REVIEW

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EphB receptors and ephrinB ligands: regulators of vascular assembly and homeostasis

Abstract Eph receptors comprise the largest family of receptor tyrosine kinases consisting of eight EphA receptors (with five corresponding glycosyl-phosphatidyl-inositol-anchored ephrinA ligands) and six EphB receptors (with three corresponding transmembrane ephrinB ligands). Originally identified as neuronal pathfinding molecules, genetic loss of function experiments have identified EphB receptors and ephrinB ligands as crucial regulators of vascular assembly, orchestrating arteriovenous differentiation and boundary formation. Despite these clearly defined rate-limiting roles of the EphB/ephrinB system for developmental angiogenesis, the mechanisms of the functions of EphB receptors and ephrinB ligands in the cells of the vascular system are poorly understood. Moreover, little evidence can be found in the recent literature regarding complementary EphB and ephrinB expression patterns that occur in the vascular system and that may bring cells into juxtapositional contact to allow bi-directional signaling between neighboring cells. This review summarizes the current knowledge of the role of EphB receptors and ephrinB ligands during embryonic vascular assembly and discusses recent findings on EphB/ephrinB-mediated cellular functions pointing to the crucial role of the Eph/ephrin system in controlling vascular homeostasis in the adult.

Keywords Endothelial cells · Angiogenesis · Eph · Ephrin · Homeostasis

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Introduction

Our impression of the angiogenic cascade has become increasingly complex. The simplistic view of an invading capillary sprout with matrix-degrading, migrating, and proliferating endothelial cells is increasingly being replaced by a complex morphogenic cascade of events that includes processes of network formation, acquisition of arteriovenous identity, and vessel maturation characterized by the orchestrated recruitment of mural cells (pericytes, smooth muscle cells; Fig. 1). The first generation of angiogenic cytokines, such as the vascular endothelial cell growth factors (VEGFs) and the fibroblast growth factors (FGFs), fit well into the concept of a sprouting capillary. More recently, the angiopoietins and their Tie receptors have been identified as a vessel assembly and maturation-mediating ligand-receptor system. Eph receptors and their corresponding ephrin ligands constitute the youngest family of essential vascular tyrosine kinases that regulate vessel assembly and differentiation during complex vascular morphogenesis.

