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Review

Angiogenesis and signal transduction in endothelial cells

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Abstract. Endothelial cells receive multiple information from their environment that eventually leads them to progress along all the stages of the process of formation of new vessels. Angiogenic signals promote endothelial cell proliferation, increased resistance to apoptosis, changes in proteolytic balance, cytoskeletal reorganization, migration and, finally, differentiation and formation of a new vascular lumen. We aim to review herein the main signaling cascades that become activated in angiogenic endothelial cells as well as the opportunities of modulating angiogenesis through pharmacological interference with these signaling mechanisms. We will deal mainly with the mitogen-activated protein kinases pathway, which is very important in the transduction of proliferation signals; the phosphatidylinositol-3-kinase/protein kinase B signaling system, particularly essential for the survival of the angiogenic endothelium; the small GTPases involved in cytoskeletal reorganization and migration; and the kinases associated to focal adhesions which contribute to integrate the pathways from the two main sources of angiogenic signals, i.e. growth factors and the extracellular matrix.

Key words. Endothelium; angiogenesis; signal transduction; cell proliferation; cell migration; cell survival.

Introduction

Angiogenesis is the process of vascular growth by sprouting of preexisting vessels. Angiogenesis is a main mechanism of vascularization during embryonic development, growth, formation of the corpus luteum and endometrium, regeneration and wound healing. Abnormal angiogenesis is also involved in many pathological processes, including tumor growth, metastasis, diabetic retinopathy and arthritis.

The molecular and cellular mechanisms leading to the angiogenic response in the endothelium have been extensively studied in the past 2 decades due to their therapeutic potential and their clinical implications. Biosignaling involved in angiogenic activation of endothelium is relatively well known. Extracellular signals involved in these processes are mainly secreted paracrine factors – frequently ligands of surface transmembrane receptors – and extracellular matrix components that usually bind to integrins and to specialized receptors. The main transmembrane receptors that transduce angiogenic signals are tyrosine-kinase receptors, G-protein-coupled receptors, tyrosine-kinase-associated receptors and serine-threonine kinase receptors.

Angiogenic endothelial cells must proliferate, produce molecules able to degrade the extracellular matrix, change their adhesive properties, migrate, avoid apoptosis and, finally, differentiate in new vascular tubes. All these processes are controlled by the signals received by endothelial cells from their environment, signals whose transduction pathways form cascades leading to gene transcription and a network of cross-talks determining the final behavior of the cell. The signal transduction pathways between the surface receptors involved in angiogenesis and the final effectors of the modified cellular behavior are only fragmentarily known. However, the knowledge of these pathways and their interrelationships is extremely impor-

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tant, since specific and essential signaling pathways in angiogenic endothelium may provide new targets of antiangiogenic or proangiogenic therapies.

In this review, we aim to collect the available information about the transduction signal pathways that are or may be involved in transformations between the quiescent and angiogenic phenotypes of the endothelium. We analyze the transduction of signals leading to proliferation, survival, degradation of extracellular matrix, migration and differentiation/morphogenesis of endothelial cells. We also describe how pharmacological interference with some of these signaling pathways might provide therapeutic tools for angiogenic-associated diseases.

Transduction of proliferation signals

The adult endothelium is a very quiescent cell type. Only a 0.01 % of all the endothelial cells of a normal adult are dividing at any given moment [1]. However, in response to angiogenic stimulation, endothelial cells enter into an actively proliferative state. The pathways leading to entry into the cell cycle depend on the angiogenic signals received (fig. 1).

VEGF receptors

Binding of VEGF to the endothelial-specific receptor VEGFR2 is the main extracellular signal triggering an angiogenic response. This binding leads to receptor dimerization and autophosphorylation of the intracytoplasmic domains in specific tyrosine residues. The activated receptor induces the recruitment and phosphorylation of SH2-domain-containing proteins. At least 12 proteins are phosphorylated in this way [2], and some of these proteins are involved in the transduction of mitogenic signals.

The main VEGFR2-induced proliferative pathway is mediated by the extracellular signal-regulated kinases-mitogen-activated protein kinases (ERK-MAPK) cascade, which can be activated in two ways. VEGFR2 can directly activate phospholipase C-γ, which cleaves phosphatidylinositol-4,5-bisphosphate and produces diacylglycerol and inositol-trisphosphate. These water-soluble products release Ca^{2+} from endoplasmic reticulum and activate protein kinases C (PKC, mainly the β2 isoform), activators of the ERK-MAPK pathway. CGP41251 (a staurosporine derivative), inhibits several PKC isoforms, including PKCβ2, and shows a strong inhibitory effect on both retinal and choroidal neovascularization [3]. Calcium signaling is necessary for VEGF-induced proliferation, since MAPK activation is not sufficient to induce endothelial proliferation in the absence of calcium release. This explains the antiangiogenic properties of carboxyamidotriazol, a cytostatic inhibitor of non-voltage-operated calcium channels [4]. Calcium-dependent synthesis of nitric oxide (NO) may be the link between calcium signaling and endothelial proliferation [5].

Phosphorylated VEGFR2 can also recruit and activate adaptor proteins, such as Shc or Skc, which induce the coupling of Grb2 to the nucleotide exchange factor Sos. In this way Sos activates the small GTP-ase Ras, a main activator of Raf. This serine-threonine kinase, when activated, triggers a phosphorylation cascade that succesively activates MEK1/2 and the ERK-MAP kinases, which

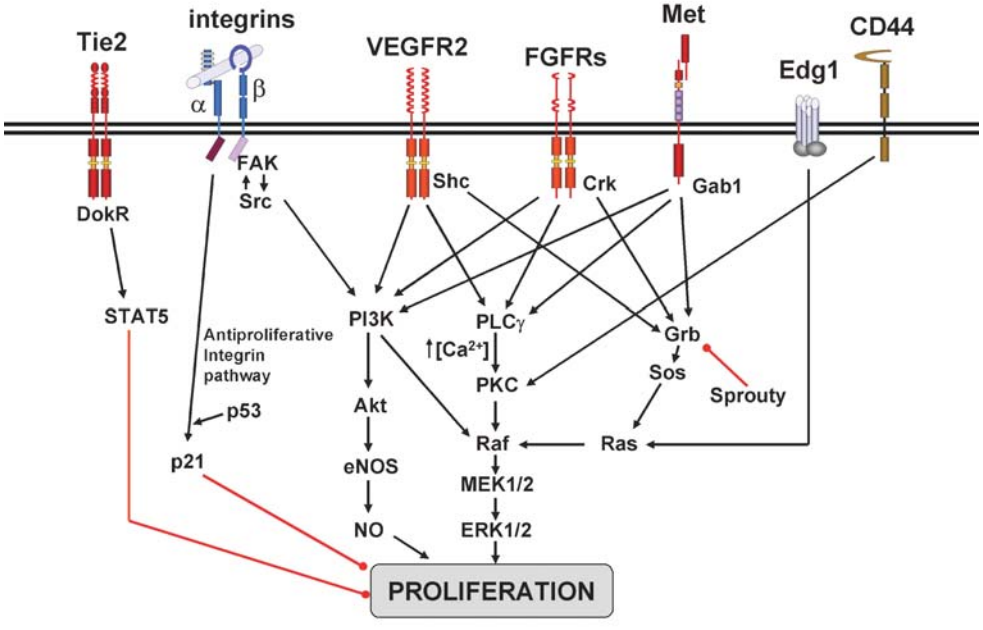

Figure 1. Main signaling pathways involved in proliferation of angiogenic endothelial cells.

translocate to the nucleus and activate transcription factors involved in cell proliferation, such as Elk-1, c-Myc, c-Fos, Ets-1, SRF and so on [6, 7].

