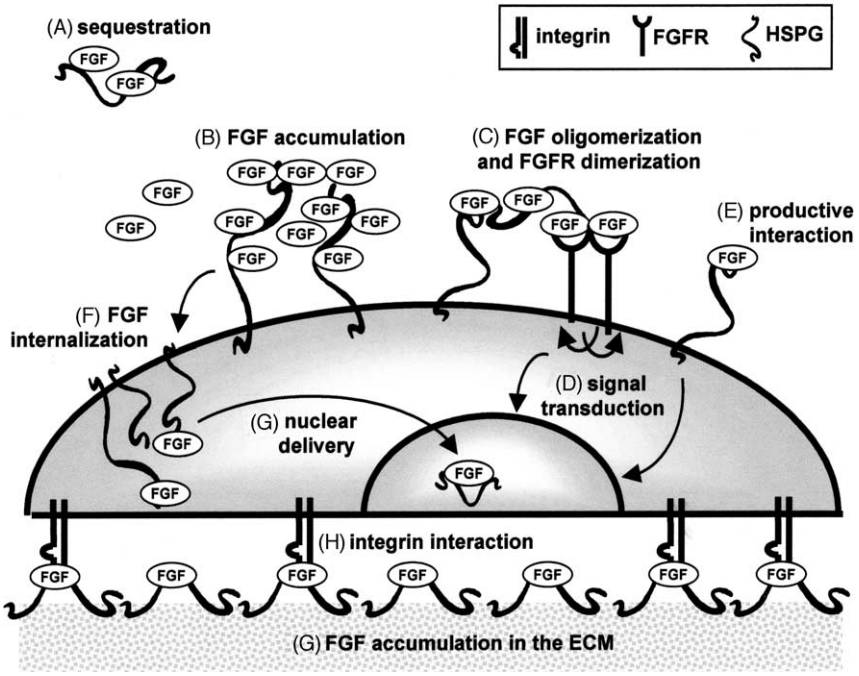

Fig. 3. Biological consequences of FGF/HSPG interaction. (A) FGF bound to free heparin/HSPGs is sequestered in the extracellular environment. (B) FGF binds to HSPGs of the endothelial cell surface, increasing its concentration in the microenvironment. (C) HSPGs promote FGF oligomerization that, in turn, triggers FGFRs dimerization and signal transduction (D) that can be activated also by a direct FGF/HSPG interaction (E). (F) HSPGs mediate cell internalization of FGF and, possibly, its nuclear delivery (G). (H) HSPGs of the ECM can present FGF to endothelial cell integrins to mediate substrate-adhesion.

HSPGs bound to fibronectin present FGF2 in a biologically active form [140]. Thus, HSPGs may facilitate the interaction of ECM components with FGF2 that, in turn, promotes endothelial cell adhesion and activation via $\alpha_v\beta_3$ (Fig. 3).

Integrin $\alpha_v\beta_3$ is expressed also at the luminal aspect of endothelium [141] suggesting that it may also mediate the biological affects exerted by free FGF2. Actually, anti-$\alpha_v\beta_3$ antibodies [136] and RGD- or DGR-containing peptides [142] inhibit mitogenesis and protease upregulation triggered by free FGF2 in cultured endothelial cells. Accordingly, RGD-peptidomimetic inhibits FGF2-dependent neovascularization in the CAM assay and tumorigenesis in vivo [143].

3.1.3. Gangliosides

Gangliosides are neuraminic acid (NeuAc)-containing glycosphingolipids. Under physiological conditions, gangliosides are mainly associated to the membrane of different cell types, including endothelium, where they modulate cell growth, adhesion, and cell–cell interaction [144]. During tumor growth and metastatization, gangliosides shed in the microenvironment [145] with a consequent increase of their serum levels (Table 1) and accumulation in the tumor microenvironment. For instance, gangliosides are highly expressed in the hyper-vascularized areas of gliomas where they regulate angiogenesis [146].