EphB receptors and their ephrinB ligands have been identified as crucial mediators in arteriovenous differentiation during embryonic angiogenesis (for recent reviews, see Adams 2002; Cheng et al. 2002). Genetic experiments have put the EphB/ephrinB system on the vascular biology map. However, an in-depth understanding of these molecules at the cellular level is largely hampered by the limited availability of suitable experimental models to study complex vascular morphogenesis. For example, an almost inherent limitation is that the process of arteriovenous differentiation can only be studied in vivo with all of the complexity of in vivo experimentation.
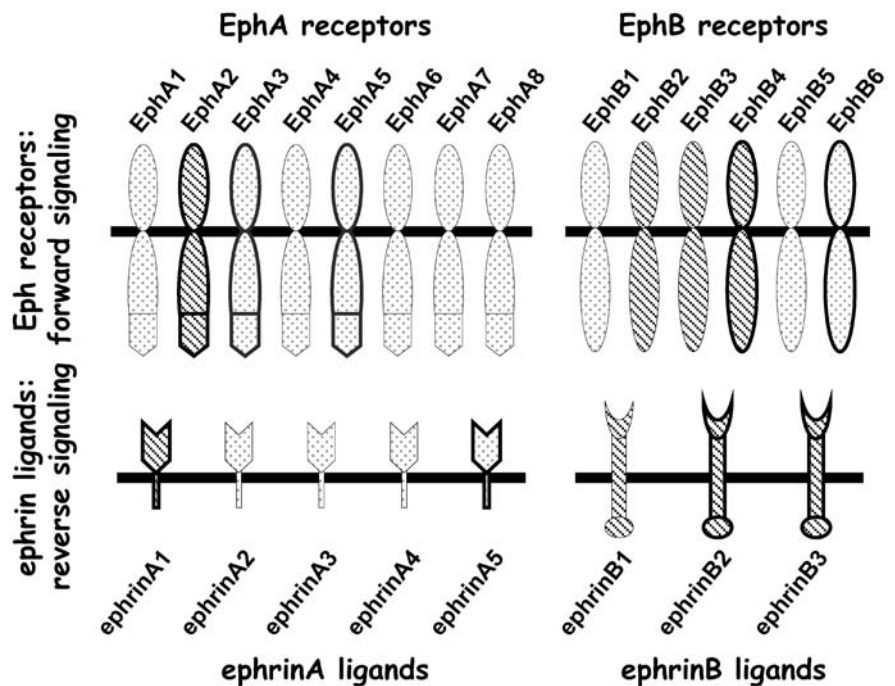
The present review aims at summarizing current knowledge about the role of the EphB/ephrinB system in orchestrating vascular assembly and the acquisition of arteriovenous identity. We will discuss recent data concerning the role of EphB receptors and ephrinB ligands in controlling cell-cell interactions in the vascular system and introduce a model of EphB/ephrinB function in controlling vascular homeostasis in the adult. We will
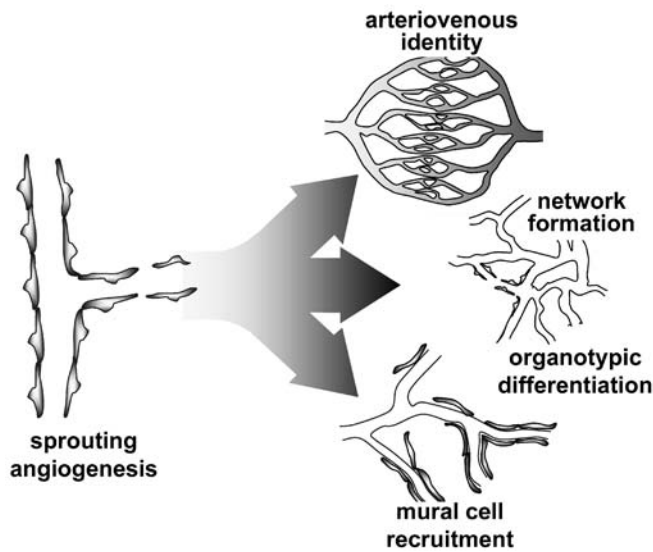
Fig. 1 Change of paradigm in angiogenesis research. The simplistic model of an invading capillary sprout with migrating and proliferating endothelial cells is increasingly being replaced by a complex morphogenic cascade of events that includes mechanisms of vascular assembly and maturation. Capillary sprouts need to establish anastomoses in order to form a complex differentiating capillary network. Vessels acquire their arteriovenous identity concomitantly with the establishment of directional blood flow. The recruitment of mural cells (pericytes, smooth muscle cells) marks a crucial step in the maturation of blood vessels. Eventually, endothelial cells establish their organotypic properties to form a continuous or discontinuous (fenestrated or non-fenestrated) endothelial cell lining

not focus on mechanisms of EphB forward and ephrinB reverse signaling in the vascular system for which the reader is referred to excellent recent literature (Kullander and Klein 2002; Palmer et al. 2002).

Characteristics of Eph receptors and ephrin ligands

Eph receptors and their corresponding ephrinB ligands are grouped into A and B subfamilies based on distinct structural properties of the ephrin ligands (Fig. 2). EphrinA ligands are glycosyl-phosphatidyl inositol (GPI)-anchored peripheral membrane molecules. EphrinB ligands are transmembrane molecules whose cytoplasmic domain is capable of engaging in various signaling activities. The corresponding Eph receptors act as classical transmembrane tyrosine kinases. Correspondingly, the activation of Eph receptors by ephrin ligands is referred to as "forward signaling", whereas the EphB receptor-mediated activation of ephrinB ligands is designated as "reverse signaling". EphrinB ligands are capable of supporting different signaling mechanisms based on the presence of tyrosine and serine phosphorylation sites and a PDZ domain-binding site in their intracellular domain.

Eph receptors and ephrin ligands were originally described as neuronal pathfinding molecules. Given that both receptor and ligand are transmembrane molecules, Eph/ephrin signaling is dependent on the juxtapositional contact of neighboring cells. As such, they elicit propulsive and repulsive activities on outgrowing axons and are, thus, crucial mediators of neuronal network formation (for reviews, see Flanagan and Vanderhaeghen 1998; Wilkinson 2001; Palmer and Klein 2003).