Experimental evidence demonstrates the importance of this mitogenic pathway in angiogenesis. A variety of inhibitors of the kinase insert domain of VEGFR2, including SU-5416, SU-6668, PTK-787, midostaurin, ZD4190 and ZD6474, have progressed to the clinical testing stage [8]. Therapeutic inhibition of retinal neovascularization has been obtained with oligodeoxynucleotides targeting Raf [9], while inactivation of ERK1/2 and MEK reduces endothelial proliferation induced by VEGF [10]. The implication of Ras seems to be essential for proliferation of endothelial cells, since inhibitors of Ras block the ERK-MAPK pathway, but not other non-mitogenic MAPK pathways such as that mediated by p38 [11]. Resveratrol, a polyphenolic antiangiogenic compound found in red wine and grapes, has been shown to inhibit MAPK phosphorylation induced by growth factors [12].

The mitogenic response induced by VEGF is amplified by NO, probably by potentiating ERK activation. In fact, inhibitors of the production of NO greatly decrease the mitogenic effects of VEGF [13, 14]. VEGF increases the activity of eNOS, the endothelial-specific isoform of NO-synthase, through a pathway mediated by Src [15]. These are two results stressing the important role that NO plays in angiogenesis [16]. NO can trigger and modulate cellular responses (involving proliferation, migration and apoptosis) mediated by activation of soluble guanylate cyclase, with production of cyclic GMP, or by redox-sensitive regulation of transcription factors and enzymes [17].

VEGFR1 biosignaling is still poorly known, and contradictory results have been obtained in different systems [18]. Some evidence suggests that VEGFR1 does not induce tyrosine phosphorylation, proliferation or migration in response to VEGF [19, 20]. Although deficiency in VEGFR1 is lethal due to vascular malformations, when the deficiency affects only the intracellular domain of the receptor, the homozygous mice are viable and fertile [21]. Swapping of the intercellular domains between VEGFR1 and VEGFR2 has shown that a small juxtamembranal sequency of the former inhibits VEGFR2 signal transduction. All these data suggest that VEGFR1 does not transduce VEGF signals, being probably a negative regulator of VEGFR2 [22].

FGF2 receptors

Another main pathway leading to proliferation of endothelial cells is initiated by the dimerization and autophosphorylation of fibroblast growth factor receptors (particularly FGFR1) after FGF2 binding. This signal also promotes activation of the ERK-MAPK pathway, either through activation of PLC-γ/PKC [23] or by recruitment of the adaptor protein Crk, which induces Grb2/Sos coupling and activation of Ras [24]. The FGF2 mitogenic pathway can also be initiated by other adaptor proteins [25]. The importance of this signalization route can be demonstrated by experiences of pharmacological inactivation of some of their elements. For example, inhibition of MEK results in inhibition of FGF2-induced angiogenesis [26].

Physiological inhibitors of the tyrosine-kinase receptor/ERK-MAPK pathway have also been described. Sprouty and sprouty-related proteins are inhibitors of this signaling pathway, and they play a key role in lung branching morphogenesis and the development of other tissues. In endothelial cells, upon FGFR and VEGFR activation, sproutys translocate to the plasma membrane, become phosphorylated and bind to the adaptor protein Grb2, avoiding the recruitment of the Grb2/Sos complex, thus blocking the Ras-MAPK pathway and inhibiting cell proliferation [27, 28]. The p38 MAP kinase pathway, which is stimulated by FGF2, also negatively regulates endothelial cell proliferation in FGF2-induced angiogenesis [29].

HGF receptor

Hepatocyte growth factor (HGF) receptor (Met) is another tyrosine-kinase receptor that can induce endothelial cell proliferation and angiogenesis. The scaffolding adaptor Gab1 is the most crucial substrate for Met signaling. Phosphorylated Gab1 binds signal-relay molecules, such as Shp2, Grb2, PI3K, phospholipase C and Crk [30]. ERK-MAPK is involved in this mitogenic pathway, as demonstrated by specific inhibitors [31].

Angiostatin, an antiangiogenic agent derived from proteolytic cleavage of plasminogen, has structural similarities to HGF. Angiostatin might block HGF-induced signaling in endothelial and smooth muscle cells through its inhibition of HGF-induced phosphorylation of Met, Akt and ERK1/2 [32].

Integrins

Endothelial cells express at least 11 integrins, namely combinations of β_1 with α_1, α_2, α_3, α_5, α_6, α_8, α_9 and α_v, as well as $\alpha_6\beta_4$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Among them, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ are upregulated during angiogenesis [33]. Integrins associate with growth factor receptors in lipid rafts of the cell membrane together with other signaling proteins. The interactions between these proteins lead to activation of a number of intracellular signaling pathways. In fact, an efficient MAPK activation is only possible when both growth factor receptors and integrins contribute. FGF2-induced angiogenesis in chick chorioallantoic membrane, for example, requires of two waves of ERK activation, induced by FGFR and $\alpha_v\beta_3$ integrin, respectively. Inhibition of the second wave blocks angiogenesis [34]. On the

other hand, blockade of $\alpha_v\beta_5$ integrin in the same model inhibits VEGF-induced activation of Ras and Raf [35].

A specially controversial question is that of the proliferative or antiproliferative signals mediated by binding of endothelial integrins to their ligands. Some integrins such as $\alpha_1\beta_1$ and $\alpha_2\beta_1$ support signal transduction and potentiate the mitogenic effect of VEGF. Their inhibition with blocking antibodies suppresses VEGF-induced angiogenesis [36].

The observation of a proliferation-promoting function of endothelial integrins would agree with the reported inhibition of angiogenesis induced by α_v antagonists. Antibodies directed against $\alpha_v\beta_3$ inhibit FGF2 and tumor necrosis factor α (TNFα)-induced angiogenesis, while antibodies targeting $\alpha_v\beta_5$ inhibit the VEGF angiogenic effect [37]. From these data, two independent pathways of endothelial activation mediated by α_v integrins seemed to be revealed. However, α_v integrin-deficient mice show no defect in embryonic angiogenesis, although this mutation is lethal due to hemorrhages and placental defects [38]. Mice deficient in β_3 and/or β_5 integrin are viable, even showing an increased angiogenic response [39, 40]. Binding of $\alpha_v\beta_3$ integrin to its substrate induces an increase in the Bcl-2:Bax ratio, producing an antiproliferative and antiapoptotic effect [41]. Moreover, α_v antibodies induce an increase of the level of the cell cycle inhibitor $p21^{waf1/cip1}$. Interestingly, the integrin antiproliferative pathway seems to require the product of the tumor suppressor gene *p53*, since α_v antagonists cause no effect in p53 null mice [42]. This antiproliferative pathway is probably the target of the antiangiogenic drug TNP-470, which blocks the cell cycle in endothelial cells. It has been shown that TNP-470 activates the p53 pathway and causes an accumulation of $p21^{waf1/cip1}$. TNP-470 has no effect on cells deficient in p53 or $p21^{waf1/cip1}$ [43, 44].

These data suggest that blocking peptides and anti-α_v blocking antibodies do not act as 'antagonists', but as ligands (i.e. agonists) of integrins by occupation of their active sites, thus explaining the antiproliferative and antiangiogenic effect of the supposed integrin 'blockade'. The phenotype of the α_v-deficient mice might be explained by functional compensation by other integrins such as $\alpha_5\beta_1$ that are essential for vascular development [45, 46].

Recent results have made the issue even more complex. Del-1 is a novel extracellular matrix protein that accumulates around angiogenic blood vessels. Occupation of $\alpha_v\beta_5$ integrin by Del-1 induces expression of the homeobox gene HoxD3 and $\alpha_v\beta_3$ integrin, promoting angiogenesis without growth factor signaling [47]. On the other hand, the interaction of $\alpha_v\beta_3$ integrin with the VEGFR2 receptor seems to be necessary for activation of FAK and p38 MAPK [48]. Thus, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins can be positive and negative regulators of angiogenesis, depending on the substrate and the cellular context [33, 49, 50].