Gangliosides bind FGF1, FGF2, and FGF4 via negatively charged Neu-Ac residues [147,148]. In the extracellular environment, gangliosides compete with free heparin for the binding to the growth factor. On endothelial cells, free gangliosides prevent the binding of FGF2 to FGFRs and HSPGs, thus inhibiting FGF2-mediated cell proliferation [147].

Ganglioside GM_1 is expressed on the endothelial cell surface and binds FGF2 with an affinity that is significantly higher than that of its free counterpart (Table 1). Under these conditions, GM_1 acts as a functional FGF2 co-receptor. Indeed, inhibitors of ganglioside synthesis or GM_1-binding cholera toxin b-subunit impair the capacity of endothelial cells to proliferate when stimulated by FGF2. On the contrary, GM_1 overloading of the cell membrane increases the responsiveness of endothelial cells to FGF2 [148].

3.1.4. Thrombospondin-1

Thrombospondin-1 (TSP-1) is a modular glycoprotein secreted by different cell types, including endothelial cells. It is composed of multiple active domains that bind to soluble factors, cell receptors, and ECM components [149]. In particular, TSP-1 associates to HSPGs of the ECM and binds integrin receptors [149]. TSP-1 was the first endogenous inhibitor of angiogenesis to be identified and its effect is due, at least in part, to its capacity to bind FGF2 [150]. The interaction is mediated by the COOH-terminal, anti-angiogenic 140 kDa fragment of TSP-1. TSP-1 prevents the interaction of FGF2 with soluble heparin and with endothelial cell HSPGs and FGFRs. Accordingly, TSP-1 inhibits the mitogenic and chemotactic activity of FGF2 in endothelial cells. TSP-1 also prevents the accumulation of FGF2 in the ECM and favors the mobilization of matrix-

bound FGF2, generating inactive TSP-1/FGF2 complexes [151]. These observations suggest that free TSP-1 can act as a scavenger for matrix-associated FGFs, affecting their location, bioavailability and function, whereas ECM-associated TSP-1 may act as a ''FGF decoy'', sequestering the growth factor in an inactive form.

3.1.5. Fibstatin

Fibstatin is a fibronectin fragment that binds FGF2 but not FGF1, FGF3, FGF6, or FGF12 [152]. Fibstatin inhibits FGF2-dependent proliferation, migration and tubulogenesis of endothelial cells in vitro and angiogenesis and tumor growth in vivo with high efficiency [152]. Like other FGF-binding partners (see above), fibstatin is endowed of the capacity to bind heparin and integrin receptors, suggesting that multiple interactions are responsible for the anti-angiogenic activity of this molecule.

3.1.6. FGF-binding protein

FGF-binding protein (FGF-BP) is a secreted protein that binds FGF1 and FGF2 [153]. FGF-BP may act as a chaperone molecule that competes with HSPGs for growth factor binding and mobilizes FGFs from ECM, thus facilitating their interaction with FGFRs. FGF-BP can serve as an angiogenic switch for different tumor cell lines, including squamous cell carcinoma and colon cancer cells [154]. FGF-BP interacts also with the protein core of the pro-angiogenic, FGF2-binding HSPG perlecan [155].

3.2. Serum components

3.2.1. Soluble FGFR1

A soluble form of the extracellular portion of FGFR1 (xcFGFR1) is present in blood, in the cerebral spinal fluid, and in the vitreous fluid [see [156] and references therein]. Also, xcFGFR1 interacts with endothelial ECM [157]. xcFGFR1 binds FGF2 with an affinity that is lower than that of the intact receptor (Table 1), but sufficient to prevent FGF2/FGFR interaction when administered at high concentrations [158]. Also, xcFGFR1 may inhibit the biological activity of FGF1, FGF2, and FGF3 by forming heterodimers with cellular FGFR1, thus blocking the process of signal transduction [159]. Nevertheless, the impact of xcFGFR1 on angiogenesis remains to be investigated.