Vascular expression of Eph receptors and ephrin ligands

Although the Eph/ephrin system was originally identified in the nervous system, knockout studies have

Fig. 2 Schematic presentation of Eph receptors and ephrin ligands. Eph receptors are classical receptor tyrosine kinases. EphrinA ligands are GPI-anchored peripheral membrane molecules. EphrinB receptors are transmembrane molecules with a cytoplasmic signaling domain. There is considerable receptor ligand promiscuity, but little crosstalk between the A and the B subfamilies. Eph receptors and ephrin ligands whose expression has been demonstrated in the vascular system are *striped*. Eph and ephrin molecules identified as being expressed by tumor cells are outlined in *bold*

revealed important functions during vascular development (Wang et al. 1998; Adams et al. 1999; Gerety et al. 1999). EphrinB2 is an early marker of arterial endothelial cells, and EphB4 reciprocally marks venous endothelial cells as revealed by gene-targeting experiments (Wang et al. 1998; Adams et al. 1999, 2001; Gerety et al. 1999). These genetic experiments have opened a whole new field of research in vascular biology, as EphB4 and ephrinB2 were the first molecules with a clearly defined asymmetric expression pattern in arteries and veins. These findings have stimulated research focusing on the identification of other molecules with an asymmetric expression pattern in the vascular system. The recent characterization of additional arterial markers, such as DeltaC (zebrafish), Dll4 (mouse), gridlock (zebrafish), VEGFR-2 (zebrafish), notch1 (mouse), notch3 (mouse), notch5 (zebrafish), tbx20 (zebrafish), BMX (mouse), and neuropilin-1 (mouse, chick), and venous endothelial cell markers, such as neuropilin-2 (mouse, chick), Tie-2 (mouse, chick), VEGFR-3 (zebrafish, mouse), has stimulated experimental work aimed at further understanding the molecular mechanisms of arteriovenous differentiation (Lawson and Weinstein 2002).

Prior to the identification of vascular EphB4 and ephrinB2 functions, ephrinA1 (previously called B61) was the first vascular ephrin molecule that was characterized as a tumor necrosis factor α-inducible gene in endothelial cells (Holzman et al. 1990; Pandey et al. 1995). However, these early experiments did not lead to a systematic analysis of Eph and ephrin molecules in the vascular system. This is largely because research into vascular Eph/ephrin molecules has to date been hampered by the limited availability of adequate protein reagents to study these molecules. Few antibodies have been raised that are suitable for immunohistochemical expression profiling experiments. Likewise, only few in situ hybridization studies have been published, and Fc fusion proteins (receptor bodies) are of limited use for analytical binding experiments in vivo. The recent publication of EphB and ephrinB staining protocols for paraffin-embedded tissues with polyclonal reagents (Batlle et al. 2002) has stimulated expression-profiling experiments, and additional data including tumor expression data can be expected in the near future.

Given the limited availability of tools and techniques, the hitherto most relevant expression data originate from lacZ staining in heterozygous mice (Gale et al. 2001; Shin et al. 2001). These studies have substantiated the reciprocal expression of arterial ephrinB2 and venous EphB4. They have also indicated that vascular EphB/ephrinB expression is not limited to endothelial cells, as lacZ staining is also observed in smooth muscle cells and pericytes. Likewise, these studies have demonstrated the intense expression of ephrinB2 by angiogenic endothelial cells in tumor microvessels and during reproductive angiogenesis. The prominent expression of ephrinB2 by arterial and by angiogenic endothelial cells has prompted the above-mentioned authors to speculate that the widely anticipated view of angiogenic sprouting originating in postcapillary venules (Gimbrone et al. 1974; Burger et al. 1983) may require reconsideration toward an arterial origin of angiogenesis (Gale et al. 2001; Shin et al. 2001). This conceptually important issue raises the question of whether EphB4 and ephrinB2 are intrinsic markers of arterial and venous endothelial cells or whether their expression is controlled by microenvironmental cues. The intrinsic regulation of ephrinB2 expression would support an arterial origin of ephrinB2-positive angiogenic endothelial cells. In contrast, the microenvironmental regulation of endothelial ephrinB2 expression would be compatible with a postcapillary venule origin of angiogenesis with ephrinB2-negative cells or even EphB4-positive endothelial cells changing their phenotype upon angiogenic activation. Lineage-tracing experiments have provided some evidence that asymmetric endothelial cell EphB4/ephrinB2 expression could be established prior to the formation of arteries and veins suggesting that the Eph/ephrin system may indeed act as a maker of arteriovenous differentiation and not just as a set of marker molecules (Zhong et al. 2001). This view has been challenged by recent cell transplantation experiments in the chick embryo indicating that arterial expression of ephrinB2 is under the control of local microenvironmental cues rather than being a lineage-determined intrinsic property of endothelial cells (Othman-Hassan et al. 2001). The concept of the microenvironmental control of ephrinB2 expression is also supported by expression-regulation experiments that have identified VEGF as a major regulator of angiogenic endothelial cell ephrinB2 expression (Korff et al., submitted). These observations are in agreement with the recently established crucial role of VEGF as an arteriolizing molecule (Mukouyama et al. 2002).