$\alpha_v\beta_3$ integrin could affect all the steps of angiogenesis by its binding to members of the CCN family of extracellular matrix-associated signaling molecules such as Cyr61 and connective tissue growth factor (CTGF) [51]. Expression of these factors by endothelial cells is upregulated by FGF2 and VEGF. This $\alpha_v\beta_3$ integrin function is stressed by the vascular defects shown by mice deficient in these factors [52, 53].

The function of $\alpha_v\beta_3$ integrin as a main regulator of angiogenesis accounts for the antiangiogenic effects of Cilengitide (a cyclic peptide mimicking the arginine-glycine-aspartic acid (RGD) recognition peptidic domain common to α_v integrin ligands) and Vitaxin (a humanized antibody acting as an α_v binding antagonist). These substances have shown a low toxicity in phase I [54] and are currently in phase II trials.

S1P receptor

The phospholipid sphingosine-1-phosphate (S1P) stimulates endothelial proliferation most probably through binding to the G-protein-coupled receptor EDG1 (endothelial differentiation gene-1, also called S1P1). This response is blocked by pertussis toxin. S1P induces Ras activation [55] and ERK and p38 MAPK phosphorylation, although endothelial proliferation is blocked only by ERK-MAPK inhibitors [56].

Hyaluronate receptor

CD44, a hyaluronate-binding protein which is stimulated by oligosaccharides resulting from hyaluronate degradation, is another receptor that can activate the ERK-MAPK proliferative pathway. Activated CD44 induces PKC translocation to the cell membrane and activation of the ERK-MAPK pathway in endothelial and tumor cells [57–59].

The STAT pathway

In endothelial cells, there are also MAPK-independent proliferative/antiproliferative signaling pathways. For example, VEGF and Tie receptors activate the signal transducers and activation of transcription proteins STAT3 and 5, which are latent cytoplasmic transcription factors that dimerize when phosphorylated and translocate to the nucleus, where they transactivate a number of genes [60]. In the case of the Tie2 receptor, this activation is mediated by the specific adaptor protein Dok-R [61]. Phosphatidyl-inositol-3-kinase (PI3K) activity is required for Tie2/Dok-R association, since the latter bears a pleckstrin homology domain [62]. On the other hand, increased expression of the cell cycle inhibitor $p21^{waf1/cip1}$ mediated by STAT5 and induced by VEGFR1, Tie1 and Tie2 signaling has been reported [60].

Janus kinase (JAK), a main activator of the STAT proteins that can be recruited by some membrane receptors, could

also play a significant role in angiogenesis. For example, endothelial cells express the granulocyte-macrophage colony stimulating factor (GM-CSF) receptor, which is capable of recruiting and activating JAK-2, leading to phosphorylation of STAT3 but not STAT5 [63]. This observation may account for the angiogenic properties of GM-CSF.

Finally, a novel STAT-related signaling pathway based on eicosanoids was recently reported. Eicosanoids are autoacoids derived from arachidonic acid metabolism. One of them, 5(S)-hydroxyeicosatetraenoic acid (5(S)-HETE) stimulates JAK-2, STAT-1, STAT-3, PI3K, Akt and DNA synthesis in endothelial cells [64]. Since 12(S)-HETE also stimulates angiogenesis and activates, in carcinoma cells, the Raf/MEK/ERK-MAPK and other angiogenesis-related pathways [65, 66], arachidonic acid metabolites might be important in the establishment of cross-talking between different angiogenic pathways (JAK/STATs, ERK-MAPK and PI3K/Akt).

Transduction of survival signals

Two signaling pathways promote survival in endothelial cells

Apoptosis is a normal process of programmed cell death involved in morphogenesis, vascular remodeling and elimination of neurons or cells from the immune system. Apoptosis seems to be essential in initiation of the angiogenic process [67]. Caspases, a family of cysteine proteases, regulate apoptosis. Initiator caspases (caspases 2, 8, 9, 10, 11 and 12) are activated by pro-apoptotic signals such as DNA damage, cytochrome c release and activation of death receptors. These initiator caspases cleave and activate effector caspases (caspases 3, 6 and 7), which in turn cleave cellular proteins executing apoptosis. The caspase cascade is controlled by apoptosis promoting and inhibitory factors [68].

Angiogenic cells degrading the extracellular matrix should reinforce the mechanisms of apoptosis inhibition to avoid the risk of anoikis, i.e. apoptosis induced by lack of adhesion to the substrate [69]. For that reason, signals inducing endothelial cell migration must also promote cell survival. It is not surprising that inhibition of these signals prevents invasion and triggers apoptosis in activated endothelium. Unligated integrins, for example, promote caspase 8 activation [70]. Endothelial cells repress their apoptogenic program through two main signaling pathways initiated from integrin-mediated attachment to the extracellular matrix and from survival factors such as VEGF and FGF2 (fig. 2). Akt is a convergence point for both pathways [71]. NO also plays a role as a survival factor potentiating the effects of VEGF [13, 14]. In fact, inhibition of eNOS increases apoptosis in endothelial cells [72, 73].

Endothelial cell apoptosis can also be promoted by TGFβ [74–76] and TNFα [77, 78]. The TNF receptor (TNFR1) is a membrane protein that binds TRADD (TNFR-associated death domain) as an adaptor that recruits signaling complexes leading to activation of caspase 8.

The PI3K/Akt pathway

The main endothelial pathway that transduces the survival signals initiated by VEGF binding to VEGFR2 is mediated by PI3K, the main activator of Akt. PI3K is a main effector of tyrosine-kinase receptors [79, 80] that induces the phosphorylation of phosphoinositides, lig-

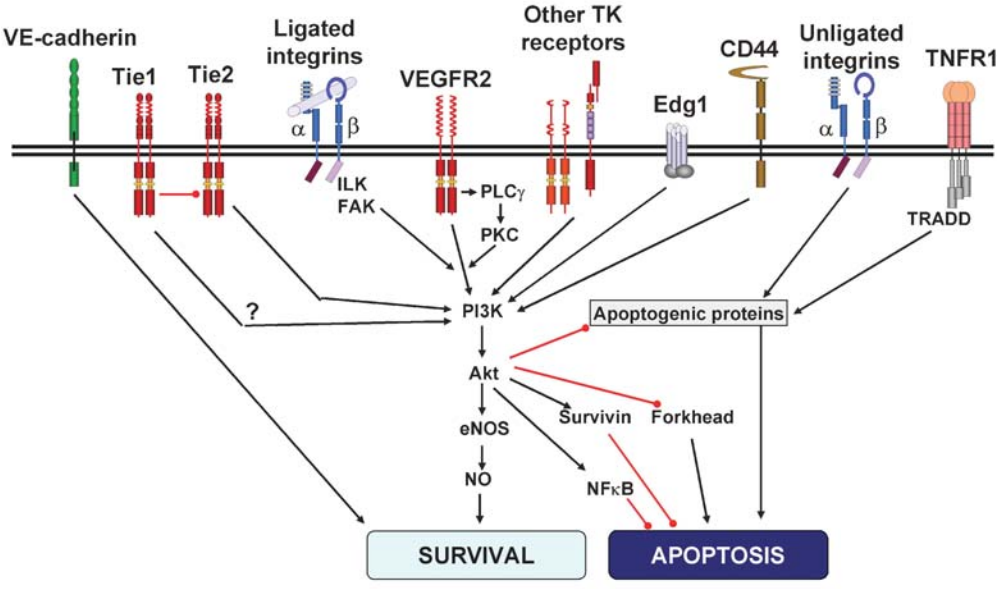

Figure 2. Main signaling pathways involved in survival of angiogenic endothelial cells.

ands of proteins with pleckstrin domains. In this way, essential molecules for survival and other cellular processes are recruited to the cell membrane and activated. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate to produce $PI(3,4,5)P_3$, a membrane recruitment signal enabling association between Akt and the constitutively active kinase PDK1 (3-phosphoinositide-dependent protein kinase). This association phosphorylates Akt, which comes back to the cytoplasm in an activated form [81].