3.2.2. Fibrinogen

Fibrinogen is a 340 kDa glycoprotein found in blood or immobilized on the blood vessel wall. Following blood vessel injury, fibrinogen is converted to fibrin that represents the temporary substrate for endothelial cell adhesion and migration in the initial phases of the healing process. Fibrin(ogen) binds FGF2, but not FGF1, with high affinity [118] without affecting FGF2/FGFR1 interaction. Indeed, FGF2 bound to immobilized fibrin(ogen) supports endothelial cell proliferation [118] and protease production [160]. The FGF2-potentiating effect of fibrin(ogen) requires $\alpha_v\beta_3$ that, in the presence of the adhesive protein, interacts with FGFR1 [138]. These observations, together with the capacity of fibrin(ogen) and FGF2 to bind $\alpha_v\beta_3$, underlay the complex interplay among FGF, cell-surface receptors, ECM components, and diffusible molecules.

3.2.3. α_2-Macroglobulin

α_2-Macroglobulin (α_2M) is a 718 kDa homotetrameric protein present in human plasma where it acts as a broad-specific proteinase inhibitor. To exert its activity, α_2M undergoes major conformational changes that lead to the activated form α_2M^*. Both α_2M and α_2M^* bind a variety of cytokines and growth factors, including FGF1, FGF2, FGF4, and FGF6, but not FGF5, FGF7, FGF9, and FGF10 [161]. The binding of α_2M to FGF2 occurs with high affinity (Table 1) and is primarily hydrophobic in nature [162]. α_2M sequesters FGF2 in the extracellular environment and inhibits its cell interaction, protease-inducing activity [163] and mitogenic capacity [161]. Interestingly, both TGF-β [162] and PDGF [163] compete with FGF2 for the binding to α_2M. Also, α_2M competes with ECM components for FGF2 interaction [161].

3.2.4. Pentraxin 3

Pentraxin 3 (PTX3) is a 45 kDa glycosylated protein predominantly assembled in 10–20 mer multimers [164]. Its COOH-terminal domain shares homology with the classic short-pentraxin C-reactive protein whereas its NH_2-terminal portion does not show significant homology with any other known protein [165]. PTX3 is synthesized and released by activated mononuclear phagocytes and endothelial cells [165] and acts as a soluble pattern recognition receptor with unique functions in various physiopathological conditions. These functions relay, at least in part, on the capacity of PTX3 to bind different structures (see [166] and references therein). PTX3 binds FGF2, but not FGF1 and FGF4, with high affinity [166]. In endothelial cells, PTX3 prevents the binding of FGF2 to cell surface FGFRs and HSPGs, with a consequent inhibition of cell proliferation and migration, and inhibits FGF2-dependent neovascularization in the CAM assay. Also, PTX3 overexpression in FGF2-transformed endothelial cells inhibits FGF2-dependent proliferation and invasion in vitro and tumorigenesis in vivo [166].

PTX3 exists both as a free or ECM-immobilized molecule [167]. Relevant to this point, FGF2 and PTX3 retains their binding capacity independently of their free or immobilized status [166]. Thus, as described for TSP-1, free PTX3 may have access to ECM-bound FGF2 by acting as a scavenger for the stored growth factor, whereas ECM-associated PTX3 may act as a ''FGF2 decoy'', sequestering the growth factor in an inactive form.

3.3. Cytokines

Platelet-derived growth factor BB (PDGF-BB) binds FGF2 in a 1:2 stoichiometry [168]. This interaction may

contribute to the inhibitory effect exerted by PDGF-BB on FGF2-dependent neovascularization [169].

The heparin-binding C-X-C chemokine platelet factor 4 (PF4) is a well known inhibitor of angiogenesis ([170] and references therein). PF4 binds FGF1 [171] and FGF2 [170]. In endothelial cells, PF4 inhibits FGF2 interaction with HSPGs and FGFR1, FGF2 internalization and mitogenic activity [170]. Heparin stabilizes FGF2/PF4 interaction by forming a ternary complex [172]. On the other hand, PF4 binds and masks cell surface or ECM-associated HSPGs, hindering these receptors to FGF2 and FGF1 binding [173].

