Inflammatory cells and chemokines sustain FGF2-induced angiogenesis

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ABSTRACT. Angiogenesis and inflammation are closely integrated processes in a number of physiological and pathological conditions, including wound healing, psoriasis, diabetic retinopathy, rheumatoid arthritis, arteriosclerosis, and cancer. Fibroblast growth factor-2 (FGF2) belongs to the family of the heparin-binding FGF growth factors. FGF2 exerts its pro-angiogenic activity by interacting with various endothelial cell surface receptors, including tyrosine kinase receptors, heparan-sulfate proteoglycans, and integrins. Elevated levels of FGF2 have been implicated in the pathogenesis of several diseases characterized by a deregulated angiogenic/inflammatory response. FGF2 induces the expression of a wide repertoire of inflammation-related genes in endothelial cells, including pro-inflammatory cytokines/chemokines and their receptors, endothelial cell adhesion molecules, and components of the prostaglandin pathway. Consistent with this pro-inflammatory signature, in vivo evidence points to a non-redundant role for chemokines and infiltrating monocytes/macrophages in FGF2-driven neovascularization. This review will focus on the cross-talk between FGF2 and the inflammatory response in the modulation of blood vessel growth.

Keywords: angiogenesis, endothelium, FGF2, inflammation, chemokines, macrophages

FGF2 AS AN ANGIOGENIC GROWTH FACTOR

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 in the production of natural angiogenic inhibitors, with a consequent alteration of the angiogenic balance [2], are responsible for the uncontrolled endothelial cell (EC) 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 EC that will migrate and proliferate, leading to the formation of solid EC sprouts into the stromal space. Then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of a 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 members of the fibroblast growth factor (FGF) family.

In 1984, J. Folkman and coworkers discovered a rodent tumor-derived factor able to bind with high affinity to heparin so that it could be purified 200,000-fold by a single passage over a heparin-affinity column. This protein, identified as basic fibroblast growth factor (FGF2), had a molecular weight of 14.8 kD, and stimulated capillary EC proliferation in vitro and angiogenesis in vivo [5, 6]. FGF2 was then purified from human normal cells/tissues [7] and from their transformed counterparts [8]. Purified FGF2 stimulated DNA synthesis, motility, and protease production (urokinase-type plasminogen activator and metalloproteases) in cultured ECs [8]. Also, FGF2 regulates the expression of cadherins, integrins and various extracellular matrix (ECM) components that contribute to the maturation of the new blood vessels regulating lateral cell-cell and substrate adhesions of ECs [9]. In vivo, FGF2 has been shown to induce neovascularization in a variety of animal models, including the chick embryo chorioallantoic membrane (CAM) assay, the rodent cornea assay, the subcutaneous Matrigel plug assay in mice, and the yolk membrane zebrafish embryo (ZFYM) assay [9, 10].

The single copy, human FGF2 gene encodes four co-expressed isoforms (24, 22.5, 22, and 18 kD). The latter isoform is translationally initiated at a classical AUG codon, while the high molecular weight FGF2 isoforms are colinear NH2-terminal extensions initiated at novel
CUG codons [11]. At variance with other FGFs, FGF2 isoforms lack a leader sequence for secretion and are released in limited amounts by an alternative secretion pathway, via membrane vesicle shedding, or following cell damage [12]. Even though experimental evidence points to significant differences in intracellular and extracellular fate and mechanisms of action of low and high molecular weight FGF2 isoforms [13], both 24 kD and 18 kD isoforms induce neovascularization in vitro and in vivo [14].

Twenty-three, structurally-related members of the FGF family have been identified [15]. FGFs are pleiotropic factors acting on different cell types, including EC, following interaction with heparan-sulfate proteoglycans (HSPG) and tyrosine kinase FGF receptors (TK-FGFR). Four TK-FGFR have been so far identified, whose structural variability is increased by alternative splicing: FGFR1 (fıl), FGFR2 (bek), FGFR3, and FGFR4. They belong to subclass IV of the membrane-spanning receptor family during angiogenesis, lymphangiogenesis, and vasculogenesis. An intimate cross-talk exists among FGF2 and the different members of the VEGF family [33]. An intimate cross-talk exists among FGF2 and the different members of the VEGF family [33].