Little is known about the expression of EphB receptors and ephrinB ligands in the adult. Endothelial cells in the adult maintain their asymmetric arteriovenous expression pattern suggesting that the EphB/ephrinB system plays a role in controlling vascular homeostasis (Fig. 3A; Gale et al. 2001; Shin et al. 2001). It is tempting to speculate about the vascular cell-cell interactions engaging in EphB/ephrinB signaling. Likewise, receptor-body staining experiments have shown that endothelial cells in quiescent vessels express ephrinB2 on their luminal surface (Korff et al., submitted). This observation suggests that, at least in the adult, EphB4/ephrinB2 signaling is likely to control interactions of endothelial cells, with corresponding receptor-expressing cells in the circulation rather than with abluminally located mural cells that similarly express Eph receptors and ephrin ligands (Gale et al. 2001; Shin et al. 2001).
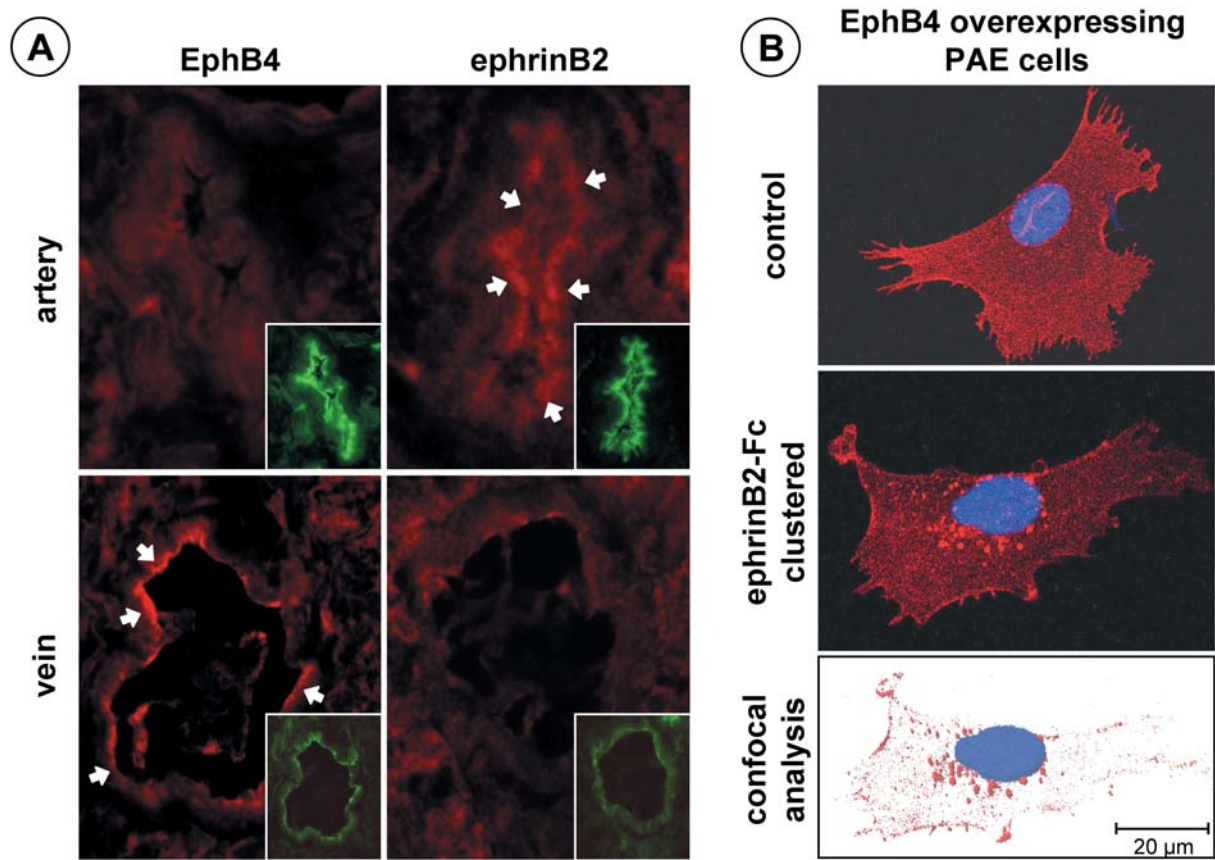
Fig. 3A, B Endothelial cell expression of EphB4 and ephrinB2 in vivo (**A**) and in vitro (**B**). **A** The arteriovenous asymmetric expression of EphB4 and ephrinB2 is maintained in the adult as demonstrated by ephrinB2-positive arteries and EphB4-positive veins (detected immunohistochemically in the mouse pancreas). *Inserts*: Endothelial staining for CD31. **B** Stimulation of EphB4-expressing endothelial cells with ephrinB2-Fc leads to rapid endocytotic internalization of the receptor ligand complex (*top* uniform surface EphB4 expression of transfected PAE cell, *middle* perinuclear accumulation of EphB4/ephrinB2 receptor ligand complexes within 30 min of ephrinB2-Fc stimulation, *bottom* tilted confocal microscopic analysis of the cell shown in the *middle* to demonstrate EphB4/ephrinB2 receptor complexes in the plane of the nucleus)