There are three Akt genes in mammals (Akt-1,2,3, also called PKBα,β,γ). Akt-1 is highly expressed in endothelial cells, phosphorylating (and, therefore, inhibiting) apoptogenic proteins such as Bad, Bax and caspase-9 [71, 82, 83]. At the same time, it increases the levels of the antiapoptotic proteins A1 and Bcl-2 [71]. In tumor cells, Akt phosphorylates and inhibits other proteins such as the apoptogenic transcription factor Forkhead and the glycogen-synthase kinase GSK3, a main regulator of β-catenin degradation [84]. Akt also activates IKK, leading to dissociation of the NFκB/IκB complex and nuclear translocation of the transcription factor NFκB, a promoter of antiapoptotic molecules such as the inhibitor of apoptosis proteins (IAPs) [85]. Akt also stimulates NOS in a Hsp90-dependent way, and the NO produced potentiates caspase inactivation [86, 87]. Finally, Akt also stimulates the expression of survivin, an anti-apoptotic protein [88, 89] with cell cycle-modulating effects [90].

PKC inhibitors block PI3K activation by VEGFR2 [91]. This activation is also dependent on integrin ligation [92] and FAK, since overexpression of a dominant-negative form of FAK decreases PI3K activation in response to VEGF [93].

Other tyrosine kinase receptors (FGFRs, Tie2, insulin receptor, IGFR and Met) can also activate the PI3K pathway in endothelial cells. Insulin, for example, is a potent antiapoptotic molecule preventing TNFα-induced apoptosis in endothelial cells through activation of Akt, leading to inhibition of caspase-9 [83]. On the other hand, an antiapoptotic effect of HGF on endothelial cells has been reported to be mediated by activation of ERK1/2 and PI3K/Akt, independent of NFκB activation [94, 95].

The PI3K/Akt pathway can also be activated by integrin mediated adhesion to the extracellular matrix. Other PI3K-activating pathways are mediated by estrogens, corticosteroids and shear stress due to blood flow [87, 96], as well as by the hyaluronate receptor CD44 [57, 58], eicosanoids and S1P. The latter upregulates eNOS through the PI3K/Akt/eNOS pathway [97]. On the other hand, CD40-dependent activation of PI3K/Akt in endothelial cells seems to regulate all the steps of the angiogenic process [98]. CD40 is a potent immune mediator belonging to the TNFR family. The binding of CD40 with its ligand CD154 or with antibodies that mimic CD40 ligation induces production of cytokines, growth factors (including VEGF) and growth factor receptors in endothelial cells [99, 100].

Activation of Akt by VEGF signaling is dependent on integrin-mediated substrate adhesion [18, 101]. The integrin cytoplasmic domain is connected to the actin cytoskeletal microfilaments by adapter molecules (talin, vinculin, α-actinin). The integrin survival pathway of PI3K and Akt activation is mediated by kinases associated to these integrin-cytoskeletal connections. This is the case of tyrosine kinase FAK (focal adhesion kinase) and ILK (integrin-linked kinase) [102, 103]. We will further deal with FAK in the section devoted to endothelial cell migration. ILK is a serine-threonine kinase that has been proposed as a key regulator of the convergence point between survival factor and substrate adhesion pathways [104].

Survival signals transduced through Tie receptors

Tie2 is the endothelial-specific angiopoietin-1 tyrosine kinase receptor. Early reports pointed to mitogenic functions for Tie2 [105, 106]. However, other studies have shown that the Ang-1 signal transduced by Tie2 is mainly related to endothelial cell survival [107–109]. In fact, activation of the Tie2-specific adaptor protein Dok-R leads to upregulation of the cell cycle inhibitor $p21^{waf1/cip1}$ mediated by STAT5 [60]. The Tie2 signal is also transduced through activation of the PI3K/Akt pathway, and this signal induces a decrease in the levels of Bad and caspase-9, -7 and -3, and an increase in survivin and NO. Inhibition of the PI3K pathway suppresses these effects of Tie2 activation [106, 110–112].

In contrast, Tie1 ligands are still unknown. Studies with chimeric Tie1 receptors have shown PI3K and Akt activation and apoptosis inhibition [113]. The extracellular portion of this receptor is proteolytically released in response to PKC activation or by stimulation with VEGF or inflammatory cytokines [114–116]. The intracellular domains of Tie1 are internalized and associate to a number of proteins, among others, the tyrosine phosphatase Shp2, a negative regulator of Tie2 [117]. This observation, together with the evidence of a direct union of Tie1 to Tie2 to form heterodimers, suggests that Tie1 might modulate or even suppress the signals transduced by Tie2 [117]. Tie1 also shows binding sites for Akt activators, suggesting a role in transduction of survival signals, accordingly with the lethal phenotype of Tie1-deficient mice [118].

VE-cadherin and endothelial cell survival

The function of the calcium-dependent homophilic adhesion molecule VE-cadherin (also called cadherin-5) in angiogenesis is still poorly known, but deficiency in VE-cadherin in mice is lethal, impairs survival signal transduction and leads to endothelial apoptosis [119]. In fact, VE-cadherin is spatially associated to VEGFR2 receptors [120], which induce a Src-dependent phosphorylation of VE-cadherin. This process is inhibited by resveratrol

[121]. Antibodies against the extracellular portion of VE-cadherin inhibit the formation of capillary structures in vitro and induce apoptosis [122].

Transduction of migration signals

Focal adhesion-associated proteins

Focal adhesions are specific areas of the cell surface where stress fibers of polymerized actin, substrate adhesion molecules (basically integrins) and adaptor proteins gather. Focal adhesions are not only mechanically involved in cell attachment, since they are the origin of signals that activate or inhibit cellular processes such as proliferation, survival and migration of endothelial cells (fig. 3). For example, shear stress induces endothelial migration through a signal initiated by $\alpha_5\beta_1$ integrin binding to fibronectin and involving the adaptor protein Shc, PI3K and ERK-MAPK1/2 [123]. Substrate adhesion properties of integrins can be used to modulate migration. The Tie receptor ligands angiopoietin-1 and, to a lesser extent, ang-2 are substrates of α_5 integrins, playing a role in endothelial cell migration [124]. A liver-specific secreted factor of the angiopoietin family, AngPTL3, does not bind Tie2, but it is substrate for $\alpha_v\beta_3$ integrin and promotes adhesion, migration and phosphorylation of Akt, MAPK and FAK [125].

VEGF stimulation of endothelial cells induces accumulation of stress fibers and formation of focal adhesions. This response, mediated by VEGFR2, involves phosphorylation of p38-MAPK, which in turns activates MAPKAPK2/3 (MAPK-activated protein kinase 2 and 3), leading to phosphorylation of Hsp27, a modulator of F-actin polymerization [126]. On the other hand, VEGFR2 activates FAK. This non-receptor cytoplasmic tyrosine kinase regulates the organization of the cytoskeleton and activates diverse signaling pathways involved in the localized adhesion of the cell surface and in cell motility [127]. Activated FAK recruits SH2-containing proteins such as Src, another cytoplasmic tyrosine kinase, which phosphorylates FAK in additional sites, inducing the association of signaling molecules (such as the Ras-activating protein Sos, PI3K, p130Cas and paxillin) to focal adhesions. In this way FAK activates ERK-MAPK and the PI3K/Akt pathways. NO modulates the phosphorylation of many of these proteins, including FAK [128], and plays an important role in migration [129].