4. FGF/FGFR system in tumor angiogenesis

4.1. Experimental tumors

Various tumor cell lines express FGF2 [174,175] and the appearance of an angiogenic phenotype correlates with the export of FGF2 during the development of fibrosarcoma in a transgenic mouse model [176]. Antisense cDNAs for FGF2 and FGFR1 inhibit neovascularization and growth of human melanomas in nude mice [177]. Also, the anti-angiogenic activity of IFN-α/β appears to be related, at least in part, to the capacity to down-regulate FGF2 expression [178]. These data suggest that FGF2 production and release may occur in vivo and may influence the growth and neovascularization of tumor xenografts. Indeed, neutralizing anti-FGF2 antibodies and soluble FGFRs affect tumor growth under defined experimental conditions [179–182]. Accordingly, targeting FGF-BP with specific ribozymes inhibits the growth and vascularization of xenografted tumors in mice [154] despite the high levels of VEGF produced by these cells [183]. Interestingly, FGF-BP may exert its biological function via a paracrine stimulation on both tumor and endothelial cells [184]. Indeed, given the pleiotropic activity of FGFs, it is not always possible to dissociate the effect of FGFs on tumor angiogenesis from those exerted directly on tumor cells. For instance, S115 breast cancer cells transfected with FGF8b, but not with FGF8a or FGF8e, originate highly vascularized tumors when injected in nude mice. However, FGF8b also affect their ancorage-independent growth in vitro in an autocrine manner [17]. Similar results were reported for FGF4-transfected human breast carcinoma cells [185]. Also, inhibition of FGF/FGFR system in glioma cells by dominant negative FGFR transfection [99] or in prostate cancer cells by *fgf*2 gene knockout [186] results in inhibition of tumor growth by both angiogenesis-dependent and -independent mechanisms.

Relevant to this point, constitutive [187,188] or tetracycline-regulated [189] FGF2 overexpression causes a significant increase in the angiogenic activity and tumorigenic capacity of a VEGF-producing human endometrial adenocarcinoma cell line without affecting tumor cell proliferation in vitro [189]. These data suggest that modulation of FGF2 expression may indeed have a direct effect on angiogenesis and may allow a fine tuning of tumor vascularity even in the presence of VEGF (see above). Accordingly, simultaneous expression of FGF2 and VEGF in these tumor cells results in fast growing tumor xenografts in nude mice characterized by high blood vessel density, patency and permeability [106]. Inhibition of FGF2 production causes a significant decrease in the growth and vascularization of these lesions, without affecting vessel patency and permeability, pericyte recruitment, tumor necrosis, and oxygenation (as evaluated by HIF-1α immunostaining). In contrast, the decrease in tumor growth and vascularization consequent to antisense VEGF cDNA transfection is also paralleled by a significant decrease in monocyte infiltrate, pericyte organization, vascular patency, and permeability. This results in an increase in HIF-1α immunoreactivity and tumor necrosis. An additional inhibitory effect is exerted by FGF2 down-regulation in antisense VEGF cDNA transfected lesions. Thus, FGF2 and VEGF factors exert a synergistic effect on tumor blood vessel density in this model. However, FGF2 and VEGF differently affect blood vessel maturation and functionality (see also [112]).

In keeping with these observations, adenoviral expression of a soluble form of VEGFR-1 in spontaneous β-cell pancreatic tumors in Rip1 Tag2 mice affected the initial stages of tumor angiogenesis whereas soluble FGFR2 appeared to impair the maintenance of tumor angiogenesis. The combination of the two soluble receptors exerted a synergistic effect [182]. In addition, expression of a dominant-negative FGFR1 in the retina of *Tryp*1-Tag mice that develop early vascularized tumors of the retinal pigment epithelium results in a significant decrease in tumor burden and vascularity [190].

4.2. Human tumors

The possibility that FGFs may play a role in human tumor vascularization represents an important issue in FGF biology and for the development of anti-angiogenic therapies. Numerous studies have attempted to establish a correlation between intratumoral levels of FGF2 mRNA or protein and intratumoral microvessel density (MVD) in cancer patients. Table 2 summarizes the results from 53 independent studies that investigated the correlation between intratumoral FGF2 levels and MVD and between these two parameters and cancer progression/prognosis. Clearly, the bulk of data highlight a marked heterogeneity among different tumors and also among different studies within the same tumor type. With a few exceptions (e.g. melanomas) FGF2 levels do not correlate persistently with MVD. This is in sharp contrast with what observed for VEGF levels that more systemically correlate with MVD.

