Ephrin-B2 Selectively Marks Arterial Vessels and Neovascularization Sites in the Adult, with Expression in Both Endothelial and Smooth-Muscle Cells

Nicholas W. Gale,*1 Peter Baluk,† Li Pan,* Marilyn Kwan,† Jocelyn Holash,* Thomas M. DeChiara,* Donald M. McDonald,† and George D. Yancopoulos*

*Regeneron Pharmaceuticals Inc., 777 Old Saw Mill River Road, Tarrytown, New York 10591-6707; and †Cardiovascular Research Institute and Department of Anatomy, University of California, San Francisco, California 94143-0130

The Eph receptor tyrosine kinases and their membrane-tethered ephrin ligands provide critical guidance cues at points of cell-to-cell contact. It has recently been reported that the ephrin-B2 ligand is a molecular marker for the arterial endothelium at the earliest stages of embryonic angiogenesis, while its receptor EphB4 reciprocally marks the venous endothelium. These findings suggested that ephrin-B2 and EphB4 are involved in establishing arterial versus venous identity and perhaps in anastomosing arterial and venous vessels at their junctions. By using a genetically engineered mouse in which the lacZ coding region substitutes and reports for the ephrin-B2 coding region, we demonstrate that ephrin-B2 expression continues to selectively mark arteries during later embryonic development as well as in the adult. However, as development proceeds, we find that ephrin-B2 expression progressively extends from the arterial endothelium to surrounding smooth muscle cells and to pericytes, suggesting that ephrin-B2 may play an important role during formation of the arterial muscle wall. Furthermore, although ephrin-B2 expression patterns vary in different vascular beds, it can extend into capillaries about midway between terminal arterioles and postcapillary venules, challenging the classical conception that capillaries have neither arterial nor venous identity. In adult settings of angiogenesis, as in tumors or in the female reproductive system, the endothelium of a subset of new vessels strongly expresses ephrin-B2, once again contrary to earlier views that such new vessels lack arterial/venous characteristics and derive from postcapillary venules. While earlier studies had focused on a role for ephrin-B2 during the earliest embryonic stages of arterial/venous determination, our current findings using ephrin-B2 as an arterial marker in the adult challenge prevailing views of the arterial/venous identity of quiescent as well as remodeling adult microvessels and also highlight a possible role for ephrin-B2 in the formation of the arterial muscle wall.© 2001 Academic Press

Key Words: artery; vein; capillary; Eph; ephrin; receptor tyrosine kinase; pericyte; angiogenesis; vasculogenesis; PECAM; CD31; alpha-smooth muscle actin.

INTRODUCTION

The Eph receptor tyrosine kinases comprise the largest known family of growth factor receptors and utilize the similarly numerous ephrins as their ligands (Flanagan and Vanderhaeghen, 1998; Gale and Yancopoulos, 1997). The ephrins are unlike ligands for other receptor tyrosine kinases in that they must be membrane-tethered in order to activate their Eph receptors (Davis et al., 1994; Gale and Yancopoulos, 1997). The obligate membrane-attachment of the ephrins provided the first clue that they might act precisely at points of cell-to-cell contact. Based on their means of tethering to the cell membrane, the ephrins can be subdivided into two subclasses. The five members of the ephrin-A subclass (ephrin-A1 to -A5) are attached to the
The ephrins and Ephs were initially studied for their actions in the nervous system, where they seem to play important roles in axonal guidance and in neuronal patterning (Flanagan and Vanderhaegen, 1998; Gale and Yancopoulos, 1997). More recent studies have begun to focus on roles of these molecules outside of the nervous system. Ephrin-B2 and its cognate EphB4 receptor have recently attracted attention in the field of cardiovascular development, based on the vascular defects observed in embryonic mice bearing null mutations in the genes for this ligand and receptor pair (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Normal vascular development initiates with a vasculogenic phase that involves formation of a primitive vascular scaffold, followed by angiogenic stages during which this early vasculature undergoes remodeling and maturation (Risau, 1997). Mouse embryos lacking ephrin-B2 and EphB4 suffer fatal defects in early angiogenic remodeling (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Moreover, ephrin-B2 and EphB4 display a remarkably reciprocal pattern of distribution within the developing vasculature, that is, ephrin-B2 marks the endothelium of primordial arterial vessels while EphB4 marks the endothelium of primordial venous vessels (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). These distributions suggested that ephrin-B2 and EphB4 are involved developmentally in establishing arterial versus venous identity, perhaps in joining arterioles to venules, and that defects in these processes might account for the early lethality observed in mouse embryos lacking these proteins (Adams et al., 1999; Gale and Yancopoulos, 1999; Gerety et al., 1999; Wang et al., 1998; Yancopoulos et al., 1998).