Src seems to be involved in angiogenesis, since a dominant negative form inhibiting activity of the normal protein in endothelial cells also inhibits VEGF-induced angiogenesis [26]. The VEGFR2/Src signaling pathway has been suggested to be involved in the increase in vascular permeability mediated by NO [15]. In fact, Src-deficient mice show normal angiogenesis, but a reduced vascular permeability in response to VEGF [26].

Small GTPases and migration

The cytoskeletal changes associated to the formation of focal adhesions involve the development of stress fibers, lamellipodia and filopodia. These changes are mainly regulated by the members of the Ras superfamily of small

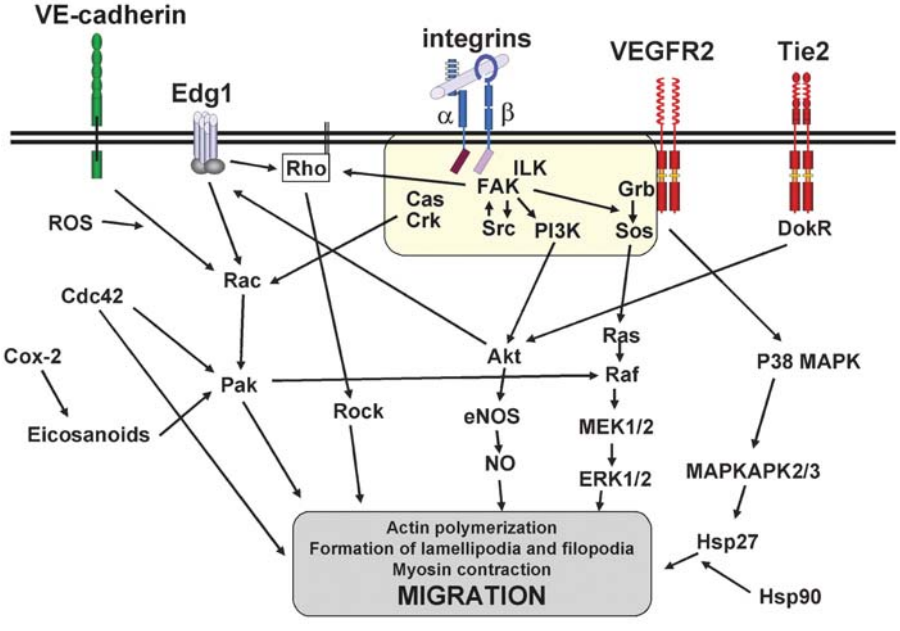

Figure 3. Main signaling pathways involved in migration of angiogenic endothelial cells. The yellow box represents the signaling complex recruited by integrins.

GTPases RhoA, Rac and Cdc42. They control the cytoskeletal dynamics and also the cadherin function in endothelium [130]. Rho and Rac are essential in the development of endothelial motility induced by VEGF [131, 132] and also control the mechanisms of polarization and migration in response to blood flow [133]. As other small GTPases, Rho, Rac and Cdc42 are tightly regulated by guanine nucleotide exchange factors (GEFs) [134].

RhoA GTPase is a key element in the induction of endothelial cell migration. Its activation requires localization to the cell membrane induced by isoprenylation of the C-terminal domain [135]. Rho promotes focal complex formation, cytoskeletal organization (by increasing actin polymerization) and myosin-mediated contraction of stress fibers [136]. Some of these effects are transduced through Rock (Rho-associated coiled-coil containing protein kinase). Specific inhibition of Rho and Rock inhibits angiogenesis in vivo and in vitro [137] and induces apoptosis in cultured endothelial cells [138]. Inhibition of Rock also prevents VEGF-induced endothelial cell migration and fibrin matrix invasion, showing the essential role that Rho proteins play in VEGF-induced angiogenesis [139]. In fact, it has been proposed that the reported antiangiogenic effect of statins (inhibitors of the cholesterol synthesis enzyme hydroxymethylglutaryl coenzyme A reductase) is mediated by inhibition of the geranylgeranylation and membrane translocation of RhoA [140]. Statins also inhibit proliferation by an increment of $p21^{waf1/cip1}$ that is related to inactivation of RhoA, depolymerization of actin F and inhibition of FAK and Akt [141].

Rac drives actin polymerization, formation of lamellipodia and recruitment of $\alpha_v\beta_3$ integrin to these structures [142]. Rac activates the ERK-MAPK pathway, but most of its effects are transduced through activation of the protein kinase Pak, as we will describe below. Inhibition of Rac induces cell junction disassembly [143], suggesting a role in modulation of intercellular adhesion. As Rac antagonists block formation of endothelial fenestrations, but only partially inhibits angiogenesis, Rac has been considered a key component for the increase of vascular permeability mediated by VEGF [144] and for endothelial cell migration [132, 145]. Rac may also be involved in transduction of signals initiated from VE-cadherin. It has been shown that transfection of VE-cadherin in VE-cadherin-deficient endothelial cells increases the number of focal adhesions and promotes actin reorganization and activation of Rac [146]. It is possible that another endothelial adhesion molecule (VCAM-1), which is upregulated in the endothelium during angiogenesis, also activates Rac through a still unknown mechanism [130].

Cdc42 promotes formation of actin microspikes, structural components of filopodia and cooperates with Rac in Pak activation [136] and in the formation of the vascular lumen in cultured endothelial cells [147]. In these cells, Cdc42 activation mediates reorientation of the microtubule organizing center in response to fluid shear stress [148] and regulates restoration of the adherens junctions after a disruption of the endothelial barrier [149].

Rac and Cdc42 activate the p21-activated serine-threonine kinase Pak, a key effector of the cytoskeletal reorganization in which several signal pathways such as Tie2/Dok-R, PI3K/Akt and tyrosine-kinase receptors/Ras converge. Pak controls cell shape and motility by regulation of actin polymerization, lamellipodia and filopodia outgrowth and myosin contraction. Pak also activates the ERK-MAPK and JNK pathways. Activation of Pak1 induces motility in endothelial cells [150], and its inhibition blocks angiogenesis [145, 151].

RhoA and Rac also induce the expression of cyclooxigenase-2 (Cox-2) at least in NIH3T3 cells [152]. Cox-2, in turn, synthesizes eicosanoids that stimulate endothelial cell migration and are angiogenic in vivo. Eicosanoids improve endothelial cell survival by increase of Bcl-2 and/or activation of the PI3K/Akt pathway [153]. A main Cox-2 product, prostaglandin E2 (PGE_2), promotes $\alpha_v\beta_3$-dependent endothelial cell adhesion, Rac activation and cell spreading. This effect is mediated by cyclic AMP (cAMP) and PKA [154], in a way essential for migration, at least in carcinoma cells [155]. These observations implicate prostaglandins in angiogenesis and provide a rationale for the observation that Cox-2 inhibitors are antiangiogenic [145, 156]. Recent studies have shown that non-steroid anti-inflammatory drugs (NSAIDs) also exert their antiangiogenic effects through Cox-2 independent pathways, involving PKG activation, NFκB inhibition and Bcl-XL downregulation [153].

Modulators of endothelial cell migration

Endostatin, a C-terminal fragment of collagen XVIII, is a potent inhibitor of endothelial migration and angiogenesis [157]. In normal conditions, endostatin promotes FAK and paxillin phosphorylation, but reduces the number of focal adhesions in FGF2-stimulated endothelial cells [158]. Endostatin binds to $\alpha_5\beta_1$ integrin, leading to focal adhesion and actin stress fiber disassembly mediated by Src and dependent on tyrosyl phosphatase [159]. Interestingly, the inhibition of endothelial cell migration occurs without interference with pathways mediated by PLCγ, Akt, MAPK, Rac or Pak [160], revealing a specific integrin signaling pathway involved in migration and mediated by Src. However, endostatin blocks VEGF binding, VEGFR2 phosphorylation and ERK, p38 MAPK and FAK activation in HUVEC, suggesting a direct interaction between endostatin and VEGFR2 [161].