FGF2/VEGF CROSS-TALK

For many years, FGF2 occupied a central stage in the angiogenesis field. Then, the VEGF family of angiogenic growth factors came into the limelight after the discovery of their pivotal role in vasculogenesis and angiogenesis during embryonic development and under numerous physiological and pathological conditions in adults [33]. The VEGF family comprises six members (VEGF-A denoting the originally identified VEGF) that interact differently with three, cell-surface tyrosine kinase VEGFR. To date, VEGF-A/VEGFR-2 interaction appears to play a major role in blood vessel angiogenesis, whereas VEGF-C and VEGF-D are mainly involved in lymphangiogenesis by interacting with VEGFR-3 expressed on lymphatic endothelium [33]. An intimate cross-talk exists among FGF2 and the different members of the VEGF family during angiogenesis, lymphangiogenesis, and vasculogenesis.

There is some experimental evidence that points to the possibility that FGF2 induces neovascularization indirectly by activation of the VEGF/VEGFR system (see [9] and references therein). On the other hand, EC tube formation, stimulated by VEGF in murine embryonic explants, depends on endogenous FGF2 [34]. Also, FGF2 and VEGF may exert a synergistic effect in different angiogenesis models [35-37] even though this may not be the case when the two factors are applied onto the chick embryo CAM [38]. Thus, FGF2 may require the activation of the VEGF/FGFR system for promoting angiogenesis. Conversely, VEGF may require FGF2 in order to exert its angiogenic potential under defined experimental conditions.

Nevertheless, the two growth factors retain distinct biological properties, exerting different biological effects on EC during angiogenesis [39-43]. Indeed, using a genetically-modified experimental tumor model, we have observed that FGF2 and VEGF affect tumor blood vessel maturation and functionality differently, with different consequences for tumor oxygenation and viability [41]. Inhibition of FGF2 production resulted in a signifi-
Inflammatory cells and chemokines sustain FGF2-induced angiogenesis

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. Angiogenesis and inflammation are closely integrated processes in a number of physiological and pathological conditions, including wound healing, psoriasis, diabetic retinopathy, rheumatoid arthritis, arteriosclerosis, and cancer [1, 61, 62].

**FGF2-DEPENDENT ANGIOGENESIS AND INFLAMMATION**

Inflammation may promote FGF-dependent angiogenesis. Inflammatory cells, including mononuclear phagocytes [63, 64], CD4+ and CD8+ T lymphocytes [65, 66], and mast cells [67] can express FGF2. Moreover, osmotic shock and shear stress induce the release of FGF2 from EC [68, 69]. FGF2 production and release from EC are also triggered by interferon (IFN)-α plus IL-2 [70], IL-1β [71], and nitric oxide (NO) [72]. NO is produced by vascular endothelium following stimulation by cytokines, bacterial endotoxins, inflammatory mediators, neuropeptides, and shear stress [73]. Even though FGF2-induced angiogenesis can occur independently of NO synthesis [74], the pro-angiogenic effects exerted by NO and NO-inducing molecules are due, at least in part, to NO-mediated FGF2 upregulation in EC [75]. Similarly, prostaglandin E2-induced angiogenesis is mediated by the activation of EC-surface FGR1 following mobilization of FGF2 sequestered by the ECM [76]. Thus, inflammatory mediators can activate the endothelium to synthesize and release FGF2 that, in turn, will stimulate angiogenesis by an autocrine mechanism of action. The inflammatory response may also cause cell damage, fluid and plasma protein exudation, and hypoxia. EC damage results in increased FGF2 and angiogenic activity of FGF2 is mediated by endogenous VEGF-C and VEGF-D upregulation, leading to VEGFR-3 activation [53]. Interestingly, no endothelial fenestration was observed in FGF2, VEGF-A, or VEGF-C-induced lymphatic vessels [54].