Despite the remarkably reciprocal distributions of ephrin-B2 and EphB4 during very early vascular development, little is known about the distribution or functions of these proteins as vascular development proceeds, in the quiescent adult vasculature, or when angiogenesis is reinitiated in the adult such as in tumors or in tissue remodeling. To explore these issues, we exploited a genetically engineered mouse in which the LacZ coding region was used to substitute and report for the ephrin-B2 coding region. We note that ephrin-B2 expression continues to selectively mark arteries during later embryonic development as well as in the adult. However, as development proceeds, we find that ephrin-B2 expression progressively extends from the arterial endothelium to the surrounding arterial smooth muscle and to pericytes; the precise point along the microvasculature at which ephrin-B2 expression becomes undetectable varies in different circulatory beds, but in some cases can mark capillaries to the midway point between terminal arterioles and postcapillary venules. These findings challenge the classical concept that capillaries have neither arterial nor venous identity as had previously been questioned using other approaches (Lojda, 1979; Mrazkova et al., 1986). In adult settings of angiogenesis, as in tumors or in the female reproductive system, the endothelium of a subset of new vessels strongly expresses ephrin-B2, once again contrary to earlier views that such new vessels lack arterial/venous characteristics and derive from postcapillary venules (Gimbrone et al., 1974; Grunt et al., 1986a, 1986b). While earlier studies had focused on a role for ephrin-B2 during the earliest embryonic stages of arterial/venous determination, our current findings suggest that ephrin-B2 continues to play an important role during the development of arteries, perhaps by regulating endothelial–smooth-muscle interactions involved in the formation of the muscle wall. Furthermore, our current findings using ephrin-B2 as an arterial marker in the adult challenge prevailing views of the arterial/venous identity of quiescent as well as remodeling adult microvessels and also highlight a possible role for ephrin-B2 in the formation of the muscle wall of vessels.

MATERIALS AND METHODS

Targeting Vector Construction and ES Cell Manipulations

The 5' and 3' ephrin-B2 gene fragments used in the construction of the targeting vector were isolated from a 129Sv mouse genomic library in the lambda FixII vector (Stratagene, La Jolla, CA). The 5' region of homology incorporated in the targeting construct consisted of a 6.7-Kb Eag1 restriction fragment derived from an ephrin-B2 genomic clone (the 5' Eag1/NcoI site was contributed by the lambda FixII phage from which this fragment was obtained), which terminated at its 3' end S1 nucleotides upstream of the ephrin-B2 start codon. This fragment was cloned into a NotI site upstream of a promoterless LacZ cDNA in the vector pKOVpLacZ, which comprises the LacZ gene followed by the phosphoglycerate kinase promoter driven neomycin resistance (NcoI) gene (PGK-KNeo) (Suri et al., 1996). A 3' region of homology consisting of a 2-Kb Eag1-Xba1 fragment, which terminates at its 5' end approximately 100 nucleotides downstream of intron1/exon1 boundary, was subcloned into a HindIII site of pKOVpLacZ between the PGK-KNeo and HSV-tk expression units using HindIII linkers (Suri et al., 1996). In this targeting scheme 281 nucleotides of the ephrin-B2 gene, including the transcriptional start site and signal sequence, were deleted and replaced by the LacZ and Neo genes. Gene targeting in ES cells and mice derived from them were confirmed by Southern blotting. Faithful expression of the LacZ gene, driven by the endogenous ephrin-B2 gene promoter, was confirmed by immuno-histochemical and in situ hybridization analysis. The LacZ-labeled Ephrin-B2 gene was bred into C57BL/6 and FVB/N strains of mice.