It is interesting to note that in some tumor types (e.g. breast and hepatocellular carcinomas) intratumoral levels of FGF2 correlate with the clinical outcome but not with MVD. As stated above, the pleiotropic activity of FGFs may affect

Table 2
Correlation between intratumor FGF2 or VEGF levels with tumor vascularity (MVD) or clinical outcome

Tumor type	FGF2 levels vs. MVD	FGF2 levels vs. clinical outcome[a]	VEGF levels vs. MVD[b]
Astrocytoma	+ −	N.D.	+ +
Basal cell carcinoma	−	−	+
Bladder carcinoma	+	+ +	+
Breast carcinoma	+ − − − −	+ + +	+ + +
Cardiac myxoma	+	N.D.	N.D.
Colorectal adenocarcinoma	−	− −	+ + +
Epidermoid lung carcinoma	−	+	+
Gastric carcinoma	−	−	+
Glioma	+ + −	+ − −	+ +
Hepatocellular carcinoma	−	+ +	+
Laringeal adenocarcinoma	−	−	+
Leiomyoma	−	N.D.	+
Leiomyosarcoma	−	N.D.	+
Melanoma	+ + +	+ +	N.D.
Meningioma	− −	− − −	−
Mesotelioma	+	+	+
Non-Hodgkin's lymphoma	−	+	N.D.
Non-small cell lung carcinoma	−	+	N.D.
Pancreatic adenocarcinoma	+ + −	+ + −	+ + +
Parathyroid adenoma	+	−	N.D
Pituitary adenoma	+	N.D.	+
Prostatic adenocarcinoma	+ + −	+	+ + +
Pulmonary adenocarcinoma	+	N.D.	+
Renal carcinoma	− −	+ −	−
Squamous cell carcinoma	+ + + −	+ − − −	+ + +
Thymoma	−	−	N.D.

+: correlation; −: no correlation; N.D.: not determined. Multiple symbols refer to distinct studies on the same tumor type.
[a] Clinical features analysed in the various studies were: grading/staging, metastatic status, disease recurrence, poor prognosis.
[b] Only those studies in which VEGF was directly compared to FGF2 were included.

both tumor vasculature and tumor parenchyma. Thus, at variance with the more endothelial-specific VEGF, FGF2 (as well as other FGFs) may contribute to cancer progression not only by inducing neovascularization, but also by acting directly on tumor cells. Accordingly, the co-expression of FGF7/KGF and its receptor FGFR2 IIIb/KGFR correlates with the high proliferative activity and poor prognosis in lung adenocarcinoma [191].

Evaluation of MVD may have prognostic significance in solid tumors [192,193], lymphomas [194], and leukemia [195]. Quantification of the angiogenic proteins in body fluids may represent an indirect, non-invasive way to measure angiogenic activity in cancer patients. Serum concentration of angiogenic factors increases with tumor progression [196] and decreases in response to treatment and long-term disease control [197]. Thus, apart from providing prognostic information in early detection of primary tumors or to follow tumor progression, measurement of these circulating factors may be used to monitor tumor regression during therapy and for the selection of patients at high risk of recurrences after treatment [198].

Moreover, the prognostic significance of FGF levels in biological fluids of cancer patients is controversial. Early studies showed that elevated levels of FGF2 in urine samples collected from 950 patients having a wide variety of solid tumors, leukemia or lymphoma were significantly correlated with the status and the extent of disease [199]. However, no association between increased serum levels of FGF2 and tumor type was observed in later studies on a large spectrum of metastatic carcinomas even though two-thirds of the patients showing progressive disease had increasing serum levels of the angiogenic factor compared with less than one-tenth of the patients showing response to therapy [200]. The clinical significance of circulating FGF2 in individual types of cancer has been recently reviewed [201]. Briefly, the levels of circulating FGF2 may have prognostic significance in head and neck cancer, lymphoma, leukemia, prostate carcinoma, and soft tissue sarcoma but they do not correlate with breast cancer progression and their significance in colorectal carcinoma is unclear. Also, after an encouraging report about a positive correlation between MVD and cerebrospinal fluid FGF2 in children with brain tumors [202], FGF2 levels in body fluids do not always reflect tumor vascularity. Moreover, serum FGF2 may not entirely derive from the neoplastic tissue in cancer patients [203].