Vascular functions of Eph-ephrin interactions

Genetic experiments have provided the groundwork for the identification of EphB receptors and ephrinB ligands as mediators of vascular assembly and differentiation. EphrinB2-deficient mice die around embryonic day 11 as a consequence of grossly perturbed vascular differentiation and arteriovenous remodeling. These mice are not capable of orchestrating the positioning of arterial and venous endothelial cells and fail to form a properly structured capillary network (Wang et al. 1998).

EphrinB2 interacts with EphB2, EphB3, and EphB4. EphB4-deficient mice essentially phenocopy the phenotype of ephrinB2-deficient mice suggesting that the EphB4/ephrinB2 axis is the primary EphB/ephrinB interaction controlling vascular morphogenesis (Adams et al. 1999; Gerety et al. 1999). Correspondingly, null mice for the other ephrinB2 receptors (EphB2 and EphB3) have no apparent phenotype (Adams et al. 1999). Nevertheless, a proportion of EphB2/EphB3 double knockout mice have a variable embryonic vascular phenotype indicating that the EphB4/ephrinB2 interaction is not fully capable of compensating for the loss of EphB2 and EphB3 (Adams et al. 1999).

Genetic experiments have also provided unambiguous evidence that reverse ephrinB2 signaling is required for proper arteriovenous differentiation, as was shown by the observation that mice lacking the cytoplasmic domain of ephrinB2 (ΔephrinB2 mice) essentially have the same vascular phenotype as the full length ephrinB2 null mice (Adams et al. 2001). The cytoplasmatically truncated ephrinB2 ligand can support forward EphB receptor signaling but is not capable of engaging in reverse ligand signaling, thereby demonstrating the important role of ephrinB2 reverse signaling for proper developmental angiogenesis. Future work will show whether differential reverse ephrinB2 signaling events (tyrosine phosphorylation, serine phosphorylation, PDZ domain-binding) control different vascular functions.