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Tumor Models

Lewis Lung carcinoma cells (5 × 10^6) were injected under the dorsal skin of syngeneic adult ephrin-B2 heterozygous mice. Ten to 14 days postimplantation a palpable tumor could be visualized under the skin at the injection site. In some cases subcutaneously injected tumor cells formed tumors within the thigh or flank muscle adjacent to the injection site, allowing the evaluation of both subcutaneous and intramuscular tumors. All experimental procedures were approved by the Committees on Animal Research of UCSF and Regeneron Pharmaceuticals.

Imaging the Vasculature

Generally the vasculature was made visible by in vivo labeling with biotin-labeled Lycopersicon esculentum lectin (100 μg in 100 μl 0.9% NaCl, Vector Laboratories, Burlingame, CA) as described. (Thurston et al., 1996). In some cases, the vasculature was made visible by postmortem perfusion of a solution of 1 mg/ml FITC-labeled dextran (Sigma) in PBS. Tissues were prepared as whole-mount specimens (aorta, vena cava, femoral artery and vein, trachea, cremaster, and external oblique muscles) or were infiltrated overnight with 30% sucrose, frozen in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and sectioned with a cryostat at a thickness of 100 μm. Biotin-labeled lectin was visualized as described (Thurston et al., 1996). LacZ activity was detected histochemically by X-Gal staining as described previously (Suri et al., 1996).

For immunofluorescent detection, tissues were fixed by vascular perfusion of 2% paraformaldehyde and washed with several rinses of PBS. Sections were incubated in 5% normal goat serum at room temperature for 1 h followed by 12–15 h in primary antibody solution in PBS/Triton. PECAM (CD31) immunoreactivity was detected by a rat anti-mouse CD31 monoclonal antibody (Pharminingen, San Diego, CA) diluted 1:500, followed by 4 h in Cy3-labeled goat secondary anti-rat IgG antibody (Jackson Immunoresearch, West Grove, PA) diluted 1:200 in PBS/Triton. Ephrin-B2 LacZ activity was detected by a rabbit polyclonal antibody (5 Prime → 3 Prime, Boulder, CO) diluted 1:1000, followed by 4 h in FITC-labeled goat secondary anti-rabbit IgG antibody. Alpha smooth muscle actin was detected with a Cy3-labeled mouse monoclonal antibody (Sigma) diluted 1:1000. For DAB immunohistochemistry antibodies to PECAM (see above) and αSMA/HRP (Dako Corp., Carpinteria, CA) were detected with anti-rat biotin-labeled secondary antibodies (Vector Labs, Burlingame, CA) at a 1:500 dilution.

RESULTS

Ephrin-B2 Continues to Specifically Mark Arteries in the Adult, but Is Unexpectedly Expressed by the Smooth Muscle of These Vessels