The VEGF/VEGF system is essential for the development of embryonic vasculature [55]. The situation is much less well-defined for the FGF/FGFR system. As stated above, the phenotype of fgfr knockout mice is not very informative, even though adenosivirus-driven, dominant-negative FGFR1 expression leads to severe, vascular alterations in mouse embryos [56]. Also, FGF2 promotes the proliferation and differentiation of VEGF-R2+ hemangioblast precursors from the mesoderm [57]. In embryoid bodies, embryonic stem cells can differentiate into a variety of cell lineages, including EC [58]. In this model, both VEGF and FGF2 lead to improved angioblast survival, but only VEGF supports the formation of primitive endothelial tubes [59]. Also, in embryoid bodies in which VEGF/VEGFR function is impaired, FGF2 stimulates the formation of EC clusters that fail to develop into primitive vessels. In contrast, VEGF induces the formation of a characteristic vascular plexus also in fgfr1-/- embryoid bodies [60].

Inflammation and the angiogenic response by inducing...
FGF2 INDUCES A PRO-INFLAMMATORY SIGNATURE IN ENDOTHELium

Taken together, the observations summarized above point to the existence of an intimate cross-talk between inflammatory and angiogenic responses during FGF2-driven neovascularization. In agreement with this hypothesis, gene expression profiling of FGF2-stimulated murine microvascular EC has revealed a pro-inflammatory signature [46] characterized by the upregulation of pro-inflammatory cytokote/chemokines and their receptors, EC adhesion molecules, and members of the eicosanoid pathway (table 1). Indeed, FGF2 upregulates the expression of a number of chemokines involved in the recruitment of different inflammatory cells such as monocytes/macrophages (Ccl2, Ccl7, Cx3c11, Opn), neutrophils (Cxcl1), NK cells (Cx3c11) and T lymphocytes (Cxcl16, Cx3c11). Also, FGF2-induced genes include inflammatory cytokines [IL-6, leukemia inhibitory factor (Lif), Opn] and cytokine receptors [oncostatin M receptor (Osmr), tumor necrosis factor receptor superfamily member 12a (Tnfrsf12a)/Tweak-receptor, interleukin 1 receptor accessory protein (Il1rap)], cell adhesion molecules related to leukocyte recruitment and transendothelial migration [VCAM-1, junctional adhesion molecule 2 (IAM2)], as well as key inflammatory mediators such as the cyclooxygenase Ptg2/Cox-2 and the prostaglandin E2 receptor Ptger4. Interestingly, FGF2 promotes the early upregulation (one hour after stimulation) for most of the inflammation-related genes examined, indicating that the induction of a pro-inflammatory signature represents an early event in FGF2-driven EC activation [46].

In keeping with the notion that the FGF2-induced inflammatory signature is relevant to the neovascular response triggered by the growth factor, immunofluorescence analysis of subcutaneously-implanted FGF2-containing Matrigel plugs confirms the presence of numerous CD31+ EC, together with a consistent CD45+ leukocyte infiltrate [46]. Characterization of the leukocyte subsets reveals that the inflammatory cell infiltrate consists mainly of CD11b+ monocytes and F4/80+ macrophages (figure 1), whereas only rare Gr-1+ neutrophils and CD3+ T-lymphocytes are detectable, and no CD19+ B-lymphocytes, NK1.1+ natural killer, or CD11c+ dendritic cells are found. Monocytes/macrophages are active players in pathological angiogenesis, including tumor neovascularization [99-102]. They can produce pro-angiogenic growth factors (e.g. VEGF, FGF2, IL-1β, IL-8, TNF-α) and proteases [103, 104]. Also, monocytes may contribute to new blood vessel formation by differentiating into endothelial-like cells [105, 106]. Monocytes/macrophages are frequently associated with proliferating blood vessels [107] and can open passages in existing vasculature to facilitate the development of vascular sprouts [104]. Thus, monocytes/macrophages often precede, temporally and spatially, the formation of new vascular sprouts, altering the microenvironment and promoting subsequent EC migration, proliferation, and vessel formation. Accordingly, we have observed that the early recruitment of mononuclear phagocytes (within two-three days after implantation) precedes blood vessel formation in FGF2-driven angiogenesis in the subcutaneous Matrigel plug assay [46]. Indeed, the capacity of monocytes/macrophages to cooperate with EC precursors during FGF2-driven neovascularization in a long-term Matrigel plug assay has been suggested [108]. In addition, numerous F4/80+ cells were observed surrounding the microvessels within the plug, four weeks after implantation. In this study, the authors hypothesize that monocytes/macrophages may contribute to ECM degradation, thus facilitating the invasion of Tie2+ precursors and blood vessel lumen formation.