Reactive oxygen species (ROS) also play a role in endothelial migration. The Rac-induced modulation of VE-cadherin adhesion described above is dependent on ROS,

and in fact, endothelial migration is blocked with ROS inhibitors [162]. Other reports have shown inhibition of endothelial cell migration associated with upregulated ROS production, induced for example by ligation of the CD40 molecule [163]. ROS potentiate reparative processes, modulate signal transduction and might be necessary for VEGFR2 biosignaling [164]. This explains the antiangiogenic effects of antioxidants and free-radical scavengers [165].

S1P also promotes endothelial cell migration [97, 166], mediated by the EDG1/S1P1 receptor [167] and leading to activation of the PI3K/Akt/eNOS pathway [97, 168]. EDG1/S1P1 is activated by phosphorylation mediated by Akt [169], and this event is essential for activation of Rac. In this way, EDG1/S1P1 is involved in the the assembly of the actin cortex and in endothelial chemotaxis. In fact, fibroblasts deficient for EDG1/S1P1 cannot activate Rac [170]. EDG1/S1P1 activation also occurs in response to PDGF, and the cytoskeletal rearrangement, lamellipodia extension and cell motility induced by PDGF are inhibited in EDG1-null fibroblasts. EDG1/S1P1 has been suggested as an integrator of PDGFR and Src/FAK signaling pathways. EDG1/S1P1 deficiency is lethal in mouse embryos, probably due to a defective recruitment of pericytes and smooth muscle cells [171].

Tie2 is also involved in endothelial cell migration through the PI3K pathway [110], FAK activation [111] and the Dok-R pathway. Tie-2 signals activate RhoA and Rac1 in a PI3K-dependent way [172]. Through these pathways, angiopoietin-1, the ligand of Tie2, induces activation of Pak and cell motility.

Ephrins are growth factors, ligands of the Eph receptors [173, 174], which have been shown to be endothelial cell migration inducers [175]. Ephrins are transmembrane proteins, and they can also transduce 'outside-in' signals. In fact, a soluble form of the receptor EphB1 induces migration and $\alpha_v\beta_3/\alpha_5\beta_1$-mediated extracellular matrix attachment in endothelial cells through ephrin-B1. This signal induces ERK1/2-independent JNK phosphorylation [176]. Endothelial EphB4 activation increases proliferation, probably through a PI3K/Akt/eNOS/PKG/MAPK cascade and migration through a Src-dependent pathway [177].

RHAMM, the receptor for motility mediated by hyaluronic acid, is also involved in endothelial cell migration. In fact, anti-RHAMM but not anti-CD44 antibodies block endothelial cell migration on Matrigel and inhibit FGF2-induced angiogenesis [58].

Transduction of signals leading to extracellular matrix degradation

Migration of endothelial cells is finally achieved by controlled cell adhesion, cytoskeletal reorganization and localized degradation of the extracellular matrix. In fact, the degradation of the basal lamina of the endothelium is one of the earliest events in angiogenesis. Degradation of the extracellular matrix is performed by a proteolytic arsenal exquisitely regulated and mainly composed of urokinase plasminogen activator (u-PA) and its receptor (u-PAR), matrix metalloproteinases (MMPs) and membrane-type metalloproteinases (MT1-MMPs). These proteases are inhibited by plasminogen activator inhibitors (PAIs) and tissue inhibitors of metalloproteases (TIMPs). Extracellular matrix degradation is the result of an imbalance between these activators and inhibitors.

Angiogenic signals induce an upregulation and activation of the proteolytic enzymes in endothelial cells (fig. 4). However, their transduction pathways are not well known.

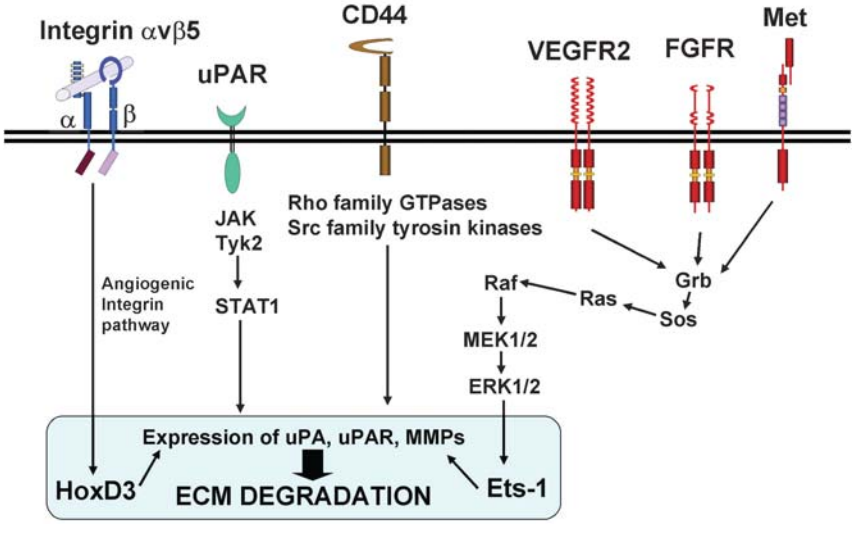

Figure 4. Main signaling pathways involved in extracellular matrix degradation induced by angiogenic endothelial cells.

The zinc-finger transcription factor Ets-1 seems to play a key role in activation of the proteolytic system by transactivation of the promoters of many of these enzymes. In fact, Ets-1 antisense oligonucleotides inhibit VEGF-induced endothelial cell migration [178].

Expression of Ets-1 is upregulated by binding of VEGFR2, FGFRs and Met to their ligands [179], and the Ras-MAPK pathway is probably involved in this induction since the *Ets-1* gene is a target of the ERK-MAPK1/2 [180, 181]. A constitutively active form of Met, Tpr-Met, activates the *uPA* gene promoter through a Grb2/Sos/Ras/ERK cascade [182]. The Ras/ERK pathway is also involved in upregulation of MMP-9, this being a necessary, but not sufficient, requirement for angiogenesis [183].

Another transcription factor, HoxD3, that is expressed in activated endothelium [184] might be involved in proteolytic activation. Antisense oligonucleotides targeting HoxD3 block the ability of FGF2 to upregulate uPA [185].

In addition to its role in the proteolytic cascade, u-PAR may be the origin of a signal transduction pathway mediated by JAK1 and Tyk2 (another member of the JAK family). This association functionally transforms u-PAR in a tyrosine kinase receptor able to transduce signals via STAT proteins. In this way, binding of uPA to its receptor causes activation of STAT1 and translocation to the nucleus in endothelial cells [186].

Other signaling pathways leading to upregulation of proteolytic enzymes might be mediated by CD44. Stimulation of this receptor by hyaluronic acid fragments leads to upregulation of uPA and uPAR in chondrosarcoma cells [59]. CD44 lacks kinase activity, but it associates with intracellular signaling components such as Rho-family GTPases and members of the Src family [187]. Finally, a membrane-associated metalloprotease called aminopeptidase N/CD13 has recently reported to be involved in the Ras/MAPK and PI3K pathways. Inhibition of these signaling pathways, which normally blocks angiogenesis, can be rescued by forced expression of CD13, which restores angiogenic potential in endothelial cells [188].