By examining the distribution of the LacZ reporter in whole mounts and in histological sections prepared from numerous adult organs, we find that ephrin-B2 expression continues to specifically mark arterial as opposed to venous vessels in the adult. In our study of adult mice, we first examined the largest vessels, including the aorta (Fig. 1A), the superior and inferior vena cavae (Fig. 1A), the pulmonary vessels, and the femoral vessels (Fig. 1B). In general, ephrin-B2 expression specifically marked large arteries but not large veins. However, unlike the situation described in early embryos, ephrin-B2 expression was not limited to the endothelial lining of the large arteries, but was prominently observed in the smooth-muscle cells circumferentially encircling these large arteries (e.g., Figs. 1C–1E). Although the endothelium and smooth-muscle cells comprising the walls of large veins did not generally express ephrin-B2, there was notable expression within the arterioles and capillaries of the vasa vasorum which nourish the walls of these large vessels (Fig. 1A). The femoral vein provided a rare exception to the general rule that ephrin-B2 was not expressed in the walls of large veins; ephrin-B2 was expressed by circumferential smooth-muscle cells in this particular vein (Fig. 1B).

We next evaluated ephrin-B2 expression in smaller adult vessels. In these cases, some of the vessels are difficult to visualize without specific markers due to their fragility and transparency. Therefore, we used FITC–dextran or lectin perfusion of the vasculature, which allowed for the visualization of the entire vasculature in whole-mount preparations. FITC–dextran perfusion of the vasculature of the external oblique muscle of the abdomen allowed for the clear demonstration that only arteries and arterioles expressed ephrin-B2, while the corresponding veins were negative (Figs. 1F and 1G). Biotinylated lectin perfusion, which reveals even finer vascular networks with high resolution, showed that ephrin-B2 expression extended into the microvasculature, marking capillaries to approximately the midway point between arterial and venous vessels, suggesting that capillaries exhibit arterial/venous characteristics. Thus, as seen within the cremaster muscle and in the trachea, smaller and smaller arterial branches remain positive until staining is lost within the intervening capillaries, midway between the arterioles and the postcapillary venules (Figs. 1H and 1I); within this intervening microvasculature, both endothelial cells and pericytes can express ephrin-B2.