Proper migration of leukocytes to chemotactic agonists in inflammatory sites is dependent upon phosphatidylinositol 3-kinase-γ (PI3Kγ) activity [109, 110]. A significant reduction in the F4/80+ cell infiltrate and in CD31+ neo-vessels is observed when FGF2-embedded Matrigel plugs are implanted in PI3Kγ−/− mice. Accordingly, macrophage depletion following intraperitoneal pretreatment with clodronate liposomes (Clodrolip) [111, 112] causes a significant reduction in the angiogenic response elicited by FGF2. Thus, monocytes/macrophages play a func-
FGF2 AS A TARGET FOR ANTI-ANGIOGENIC STRATEGIES

A significant effort has been directed towards the development of anti-angiogenic agents that prevent the growth of new blood vessels, the monoclonal anti-VEGF antibody bevacizumab representing the first FDA approved...
Inflammatory cells infiltrate the areas of FGF2-induced neovascularization in a Matrigel plug. Matrigel pellets containing vehicle or FGF2 were implanted subcutaneously in mice and examined after seven days for the presence of CD31+ endothelial cells (in red) and F4/80+ infiltrating macrophages (in green) by double-immunostaining. Nuclei are shown by DAPI counterstaining. Original magnification, x 200.

CONCLUSION

The above observations indicate that FGF2-driven angiogenesis is, at least in part, chemokine-dependent. It is tempting to speculate that chemotactic factors produced by FGF2-stimulated endothelium may recruit mononuclear phagocytes that, in turn, will amplify the angiogenic response by releasing monocyte-derived pro-angiogenic cytokines (figure 2). For instance, Opn, a cytokine endowed with a potent chemoattractant activity for monocytes, is strongly induced by FGF2 in EC [91]. In turn, Opn induces neovascularization [91] by recruiting monocytes/macrophages and promoting the release of the pro-angiogenic cytokine IL-1β [134]. Also, FGF2-induced chemoattractants may play a direct role in neovascularization. Indeed, various chemokines have been shown to function as angiogenic factors by direct interaction with specific chemokine receptors expressed on EC.