In conclusion, clinical reports have not established yet a clear relationship among FGFs, tumor angiogenesis, and tumor progression/prognosis. Further studies assessing the correlation between FGF levels at the tumor site and/or in body fluids and MVD are eagerly awaited before these growth factors, as well as other angiogenic factors, can be used as prognostic indicators, surrogate markers of angiogenesis in cancer patients, and as targets for angiostatic therapies.

5. FGF-dependent angiogenesis and inflammation

Inflammation is the response of a vascularized tissue to sub-lethal injury, designed to destroy or inactivate invading pathogens, remove waste and debris, and permit restoration of normal function, either through resolution or repair.

Inflammation may promote FGF-dependent angiogenesis (Fig. 4). Inflammatory cells, including mononuclear phagocytes [204,205], $CD4^+$ and $CD8^+$ T lymphocytes [206,207], and mast cells [208] can express FGF2. Moreover, osmotic shock and shear stress induce the release of FGF2 from endothelial cells [209,210]. FGF2 production and release from endothelial cells are also triggered by IFN-α *plus* IL-2 [211], IL-1β [212], and nitric oxide (NO) [213]. NO is produced by vascular endothelium following stimulation by cytokines, bacterial endotoxins, inflammatory mediators, neuropeptides, and shear stress [214]. Even though FGF2-induced angiogenesis can occur independently of NO synthesis [215], the pro-angiogenic effects exerted by NO and NO-inducing molecules are due, at least in part, to the NO-mediated FGF2 upregulation in endothelial cells [216]. Thus, inflammatory mediators can activate the endothelium to synthesize and release FGFs that, in turn, will stimulate angiogenesis by an autocrine mechanism of action (Fig. 4). On the other hand, PTX3, synthesized locally by endothelial cells in response to IL-1β and TNF-α, binds FGF2 and acts as a natural angiogenesis inhibitor (see above), thus allowing a fine tuning of FGF2 pro-angiogenic activity in inflammation.

The inflammatory response may also cause cell damage, fluid and plasma protein exudation, and hypoxia. Endothelial cell damage results in increased FGF2 production and release [217]; exudated fibrin(ogen) can bind FGF2 and enhances its biological activity (see above); hypoxia upregulates the production of angiogenic growth factor, including VEGF [218] and FGF2 [204]. Furthermore, hypoxia increases endothelial cell responsiveness to FGF2 by promoting HSPG synthesis [219] and upregulates FGF2 production also in vascular pericytes [220].

Conversely, by interacting with endothelial cells, FGF2 may amplify the inflammatory and angiogenic response by inducing vasoactive effects and the recruitment of an inflammatory infiltrate (Fig. 4). Indeed, FGF2, but not FGF1, causes vasodilation of coronary arterioles via an increase in NO production [221]. FGFs can also induce vascular permeability indirectly, by upregulating VEGF and proteases (see above), and directly, as suggested for FGF2 and FGF5 [222]. Transient exposure to FGF1 and FGF2