Transduction of signals leading to differentiation and morphogenesis

After proliferation and migration, endothelial cells should be able to recover quiescence, produce a new basal lamina, recruit perivascular cells and form lumenized tubules. The signaling mechanisms controlling these steps of angiogenesis are less known than the transduction pathways leading to proliferation, survival and migration (fig. 5). However, the critical involvement of EDG1/S1P1, Rho and Rac has been demonstrated [162, 189]. EDG1/S1P1 binds sphingosine-1-phosphate and, with a lesser affinity, lysophosphatidic acid [190]. Forced expression of EDG1/S1P1 in fibroblasts induces them to join and form tubules in culture [191]. EDG1/S1P1 signals are transduced through the protein $G_{i\alpha}$ and activate the ERK, phospholipase-2 and Rho pathways, inducing assembly of α_v integrins in areas of focal adhesion and the formation of intercellular junctions [192, 193].

Ephrins and Eph receptors are critically involved in angiogenesis, as demonstrated by the lethal phenotypes of the mice deficient for ephrin-B2, the receptor EphB4 and

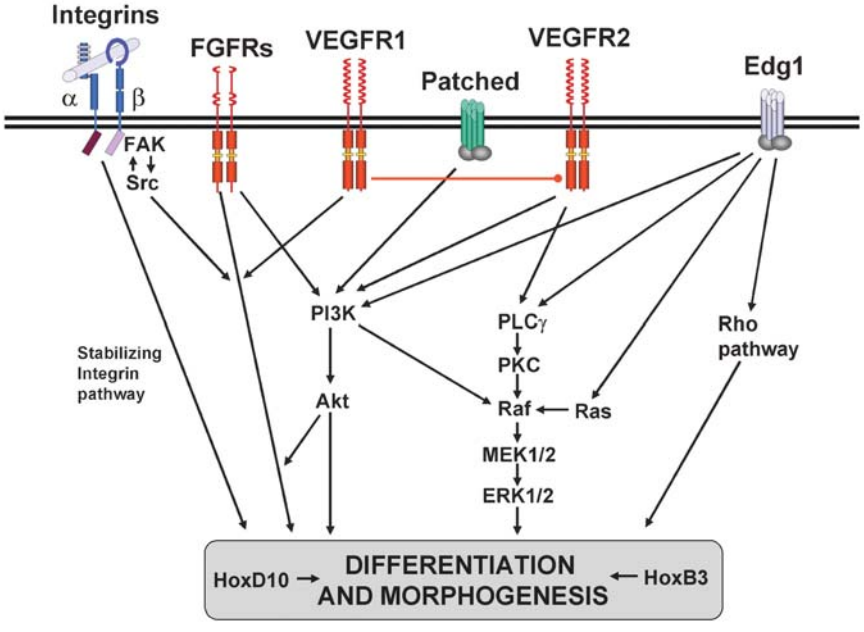

Figure 5. Main signaling pathways involved in differentiation and morphogenesis of angiogenic endothelial cells.

the double mutant EphB2/EphB3 [194]. The lethality is due to defects in vascular remodeling similar to those exhibited by Ang1- or Tie2-deficient mice, thus suggesting a role for ephrins in morphogenesis. On the other hand, ephrin-A1, whose production in endothelium is stimulated by TNFα, is angiogenic [195]. This indirectly angiogenic effect of TNFα is mediated through the TNF receptors 1 and 2, and transduced through the p38 MAPK and JNK pathways, but not through the ERK-MAPK pathway [196].

EphA signals seem to be necessary for VEGF-induced angiogenesis, since the blockade of this receptor inhibits ephrin-A1 and VEGF-induced migration in endothelial cells [197]. EphA2 receptors are overexpressed in tumor endothelium, and a soluble form of this receptor inhibits tumor angiogenesis [198]. In contrast, activation of EphA receptors attenuates the Ras/MAPK pathway and inhibits endothelial proliferation [199].

The ephrin/Eph system is involved in axon guidance, and this is not the only relationship between neural pathfinding and capillary morphogenesis. Slit is a secreted protein that acts as chemorepellent of axons through Roundabout (Robo) receptors. However, Slit2, a protein expressed by many tumors, is attractive for endothelial cells and promotes tube formation through a pathway initiated by the endothelial receptor Robo1 and involving PI3K [200]. An endothelial-specific Robo receptor, Magic Roundabout, expressed at sites of active angiogenesis, has also been reported [201].

Besides its mitogenic effects, FGF2 also plays a morphogenetic role and stimulates tube formation in cultured endothelial cells. This function apparently requires of VEGFR1 signaling and depends on Akt [202] and Src-family members [25, 203].

The Notch signaling system seems to modulate the migration and morphogenetic program of the endothelium during angiogenesis. Jagged is a transmembrane protein that acts as a ligand of the Notch receptor, which is involved in signaling mechanisms through direct cell-cell contact. The adhesion of endothelial cells to fibrin increases the expression of Jagged, limiting the response to FGF2 [204]. Notch1 and its ligand Dll4 are induced in arterial endothelium by VEGF but not by FGF2. This induction is mediated by VEGFR2/PI3K/Akt, but not by the MAPK or Src pathways. Expression of Notch1 in endothelial cells stabilizes the network of vessels in vitro [205]. On the other hand, the forced expression of endothelial-specific Notch4 in epithelial cells inhibits the formation and branching of tubules [206, 207]. Expression of constitutively active Notch4 inhibits endothelial sprouting and VEGF-induced angiogenesis in the chick chorioallantoic membrane. This effect is partially mediated by promoting β1-integrin adhesion to the extracellular matrix [208]. This observation may explain the lethal phenotype and the vascular malformations provoked by the expression of Notch4 under control of the VEGFR2 promoter [209].

Another pathway that may be involved in morphogenesis is that mediated by Sonic Hedgehog (Shh) and its receptor Patched. Treatment of HUVEC with Shh results in capillary morphogenesis, an effect blocked with pertussis toxin and PI3K inhibitors [210]. It was already known that Shh is an indirect angiogenic factor, stimulating the production of VEGF and angiopoietins by mesenchymal cells [211].

The transcriptional control of the morphogenetic program is poorly known. Hox genes, and particularly HoxB3 and HoxD10, have been related with this control [212, 213]. HoxD10 shows a higher expression in quiescent endothelial cells, and it has been proposed to control the non-angiogenic phenotype [214]. It is important to remark that VEGF, but not FGF2, modulates expression of 8 of the 10 HoxB genes expressed by HUVEC [215].

Other signaling systems and signaling modulators involved in angiogenesis

Wnts, Frizzled and β-catenin

The Wnt signaling system, constituted of transmembrane receptors Frizzled and several Wnt ligand proteins [214], has been associated with angiogenesis. A soluble Frizzled form (FrzA), able to interfere with Wnt signaling, is angiogenic and stimulates endothelial cells by a pathway independent of VEGF, FGF2, angiopoietin-1 and Akt activation, but involving downregulation of GSK3 [216], as happens in the canonical Wnt signaling pathway. On the other hand, mutations in the Wnt receptor Fzd4 have been associated with human angiogenic vitreoretinopathy [217], and the knockout of Fzd5 is lethal in mice by defects in yolk sac angiogenesis, with reduced endothelial cell proliferation [218]. Wnt2-null mice also show vascular abnormalities [219]. All these observations suggest that Wnt/Fzd/β-catenin signaling might be involved in at least some types of angiogenesis. In fact, endostatin inhibits Wnt signaling and stimulates degradation of β-catenin in a GSK-3 independent way [157, 220].

The TGFβ pathway

Growth factors of the TGFβ superfamily transduce their very diverse physiological responses through serine-threonine kinase receptors [221]. Several types of receptors have been described, belonging to two main groups, type I and type II. These receptors form oligomeric signaling complexes upon ligand binding. Endoglin is an endothelial-specific type III TGFβ coreceptor. Endoglin is upregulated during angiogenesis, and increases the TGFβ binding to the type II receptor. Endoglin is also upregulated in tumor vessels, and its deficiency in mice is lethal

by cardiac defects and impaired yolk sac angiogenesis [222]. The availability of TGFβ in angiogenesis must be important, since MMP-2 and MMP-9 upregulation during angiogenesis proteolytically activates latent TGFβ precursor stored in the extracellular matrix [223].