anti-angiogenic drug [122]. On the other hand, drug resistance to VEGF blockade may occur following reactivation of angiogenesis triggered by compensatory upregulation of the FGF2/TK-FGFR system [123]. Thus, given its potent angiogenic activity, FGF2 may represent the target for the development of novel anti-angiogenic strategies. The various approaches based on the inhibition of FGF2 have been reviewed extensively elsewhere (see [124] and references therein). Briefly, FGF2 can be neutralized at different levels by: i) inhibition of FGF2 production/release; ii) inhibition of the expression of the various FGF2 receptors in EC (including TK-FGFR, HSPG, gangliosides); iii) engagement by selected antagonists of the various FGF2 receptors (including TK-FGFR, HSPG, gangliosides, and integrins); iv) sequestration of FGF2 in the extracellular environment; v) interruption of the signal transduction pathways triggered by FGF2 in EC. Also, as stated above, FGF2 induces a complex “pro-angiogenic/pro-inflammatory phenotype” in EC. These processes are mediated by distinct effectors induced/activated by FGF2, and their blockage may result in the inhibition of FGF2-dependent angiogenesis [124]. Accordingly, in keeping with the pro-inflammatory signature triggered by FGF2 in EC, we have observed that FGF2-mediated angiogenesis is significantly reduced in the CAM assay by mechanistically distinct steroidal (hydrocortisone) and non-steroidal (ketoprofen) anti-inflammatory drugs, further implicating inflammatory cells/mediators in FGF2-dependent neovascularization [46]. Interestingly, FGF2-induced neovascularization is also inhibited by M3 protein [46], a murine gammaherpesvirus 68 protein that binds with high affinity to human and mouse CC, CXC and CX3C chemokines and inhibits their activity [125, 126], with potential therapeutic implications in inflammatory conditions [127]. Our findings suggest that M3 protein may represent the basis for the design of novel angiogenesis inhibitors for therapeutic interventions in angiogenesis-dependent pathological conditions, including tumor growth and metastasis. The bulk of experimental evidence summarized above clearly indicates that the FGF2/TK-FGFR system may represent a target for novel anti-angiogenic strategies in tumors. At present, cancer clinical trials are in progress to assess the safety and efficacy of various compounds with a potential capacity to affect the FGF2/TK-FGFR system at different levels [128, 129]. It must be pointed out however, that the ability to interact with this system may not represent the main rationale that has lead to their testing. This is the case for various heparin derivatives that have been tested in cancer patients because of their anti-thrombotic effect rather than for their potential FGF2-antagonist activity. Similarly, the humanized monoclonal anti-α,β1 antibody vitaxin [130, 131] has been investigated for its ability to affect the cell-adhesive function of this integrin receptor rather than its potential ability to act as a signaling TK-FGFR [132]. Also, numerous cytotoxic drugs can affect the FGF2 activity and angiogenesis [124]. Moreover, the impairment of receptors characterized by a broad spectrum of ligands, such as HSPG or integrins, may result in the simultaneous inhibition of various angiogenic growth factors [133]. Novel strategies aimed at inhibiting multiple targets, including FGF2, may represent an effective approach for the treatment of angiogenesis-dependent diseases, including cancer.
thus promoting proliferation, migration, and capillary tube formation [135, 136]. Among them, the FGF2-induced chemokines Ccl2, Cxcl1, Cxcl16 and Cxcl3l1 could act as enhancers of the neovascularization process elicited by the growth factor.

FGF2 expression is increased at sites of chronic inflammation [137-139], after tissue injury [140], and in different types of human cancer [9]. Cell damage, tissue injury and repair, neoplastic transformation, and inflammatory mediators can activate the endothelium, stromal, and parenchymal cells to synthesize and release FGF2 [70-72]. Released FGF2 may contribute to host defense responses by acting as a danger signal molecule, thus activating pro-angiogenic and pro-inflammatory signatures in endothelium that, by acting in concert, will lead to neovessel formation and monocyte/macrophage engagement (figure 3). Indeed, several biological features shown by FGF2 in vitro and in vivo strongly resemble those shown by the chromosomal high mobility group box-1 (HMGB1) protein (summarized in figure 3), a prototypic, damage-associated molecular pattern (DAMP) cytokine [141]. Interestingly, recent observations from our laboratory have shown the capacity of HMGB1 to trigger angiogenesis by interacting with the receptor for advanced glycation end products (RAGE) expressed by EC [142].

In conclusion, chemokines and inflammatory cells are important early mediators of FGF2-driven angiogenesis and play a relevant role in the neovascularization process elicited by the growth factor. Conversely, FGF2 may exert important functions at sites of inflammation and/or tissue injury, not only by inducing neovascularization but also by contributing to the activation of innate immune responses.

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**Figure 3**

FGF2 as a danger signal molecule in pathological contexts. Cell damage, tissue injury and repair, neoplastic transformation, and inflammatory mediators can trigger the synthesis/release of FGF2. Released FGF2 may contribute to host defense responses by acting as a danger signal molecule, thus activating pro-angiogenic and pro-inflammatory signatures in endothelium that, by acting in concert, will lead to neo-vessel formation and monocyte/macrophage engagement. Accordingly, FGF2 shares various biological features with the prototypic damage-associated molecular pattern cytokine HMGB1.
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