Transgenic overexpression of ephrinB2 under the ubiquitous and constitutive CAG promoter or endothelial cell-specifically under the Tie-2 promoter has provided

strong evidence that the EphB/ephrinB system is involved not only in arteriovenous endothelial cell positioning, but also in endothelial cell interactions with mural cells (pericytes, smooth muscle cells). CAGp-ephrin-B2 transgenic mice show sudden death at neonatal stages from aortic dissecting aneurysms attributable to defective recruitment of vascular smooth muscle cells to the ascending aorta indicating that EphB/ephrinB2 signaling between endothelial cells and surrounding mesenchymal cells is crucial for normal embryonic angiogenesis and vessel maturation. The authors conclude from these findings a three compartment system with EphB4-positive cells, ephrinB2-positive cells, and EphB/ephrinB2-negative cells (Oike et al. 2002), which is increasingly supported by emerging vascular EphB/ephrinB expression data.

Little progress has been made in translating the genetic findings into mechanistic experiments at the cellular level. This is largely because of the limited availability of suitable cellular models to study later steps of the angiogenic cascade (Fig. 1). Nevertheless, a number of cell-cell interactions and confrontation studies involving cells of the vascular system have been published in the last few years.

Corresponding to the transgenic overexpression of ephrinB2, co-culture experiments of ephrinB2-expressing OP9 stromal cells and paraaortic splanchnopleura have revealed a permissive role of stromal cell ephrinB2 on vascular network formation. EphinB2-positive endothelial cells proliferate in the presence of ephrinB2-positive OP9 cells and actively induce the recruitment and proliferation of α-smooth muscle actin (α-SMA)-positive cells. In contrast, stromal cells expressing EphB4 inhibit vascular network formation, the proliferation of ephrinB2-positive endothelial cells, and the recruitment of α-SMA-positive cells (Zhang et al. 2001). These findings suggest that mural cell EphB4 acts in a negative, repulsive manner on ephrinB2-positive endothelial cells. However, the interpretation of these findings is complicated by the prominent angiogenic ephrinB2 expression that contributes to shaping the invasive phenotype of endothelial cells during angiogenesis. Likewise, it is not trivial to understand how ephrinB2-positive stromal cells act positively on ephrinB2 endothelial cells. This may reflect the complexity of the multicellular paraaortic splanchnopleura model, which possibly allows multiple additional cell-cell interactions.

Recent endothelial cell interactions experiments support a model of reverse ephrinB2-mediated propulsive and EphB4-mediated repulsive activities during angiogenesis (Füller et al. 2003). Activation of endothelial cell ephrinB2 promotes adhesion, migration, chemotaxis, capillary network formation, and sprouting angiogenesis. In turn, forward endothelial EphB4 signaling acts in an anti-adhesive and anti-migratory manner and inhibits sprouting angiogenesis (Füller et al. 2003; Hamada et al. 2003). Forward EphB4 activation is associated with rapid receptor turnover as evidenced by the internalization of the EphB4/ephrinB2 receptor complex upon ephrinB2-Fc

ligand-body binding (Fig. 3B). Given the angiogenic and arteriogenic expression of ephrinB2, the antagonistic functions of EphB4 and ephrinB2 support an artery to vein "push and pull" model of invasive angiogenesis. Angiogenic activation (e.g., by VEGF induction) is associated with ephrinB2 expression, which supports a proangiogenic and proinvasive phenotype with an artery to vein orientation. Surrounding EphB4-positive cells may guide invasive outgrowth of ephrinB2-positive endothelial cells. In turn, the activation of endothelial cell EphB4 may transduce negative signals leading to cellular repulsion. Consequently, propulsive and repulsive ephrinB2- and EphB4-mediated effects during capillary network formation limit cellular intermingling and control boundary formation (Füller et al. 2003).

The repulsive functions of endothelial EphB4 are largely compatible with the repulsive functions of Eph receptors in neurons. Neuronal Eph receptor activation inhibits axonal outgrowth and leads to growth cone collapse (Wilkinson 2001). However, recent work also suggests that EphB receptors might act in a bimodal manner being capable of transmitting both pro-adhesive and anti-adhesive signals (Huynh-Do et al. 1999; Zou et al. 1999; Carter et al. 2002). Likewise, opposing functions of different splice variants of EphA7 have been demonstrated (Holmberg et al. 2000). Reverse ephrinB signaling has also been implicated in both attractive and repulsive functions (Kullander and Klein 2002). Thus, EphB receptors may well be capable of acting in a context-dependent, bimodal manner, being able to elicit both propulsive and repulsive activities on EphB/ephrinB-interacting cells in the vascular system.