Because different adult tissues have many differences in the morphology and in the properties of their vascular beds, we next compared the distribution of ephrin-B2 in a large number of adult organs. Despite important differences in the details of ephrin-B2 expression in different vascular beds, a consistent finding was that ephrin-B2 was expressed primarily in the arterial side of all of these vasculatures, and often prominently by the smooth muscle of these vessels. In the brain large arteries were clearly ephrin-B2 positive while veins were ephrin-B2 negative, but it was hard to detect ephrin-B2 expression in small arterioles and capillaries (Figs. 2A and 2B); in brain, numerous neurons also express ephrin-B2. In striking contrast to brain, cardiac and skeletal muscle express ephrin-B2 at high levels in their small arterioles and even in their capillaries (Figs. 2C and 2D); in these tissues, ephrin-B2 expression was clearly noted on smooth muscle in arteries and arterioles and in pericytes in capillaries. Ephrin-B2 revealed yet another pattern within the distinct vasculature of the spleen, in which the arterial circulation drains into sinusoids before being collected by the venous circulation. In the spleen, the...
arterial circulation lies mainly within the white pulp while
the venous circulation lies within the splenic red pulp, and
ephrin-B2 expression is clearly limited to the central arteri-
es, arterioles, and capillaries of the white pulp, terminating
abruptly at the marginal zone between white and red pulp
where venous sinuses originate (Fig. 2E); high-
magnification views of the splenic arterioles indicate that
ephrin-B2 is being expressed by the endothelium of these
vessels (Fig. 2F). In the liver ephrin-B2 is expressed by the
hepatic artery and not the central vein, with weak and
FIG. 1. Ephrin-B2 continues to specifically mark arteries in the adult, but is unexpectedly expressed by the smooth muscle of these
activity. The aorta expresses ephrin-B2, while the vena cava does not, except in the vasa vasorum. (B) Whole-mount views of femoral artery
and vein. Ephrin-B2 expressing smooth muscle cells within the femoral vein are indicated by arrowheads. (C–E) Sections of aorta showing
ephrin-B2/LacZ expression compared to SMA immunohistochemistry (revealing smooth-muscle cell layers) and PECAM immunohisto-
chemistry [revealing the endothelial cell lining (as indicated)]. Arrowhead in (D) indicates ephrin-B2-positive endothelial cell lining the
lumen of the aorta. (F–G) Whole-mount ephrin-B2/LacZ of external oblique muscles compared to FITC dextran perfusion showing lacZ only
in arterial structures. Arteries (A) and veins (V) are indicated. (H–I) Lectin-perfused whole-mount preparations of cremaster and trachea
showing ephrin-B2/LacZ expression within complete microvascular circuits. Arteries (A) and veins (V) and capillaries (C) are indicated.
FIG. 2. Variations in extent and limit of ephrin-B2 expression in various vascular beds revealed by comparison of ephrin-B2/LacZ expression and lectin labeling of vasculature. (A–B) Low- and high-magnification views of thick sections of brain (cerebral hemisphere). Neurons express strong LacZ activity, large arteries express ephrin-B2, but brain capillary endothelial cells (arrows) and pericytes appear negative. (C–D) Striated muscle of heart (C) and skeletal muscle (D) has strong LacZ activity around capillaries (arrows), possibly located in pericytes. (E, F) Low- and high-magnification views of spleen. Strong LacZ activity is observed in smooth-muscle cells of the central artery in splenic white pulp (arrowheads in E) and in endothelial cells of terminal arterioles (arrowheads in F). (G–H) Low- and high-magnification views of kidney. Strong LacZ activity is observed in the interlobular artery, glomeruli (Gl), afferent and efferent arterioles (Af and Ef in H), and proximal kidney tubules. Ephrin-B2 is expressed in both arterial smooth-muscle cells and endothelial cells (arrowheads in inset in G). (I–J) Low- and high-magnification views of thick sections of lung. Strong LacZ staining is observed in the endothelial cells of the pulmonary arteries (arrowheads in I and J).
scattered expression by cells within liver sinusoids (not shown). The kidney provides another unique configuration of vessels, with the initial arterial vessels eventually branching into afferent arterioles that then each further branch into a tuft of glomerular capillaries, only to then rejoin to form an efferent arteriole exiting the glomerulus; efferent arterioles then give rise to a second network of capillaries, the peritubular capillaries, which then coalesce into venous vessels. In the complex vasculature of the kidney, ephrin-B2 once again specifically marks the various arterial vessels. In afferent arteriolar walls, it appears as if ephrin-B2 is highly expressed by both arterial endothelium and smooth muscle (inset, Fig. 2G), while it is difficult to resolve the cell types that express ephrin-B2 within the glomerulus. Ephrin-B2 expression decreases in efferent arterioles and is undetectable in peritubular capillaries and the downstream venous circulation (Figs. 2G and 2H). Finally, the lung conforms to the pattern of arterial-specific expression of ephrin-B2. In the lungs, ephrin-B2 expression is clearly seen in endothelial cells of pulmonary arteries and their primary and secondary branches, with only weak expression by the smooth muscle cells of the pulmonary arteries (Figs. 2I and 2J); pulmonary venules and veins do not express ephrin-B2 (not shown).

**During Development, Ephrin-B2 Progressively Extends from Specifically Marking Arterial Endothelium to Also Marking Arterial Smooth Muscle**