TGFβR oligomerization is induced by ligand binding and activates an intracellular signaling cascade mediated by two pathways, one involving SMAD proteins [224] and the other related with pathways already described for tyrosine kinase receptors (PI3K/Akt and all the MAPK pathways). SMADs are a family of signal transducers that form hetero-oligomeric complexes able to translocate to the nucleus (always after the binding of SMAD4) and to modulate the activity of many transcription factors as well as transcriptional coactivators and corepresors. TGFβ receptor activation induces SMAD2 and 3 to bind SMAD4, forming these transcriptional modulators, while BMP receptors activate SMAD1, 5 and 8 in a similar way. SMAD6 and SMAD7 interfere with the activation of other SMADs. This complex network of interactions explains the pleiotropic effects of TGFβ in many biological processes, including angiogenesis. For example, TGF-βRII interacts with TGFβRI/ALK5 (activating SMAD2 and 3) but also with the BMP receptor ALK1 (activating SMAD1 and 5). Differential activation of these receptors in endothelial cells has been suggested to control the activation state of endothelium [225, 226]. On the other hand, TGFβ1 promotes survival in endothelial cells via an autocrine pathway, involving expression of TGFα and phosphorylation of EGFR, leading to activation of ERK and PI3K pathways [227]. However, endothelial cells from mice deficient in TGFβRI show increased proliferation, abnormal migration and decreased fibronectin production [228]. On the other hand, mutations in the gene coding for ALK1 have been related with the human vascular disease hemorrhagic telangiectasia, where endothelial cells show a defective ability to differentiate and recruit perivascular cells [229].

Other G-protein-coupled receptor pathways

Endothelin-1 is an angiogenic protein that acts as a ligand of the ET(B) protein G-coupled receptor. Endothelin modulates all the stages of the angiogenesis through signals initiated from this receptor [230].

The chemokine IL-8 is angiogenic in vitro and in vivo, and probably is a mediator of the macrophage-induced angiogenesis [231]. It induces proliferation and migration of endothelial cells in vitro through the G-protein-coupled receptors CXCR1 and 2. Expression of an other receptor from the same family, CXCR4, is increased in endothelial cells by FGF2, and it can mediate the motogenic response to this factor, since the CXCR4 ligand SDF-1 is a potent inducer of endothelial motility [232, 233]. In fact, mice deficient in CXCR4 or in SDF-1 show defective vascularization of the gut [234, 235]. The molecular pathways transducing the CXCR signals are not well characterized, although activation of FAK, paxillin, Crk, PI3K, MEK and ERK-MAPK1/2, as well as increased NF-κB activity in nuclear extracts, have been described [236].

NFκB in angiogenesis

The role of the NFκB transcription factor in angiogenesis is suggested by some observations. For example, adhesion of endothelial cells to fibronectin, mediated by $\alpha_5\beta_1$ integrin, activates NFκB through a pathway the depends on Ras, PI3K and Rho [237]. However, adhesion to the basement membrane protein laminin mediated by $\alpha_2\beta_1$ integrin has a limited effect. It has been also reported that the $\alpha_v\beta_3$ integrin-mediated survival signal generated by adhesion to vitronectin and osteopontin involves NFκB activation [238]. This signal is dependent on Ras and Src, but not on PI3K or MEK. On the other hand, the NFκB repressor IκB-2A expressed in endothelial cells inhibits FGF2-induced angiogenesis [237].

Hypoxia, ROS and angiogenesis

Hypoxia-induced expression of VEGF is the main mechanism triggering an angiogenic response in physiological conditions. This mechanism can be activated in virtually all cell types and is also functional in endothelial cells, thus providing another signaling pathway that contributes to angiogenesis. Hypoxia induces upregulation of endoglin in endothelial cells through a p38-dependent pathway [239]. Hypoxia also induces Met expression [240]. It has been reported that endothelial cells, which form capillary-like tubules when cultured on fibrin in presence of TNFα and VEGF or FGF2, become able to form these structures in hypoxic conditions with the only requirement being FGF2 [241]. A cycle of hypoxia/reoxigenation potentiates endothelial morphogenesis in vitro [242], and it has been suggested that ROS might increase the activity of diverse MAPK and the nuclear translocation of NF-κB, potentiating some signal transduction angiogenic pathways. In fact, oxygen peroxide stimulates angiogenesis and increases the expression of Ets1, a main transactivator of proteolytic enzymes [243].

Other cytokines

A novel signal transduction system involved in angiogenesis is mediated by pleiotrophin and midkine, two heparin-binding cytokines expressed by tumor cells that bind to the anaplastic lymphoma kinase (ALK) receptor and induce endothelial proliferation [244, 245]. ALK is a tyrosine kinase (unrelated to the TGFβ receptors with the same acronym) whose activation by pleiotrophin induces phosphorylation of Shc, PLCγ and PI3K [245]. Pleio-

trophin also binds and inactivates the receptor protein tyrosine phosphatase (RPTP) beta/zeta, resulting in increased phosphorylation of β-catenin [246]. Another angiogenic cytokine is TRANCE (TNF-related activation-induced cytokine), a ligand of RANK (receptor activator of NFκB) that activates ERK-MAPKs and FAK by a VEGF-independent pathway. This activation is blocked by Src inhibitors, PLC and calcium chelators [247].

Tissue factor is a transmembrane glycoprotein originally identified as a member of the coagulation cascade. Recently it was shown that tissue factor can act as a cytokine-like receptor which would be involved in angiogenesis [248].

Estrogens

Some substances can modulate angiogenesis through nuclear receptors. This is the case of estradiol and its metabolites, which have well-known effects on the vascular wall. Estrogens potentiate the effects of VEGF in tissues undergoing physiological angiogenesis during the female reproductive cycle (corpus luteum, endometrium) [249, 250]. It has been reported that ovariectomized rats show a smaller angiogenic response when injected with FGF2 containing Matrigel plugs, although this response is rescued by estrogen injection [251, 252]. The role played by estrogens must be very complex, since estradiol potentiates morphogenesis of endothelial cells cultured on Matrigel [252], but its metabolite 2-methoxyestradiol inhibits angiogenesis in animal models.

Concluding remarks

Despite the high specificity of the signaling system triggering an angiogenic response in endothelial cells, there are no specific signaling pathways or transcription factors regulating the entire angiogenic process or any of its stages [253]. That means that regulation of endothelial behavior during angiogenesis is the result of a very complex network of intracellular signaling systems that trigger, control and terminate the process. This is expected from a process leading to a radical transformation in a cell type that is extraordinarily stable in the absence of the angiogenic signal. Knowledge of this network may be at least as necessary and useful as the knowledge of the extracellular signals which initiate angiogenesis in order to find angiogenic inhibitors or activators with therapeutic utility. It is important to emphasize that several angiogenic signaling pathways, such as the MAP kinases or the PI3K/Akt cascades, are shared with those leading to tumor cell proliferation and invasion. It should not be surprising that future angiogenic inhibitors able to interfere with some intracellular signaling pathway also exhibit antitumoral activity. On the other hand, and probably more unexpectedly, some of the signaling mechanisms involved in angiogenesis are shared by developmental processes. This is the case of Notch, Sonic Hedgehog, Wnt/Fzd/β-catenin or those mediated by Hox cluster genes. This observation raises questions about the evolution of the mechanisms of angiogenesis and about the origin of the endothelium itself, an exceptional cell type among the metazoans. It is conceivable that phylogenetically primitive signaling mechanisms regulating changes between epithelial and mesenchymal phenotypes have been evolutionarily conserved and can account for the outstanding differences between the quiescent and the angiogenic endothelial cell [254]. This evo-devo approach might provide new insights about the mechanisms which trigger, regulate and terminate the angiogenic response.

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