Expression and function of Eph/ephrin molecules during tumor growth and tumor progression

Eph receptors and ephrin ligands not only regulate the migratory and networking capacity of neuronal and vascular cells. EphB receptors and ephrinB ligands have recently been shown to exert positional guidance cues during intestinal epithelial cell migration and differentiation (Batlle et al. 2002). Similarly, circulating leukocytes and bone marrow cells express Eph/ephrin molecules (Luo et al. 2002; Munoz et al. 2002; Suenobu et al. 2002). A growing list of Eph receptors and ephrin ligands has also been identified in tumors including colon and lung (Dodelet and Pasquale 2000; Ogawa et al. 2000; Liu et al. 2002). Eph/ephrin expression by tumor cells points towards a role of the Eph/ephrin system in controlling tumor cell interactions with the host microenvironment that in turn controls tumor progression and metastatic dissemination. EphA2 and its ligand ephrinA1 are expressed by both endothelial cells and various human tumor cells, thereby establishing a microenvironment that stimulates tumor neoangiogenesis by activating EphA2 receptors expressed on angiogenic endothelial cells (Ogawa et al. 2000). The blocking of EphA class receptor activation by competitively acting soluble EphA receptors

has been shown to inhibit tumor angiogenesis in two different tumor models (Brantley et al. 2002). Interestingly, the stimulation of EphA2 phosphorylation by an EphA2-specific antibody inhibits the malignant behavior of breast tumor cells, indicating that EphA2 signaling may induce different phenotypes in endothelial and tumor cells (Carles-Kinch et al. 2002). Likewise, overexpression of EphA2 has been shown to induce malignant transformation and to confer tumorigenic potential on nontransformed mammary epithelial cells (Zelinski et al. 2001).

In contrast to the EphA/ephrinA receptor ligand system (Nakamoto and Bergemann 2002), much less is known about the functions of the B class Eph/ephrin molecules during tumorigenesis. Several studies have demonstrated expression of B class Eph/ephrin molecules in a variety of tumors and suggest a functional relationship between Eph/ephrin expression and tumor progression (Nikolova et al. 1998; Vogt et al. 1998; Tang et al. 1999, 2000; Stephenson et al. 2001; Takai et al. 2001; Liu et al. 2002; Chen et al. 2003). Corresponding functional experiments in an experimental A375 melanoma model have indicated that perturbation of bi-directional EphB4/ephrinB2 signaling by overexpression of dominant-negatively acting, soluble monomeric EphB4 (sEphB4) dramatically inhibits tumor growth (G. Martiny-Baron et al., submitted). This dramatic growth inhibitory effect is observed despite sEphB4 exerting only mild effects on tumor and endothelial cells. Tumor cell proliferation and soft Agar colony formation are not affected. Instead, sEphB4-overexpressing A375 tumor cells have altered adhesive properties as evidenced by their perturbed ability to organize in three-dimensional spheroids. Likewise, total microvessel density in sEphB4-overexpressing tumors is only moderately reduced. However, the combined targeting of the tumor and the vascular compartment can massively destabilize tumor progression even if sEphB4 overall elicits only subtle effects on tumor and endothelial cells. Future work should shed more light on the individual steps that are involved in the tumor progression cascade and that are controlled by Eph/ephrin interactions.

Perspectives

Vascular Eph/ephrin research is just beginning. Genetic experiments have focused attention on the role of EphB receptors and ephrinB ligands as crucial determinants of vascular assembly and arteriovenous identity. However, the originally proposed neuronal-specific signaling system is increasingly being recognized as universal cell-cell interaction and trafficking controlling machinery. Eph/ephrin molecules are expressed by neuronal cells, endothelial cells, smooth muscle cells, epithelial cells, leukocytes, tumor cells, and possibly other cell types. The vascular endothelium forms one of the largest surfaces within the body, acting as a critical interface that controls the trafficking of cells, including the recruitment of circulating leukocytes and the metastatic dissemination of malignant tumor cells. Further insights into the complementary expression patterns of EphB receptors and ephrinB ligands in the vascular endothelium and the multiple cell types interacting with endothelial cells may lead to novel avenues of research aimed at understanding the EphB/ephrinB system as a cell trafficking and vascular homeostasis-mediating cell-cell signaling system.

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