Our finding that ephrin-B2 is highly expressed by vascular smooth-muscle cells in adult arteries was rather unexpected and differed from the endothelial-specific expression of ephrin-B2 described in early embryonic arteries (Adams et al., 1999; Wang et al., 1998). Thus, we sought to determine the progression of ephrin-B2 expression during embryonic development. Staining for the LacZ reporter of ephrin-B2 expression was compared with staining for an endothelial marker (PECAM) as well as a smooth-muscle marker (smooth muscle actin, SMA) at various embryonic stages. At embryonic day 11.5 postcoitum (E11.5), the aorta is beginning to become invested by a single layer of smooth muscle. At this stage, the endothelium of the aorta as well as of its sprouts expresses high levels of ephrin-B2 (Figs. 3A–3C). In contrast, the single layer of smooth-muscle cells around the aorta lacks detectable ephrin-B2 expression (Fig. 3C). However, it appears as if some mesenchymal cells scattered around the aorta strongly express ephrin-B2 at this stage (Fig. 3B). During the next few embryonic days, the smooth muscle of the aorta gains many more layers of smooth muscle, and these circumferential smooth muscle cells strongly express ephrin-B2, as does the endothelium (Figs. 3D–3F); once again, more distantly surrounding cells also express ephrin-B2 (Fig. 3E), and it is unclear whether these ephrin-B2-expressing mesenchymal cells are precursors that have given rise to, or will continue to give rise to, the layers of smooth-muscle cells.

While most major arteries expressed ephrin-B2 within their smooth-muscle cell layers at E14.5, there were notable exceptions. For example, ephrin-B2 was highly expressed by the endothelium, but not the surrounding smooth-muscle cells, of the umbilical artery (Figs. 3G–3I).

Taken together, these results demonstrate that initially the vascular endothelium is ephrin-B2 positive at developmental stages when there is little or no surrounding smooth-muscle layer. While the initial layer of smooth-muscle cells appears not to express ephrin-B2, closely surrounding mesenchymal cells do express it strongly. Shortly thereafter, arteries become invested in many layers of smooth muscle, all of which strongly express ephrin-B2.

**Ephrin-B2 Is Highly Expressed at Sites of Secondary Angiogenesis in the Embryo, as Well as at Sites of Normal and Pathological Angiogenesis in the Adult**

As in the adult, ephrin-B2 clearly marks the arterial vascular tree at E14.5 (Fig. 4A). At this stage, numerous initially avascular structures are being invaded by robust secondary angiogenic processes. For example, at these stages the formerly avascular heart becomes vascularized. While the cardiac muscle cells do not express ephrin-B2, the newly forming coronary arterioles strongly express ephrin-B2 (Fig. 4B) within their endothelium as well as their smooth muscle; similarly high ephrin-B2 expression marks the arterial circulation at other sites of secondary angiogenesis in the embryo, such as in the developing neural tube, kidney, and lung (not shown). In the heart, it should be noted that ephrin-B2 is also highly expressed by the endocardium, most notably overlying the newly formed atrioventricular valves as well as the septa forming between the right and left atrium (Fig. 4B, and data not shown).

Our observations of high levels of ephrin-B2 expression at sites of secondary angiogenesis in embryos prompted us to examine sites of physiologic as well as pathologic angiogenesis in the adult. Physiologic angiogenesis accompanies follicular maturation and corpus luteum formation in the adult ovary. During follicular maturation, the avascular follicle becomes surrounded by new vessels permeating its theca interna, and these vessels strongly express ephrin-B2 (Fig. 4C). After the mature follicle exudes its ovum, it undergoes further differentiation to form a highly vascularized corpus luteum, and the new vessels invading the corpus luteum also express high levels of ephrin-B2 (Fig. 4D). In the angiogenic vessels surrounding the follicle or invading the corpus luteum, ephrin-B2 appears to be expressed by the endothelium.

To examine the expression of ephrin-B2 during pathologic angiogenesis, subcutaneous tumors were examined. In a dramatic example of one such tumor that had invaded the underlying muscle, it can be seen that certain vessels growing into the tumor from the surrounding muscle express high levels of ephrin-B2 (Fig. 4E). Consistent with the notion that ephrin-B2 marks arterial and not venous ves-
sels, these tumor vessels appear to arise from previously existing ephrin-B2-expressing arterioles within the muscle (Fig. 4E). These data suggest that tumor vessels, which were previously assumed to consist of homogenous capillaries based on their small size and paucity of smooth-muscle investiture (e.g., Folkman, 1971), may be divided into microvessels with either arterial or venous identity. Further consistent with this notion, it is clear that only a subset of the new tumor vessels are ephrin-B2 positive (Figs. 4F–4H). Finally, ephrin-B2 was expressed by the endothelium of tumor vessels (Figs. 4F and 4I); smooth-muscle cells associated with tumor vessels clearly did not express ephrin-B2.