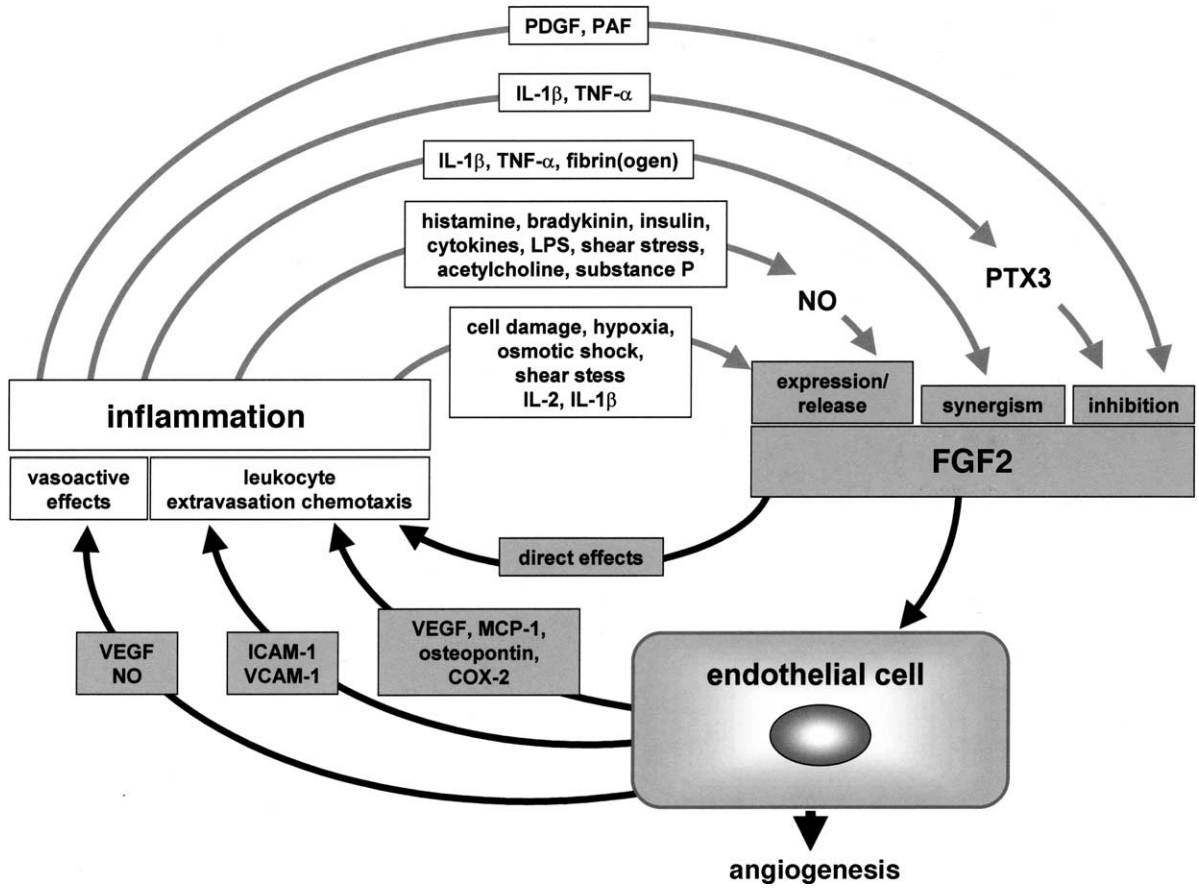

Fig. 4. Schematic representation of the interplay between FGFs and inflammation. Several inflammatory mediators can affect the biological activity of FGFs by different mechanisms. In turn, FGFs can modulate various steps of the inflammatory process by acting directly (or indirectly following endothelial cell activation) on inflammatory leukocytes. This results in the amplification of the angiogenic response triggered by FGFs on endothelial cells.

upregulates the expression of cell adhesion molecules ICAM-1 and VCAM-1 in endothelial cells, increasing polymorphonuclear leukocyte adhesion and transendothelial migration [223]. Also, FGF2-stimulated endothelial cells upregulate the synthesis of various chemoattractants, including VEGF, that may exert a chemotactic activity on monocytes [224], the angiogenic/monocyte chemotactic protein osteopontin [225], monocyte chemoattractant protein-1 [107,226], and the pro-angiogenic cyclooxygenase-2 [227]. Moreover, FGF2 exerts a direct chemotactic effect on monocytes (Presta, unpublished observations). Finally, in agreement with a possible role of inflammatory cells in FGF2-mediated neovascularization, a significant inhibition of the angiogenic response to FGF2 is observed in neutropenic mice [228].