**DISCUSSION**

The initial findings that ephrin-B2 marks the arterial endothelium at the earliest stages of embryonic angiogenesis, and that its EphB4 receptor reciprocally marks the venous endothelium, suggested that ephrin-B2 and EphB4 might be involved in the establishment of arterial versus venous identity (Adams et al., 1999; Wang et al., 1998). A puzzling aspect of these initial findings was that despite their global distributions on their respective sides of the vasculature, the ephrin-B2 ligand and its EphB4 receptor only seemed to have the opportunity to engage each other at the junctions of arterial and venous vessels. Indeed, this
FIG. 4. Ephrin-B2 is highly expressed at sites of secondary angiogenesis in the embryo, as well as at sites of normal and pathological angiogenesis in the adult. (A–I) From ephrin-B2/LacZ mice. (A) Ephrin–LacZ expression in dorsal view of thoracic organs from E14.5 day mouse reveals extensive expression in all arteries present but not in veins (not visible). Aorta (Ao), subclavian artery (sa), common carotid arteries (cca), ductus arteriosus (da), pulmonary arteries (pa), esophagus (Eo), and bronchial epithelium (Br) are indicated. (B) Section of E14.5 day heart reacted for β-Gal revealed high levels of expression of ephrin-B2/LacZ in new coronary vessels of the ventricular wall and septum. Expression was also observed in developing atrial septa and within the endocardium over the trabeculae and atrioventricular valves.
possibility led to the speculation that ephrin-B2 and EphB4 might actually be involved in regulating the proper formation of such anastomoses and in preventing inappropriate fusions between tangential aspects of arterial and venous vessels (Adams et al., 1999; Wang et al., 1998; Yancopoulos et al., 1998). Unfortunately, the early death of embryos lacking ephrin-B2 or EphB4 did not allow for analysis of the ongoing roles of this requisite ligand–receptor pair in vivo.

Our current findings suggest a much more extensive and ongoing role for EphB4 and ephrin-B2 in arterial vessels. We have found that ephrin-B2 expression continues to selectively mark arteries during later embryonic development, as well as in the adult. Furthermore, as development proceeds, we find that ephrin-B2 expression progressively extends from the arterial endothelium to surrounding arterial smooth-muscle cells and to pericytes. That is, ephrin-B2 is initially expressed by the vascular endothelium of primary vessels and their sprouts, and not by the first layer of smooth-muscle cells that begin to invest the vasculature. Soon thereafter, the multiple layers of smooth-muscle cells enveloping arterial vessels begin to highly express ephrin-B2, as do loosely associated mesenchymal cells, which may be smooth-muscle precursors surrounding these vessels; ephrin-B2 remains highly expressed in the endothelium and smooth muscle of adult arterial vessels. Altogether, these findings suggest that ephrin-B2 might be involved, in an ongoing fashion, in regulating endothelial–smooth-muscle interactions involved in the formation and maintenance of the muscular walls of arteries. Furthermore, coexpression of EphB4 with ephrin-B2 in the aorta (data not shown and Shin et al., 2000) suggests that ligand–receptor pair can act together within the arterial vessel wall and that interaction of ephrin-B2 and EphB4 is thus not strictly limited to mediating interactions between arterial and venous compartments; additional EphB receptors may be involved.

Just as the detailed architecture and properties of the vasculature differ in different tissues, the precise distribution of ephrin-B2 differs in different vascular beds, as does the relative expression levels of ephrin-B2 by endothelium as opposed to smooth muscle in these vascular beds. Also variable is the precise point, between the arterial and venous circulation, at which ephrin-B2 expression becomes undetectable. In some cases, such as in the microvasculature of particular muscles and in the trachea, we were able to show that the endothelium and pericytes of capillaries still express ephrin-B2, with expression becoming undetectable midway between terminal arterioles and postcapillary venules. These findings suggest that capillaries can be divided into an arterial subset that maintains arterial identity and a venous subset that maintains venous identity, contrary to earlier views that capillaries lack arterial/venous distinctions.

In adult settings of physiologic and pathologic angiogenesis during remodeling of the female reproductive system or in tumors, ephrin-B2 seems to recapitulate its earliest patterns of embryonic expression. That is, ephrin-B2 is highly expressed by the endothelium of some angiogenic vessels and their sprouts and is largely lacking from the few smooth-muscle cells that are associated with new vessels. The finding that angiogenic sprouts at sites of adult neovascularization have arterial identity challenges prevailing views that these sprouts largely derive from postcapillary venules and lack arterial identity (Gimbrone et al., 1974; Grunt et al., 1986a,1986b). Instead, our data suggest that adult angiogenic signals cause recapitulation of an embryonic process in which ephrin-B2 might initially be involved in arterial sprouting and perhaps in anastomoses with EphB4-expressing venous sprouts, followed by playing a role in maturation of the arterial vessels by regulating formation of the muscle wall.

Whatever the roles of ephrin-B2 during arterial sprouting and wall formation, any such roles seem likely to depend on interactions of ephrin-B2 with its cognate EphB receptors. As noted above, such interactions would presumably depend on cell-to-cell contact between cells bearing the membrane-tethered ephrin-B2 and its EphB receptors and could result in bidirectional signaling between these adjacent cells. Though initial work had focused on the reciprocal distribution of the EphB4 receptor in the venous circulation of the embryo (Gerety et al., 1999; Wang et al., 1998), more recent findings indicate that several EphB receptors are more widely expressed so as to have direct access to arterial ephrin-B2 (Adams et al., 1999) and this study; consistent with the notion that EphB2 and EphB3 receptors play normal roles during vascular development, double null mutants for EphB2/EphB3 receptors display vascular defects. Furthermore, the finding that EphB4 is expressed in the aorta (data not shown and Shin et al., 2000) indicate that it too might have direct access to ephrin-B2 within arterial muscle walls.

Some of the findings in this manuscript are corroborated...
in the simultaneous submission by Shin et al. (2000). Earlier studies had focused on a role for ephrin-B2 during the earliest embryonic stages of arterial/venous determination. Our current findings together with those of Shin et al., using ephrin-B2 as an arterial marker in the adult, challenge prevailing views of the arterial/venous identity of quiescent as well as remodeling adult microvessels. In addition, our findings also highlight a possible role for ephrin-B2 in the formation of the arterial muscle wall, perhaps by regulating endothelial-smooth-muscle interactions involved in the formation of the muscle wall. Further meaningful insight into the role of ephrin-B2 during arterial development will presumably depend on examining the requirement for ephrin-B2 in vivo. The early death of mouse embryos lacking ephrin-B2, and the poorly defined vascular defects in these embryos, has limited their usefulness for this purpose. It seems likely that further understanding the role of ephrin-B2 during later arterial vessel development, and the purpose behind its fascinating expression patterns, will depend on genetic manipulation of the ephrin-B2 system in a regulated fashion during later stages of embryonic development as well as in the adult.

ACKNOWLEDGMENTS

We gratefully acknowledge Mary Simmons, Louise Freed, and Virginia Hughes for assistance with mouse husbandry; Anthony Lucarelli for help with the generation of the knock-out animals; and Evan Burrows for help with imaging. This work was supported in part by National Institutes of Health Grant HL-24136 to (D.McD. And P.B.). We thank Shin et al., for agreeing to exchange manuscripts prior to submission.

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Received for publication August 11, 2000
Accepted November 7, 2000
Published online January 19, 2001