Even though these experimental evidences point to a possible loop of amplification of the angiogenic response triggered by FGF2 and mediated by the inflammatory infiltrate, long-lasting exposure to FGF2 down-regulates cytokine-induced ICAM-1, VECAM-1, and E-selectin expression in endothelial cells. Consequently, polymorphonuclear leukocyte adhesion and transendothelial migration are reduced [223]. Similarly, monocyte/macrophages adhesion to endothelium and the chemotactic response to various chemokines are markedly inhibited by long-term stimulation by FGF1 or FGF2, but not by VEGF [229]. Also, FGF2 suppress transendothelial migration of $CD4^+$ T-lymphocytes [230] and tissue factor expression in endothelial cells [231]. These observations suggest that the pro- or anti-inflammatory activity of FGFs may be contextual and may explain, at least in part, the reduced leukocyte adhesion and transendothelial migration observed in experimental tumors [232] that, nevertheless, are characterized by the presence of pro-angiogenic tumor-associated macrophages [233].

6. FGFs and therapeutic angiogenesis

Therapeutic angiogenesis represents a possible approach to the treatment of severe ischemic diseases in patients with coronary (CAD) or peripheral (PAD) artery injury. Aim of this therapy is to restore and maintain tissue perfusion by increasing the number of collateral blood vessels within the ischemic territories following the delivery of specific angiogenic growth factors. Different delivery methods, including intravenous, intracoronary, intramyocardial and intrapericardial routes, are normally used to administer angiogenic factors either as recombinant proteins or by gene transfer using naked DNA or vectors that encode the gene to be incorporated into the target cells.

Among the different members of the FGF family, FGF1, FGF2, FGF4, and FGF5 have been more widely investigated, with particular emphasis to FGF2. For instance, in swine and canine models of coronary occlusion, intracoronary FGF2 administration or local injection in the myocardium can reduce scar size, preserve myocardial function, and increase number of blood vessels (reviewed in [234]).

In CAD patients, slow-release FGF2 capsules implanted in the myocardium in a phase I clinical trial caused a significant reduction in size of the ischemic region and treated patients had more freedom from angina recurrence than controls [235,236]. Also, single-bolus intracoronary FGF2 infusion showed transient beneficial effects, including reduction of angina symptoms, increase of treadmill tolerance and quality of life [237]. Transient beneficial effects were observed also in the phase II trial FIRST in which FGF2 was administered via intracoronary infusion [238]. In PAD patients, a positive response was observed in a phase I trial in which patients with symptoms of claudications and advanced peripheral arterial disease where given intra-arterial FGF2 infusion [239]. An early, transient improvement in performance was observed also in the phase II trial TRAFFIC in which patients with infra-inguinal atherosclerosis and claudication received a bilateral intra-arterial infusion of FGF2 [240].

Experience with FGF1 is more limited. Early studies using a recombinant FGF1 protein reported no beneficial effects in a dog model of myocardial ischemia probably due to the short protein half-life. Indeed, administration of a FGF1 mutant with prolonged half-life showed an augmentation of blood flow and function in ischemic porcine myocardium [241]. Similar beneficial effects were observed in a hindlimb ischemia rabbit model using a single intramuscolar dose of naked DNA encoding FGF1 [242]. Phase I clinical trials have shown some beneficial effects following FGF1 protein injection in ischemic myocardium [243]. Similarly, intramuscular FGF1 gene injection in PAD patients resulted in a transient beneficial effect that was not sustained at 6 months [244].

The angiogenic potency of FGF4 and FGF5 was evaluated by gene therapy using an adenoviral vector in the rabbit hindlimb [94,245] and in the pig myocardium [246]. Adenovirus-delivered FGF4 was tested in two phase I clinical trials (AGENT and AGENT 2), involving patients with chronic stable angina. No beneficial effects were observed in both trials [246,247].

In conclusion, current clinical experience in ischemic disease suggest that FGF-based angiogenic therapy may represent a promising treatment for patients. However, further investigation is required to solve mayor problems that are critical to successful therapy: identification of the most effective delivery approach, proper selection of patients, timing and dosage of angiogenic factors used alone or in combinations [248].

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