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Expression and Function of Laminins in the Embryonic and Mature Vasculature

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Abstract

Endothelial cells of the blood and lymphatic vasculature are polarized cells with luminal surfaces specialized to interact with inflammatory cells upon the appropriate stimulation; they contain specialized trans-cellular transport systems, and their basal surfaces are attached to an extracellular basement membrane. In adult tissues the basement membrane forms a continuous sleeve around the endothelial tubes and the interaction of endothelial cells with basement membrane components plays an important role in the maintenance of vessel wall integrity. During development, the basement membrane of endothelium provides distinct spatial and molecular information which influences endothelial cell proliferation, migration and differentiation/ maturation. Microvascular endothelium matures into phenotypically distinct types: continuous, fenestrated and discontinuous, which also differ in their permeability properties. Development of these morphological and physiological differences are thought to be controlled by both soluble factors in the organ or tissue environment, and by cell-cell and cell-matrix interactions.

Basement membranes of endothelium, like those of other tissues, are composed of laminins, type IV collagens, heparan sulphate proteoglycans and nidogens. However, isoforms of all four classes of molecules exist, which combine to form structurally and functionally distinct basement membranes. The endothelial cell basement membranes have been shown to be unique with respect to their laminin isoform composition. Laminins are a family of glycoprotein heterotrimers composed of an α, β and γ chain. To date 5α, 4β and 3γ laminin chains have been identified that can combine to form 15 different isoforms. The laminin α chains are considered to be the functionally important portion of the heterotrimers, as they exhibit tissue-specific distribution patterns and contain the major cell interaction sites. Vascular endothelium expresses only two laminin isoforms and their expression varies depending on the developmental stage, vessel type and the activation state of the endothelium. Laminin 8 (composed of laminin $\alpha 4$, $\beta 1$, $\gamma 1$ chains) is expressed by all endothelial cells regardless of their stage of development and its expression is strongly upregulated by cytokines and growth factors that play a role in inflammatory events. Laminin 10 (composed of laminin $\alpha 5$, $\beta 1$, $\gamma 1$ chains) is detectable primarily in endothelial cell basement membranes of capillaries and venules commencing 3-4 weeks after birth. In contrast to laminin 8, endothelial cell expression of laminin 10 is upregulated only by strong

proinflammatory signals and, in addition, angiostatic agents such as progesterone. Other extracellular matrix molecules, such as BM40 (also known as SPARC/osteonectin), thrombospondin 1 and 2, fibronectin, nidogen 1 and 2, and collagen types VIII, XV and XVIII, are also differentially expressed by endothelium, varying with the endothelium type and/or pathophysiological state. The data argue for a dynamic endothelial cell extracellular matrix that presents different molecular information depending on the type of endothelium and/or physiological situation. This review will outline the unique structural and functional features of vascular basement membranes, with focus on the endothelium and the laminin family of glycoproteins.

1. Overview of Endothelial Cell Development, Differentiation and Maturation

Endothelial cell development occurs in several distinct steps, commencing with the formation of simple endothelial tubes via processes of vasculogenesis and angiogenesis, followed by the recruitment of mural cells and differentiation into different vessel types (e.g. capillary, venous and arterial) and, finally, tissue- or organ-specific maturation of capillaries and post-capillary venules. Tube formation has received considerable attention and several excellent reviews exist on this topic (23, 29, 79, 144). While factors have been identified that control differentiation of venous versus arterial endothelium, tissue- and organ-specific factors involved in vessel maturation remain less well studied. However, it is clear that the local environment plays a significant role in defining barrier function or permeability of endothelium, the complexity of junctional complexes, apical and basal surface specialization, as well as the supramolecular organization of the basement membrane (Fig. 1).

The nascent vascular network in the embryo arises principally by vasculogenesis, i.e. de novo vessel formation from angioblasts or stem cells, and by sprouting angiogenesis, i.e. sprouting and branching of a pre-existing endothelial sheet (141). In addition, there is evidence for non-sprouting angiogenesis or intussusception (splitting of preexisting vessels) as a mode of rapidly expanding vascular networks, without the need for endothelial cell proliferation (reviewed in (21)).

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Signaling via vascular endothelial growth factor (VEGF) (99), originally know as vascular permeability factor (VPF) (155) because of its ability to form edema, is important for several aspects of vessel formation (39, 44, 45, 119). A number of VEGFs have been identified to date and their interactions with different VEGF-receptors have been partially unraveled (45). Since most information is available for the role of VEGF-A and its receptor VEGFR2 (also known as flk-1) we describe only this interaction here and its function on initiation of vessel formation and the series of molecular and cellular events that lead to vessel maturation (24, 25, 144, 200). $CD31^+/CD34^+/$ VEGFR2 positive angioblasts form a vascular plexus that gives rise to the the yolk sac arteries and veins and, within the embryo, to the dorsal aorta and the cardinal vein (41, 46, 126, 195). Sprouting of this primary vasculature by angiogenesis results in extension of the vascular tree and is associated with the upregulation of genes involved in vessel branching and maturation, including angiopoietin-2 (Ang-2), nitric oxide (NO) synthase and VEGF-A (23, 200). The current model of vascular sprouting hypothesizes that the vessels dilate in response to nitric oxide (NO), a product of NO synthase, and become leaky due to the action of VEGF (79). Activation of proteases, such as matrix metalloproteinases (MMPs) and suppression of protease inhibitors (tissue inhibitor of matrix metalloproteinases- TIMPs), results in proteolytic processing of the underlying basement membrane and surrounding interstitial matrix (79, 82). Plasma proteins, such as fibrinogen and fibronectin, leak from the nascent vessels and provide a "provisional matrix" for migration of endothelial cells to form a new sprout. The endothelial cells migrate via specific interactions with the provisional matrix and respond to the chemotactic and mitogenic effects of VEGF and other growth factors (Fig. 1) (29, 79).

The nascent vessels are subsequently stabilized by the recruitment of pericytes and smooth muscle cells, and the associated formation of stable basement membranes. Several factors have been identified that are essential for recruitment of mural cells, including a) platelet derived growth factor B (PDGF-B) and its receptor, PDGFRβ {   o   1999 #692}; b) sphingosine-1-phosphate-1 (S1P-1) and endothelial differentiation sphingolipid-G-protein-coupled receptor-1 (EDG-1) (87); c) angiopoietins and Tie1 and Tie2 receptor tyrosine kinases (104) and d) transforming growth factor-β (TGFβ) and associated receptors RI, RII and endoglin (62, 132). PDGF-B, PDGFRβ and EDG-1 null mice all lack recruitment of pericytes (65, 103), resulting in vessel instability and perinatal death. Both PDGFRβ and EDG-1 are expressed on

pericytes and while PDGFRβ is responsible for the pericyte response to the chemotactic and mitogenic signals of PDGF-B released from endothelial cells. The role of S1P-1-EDG1 interactions are less well defined. It is hypothesized that EDG-1 signaling occurs downstream of PDGF and may be involved in extracellular matrix production (74).

The Tie1 and Tie2 receptor tyrosine kinases, and the two Tie2 ligands, Ang1 and Ang2, produced by endothelial cells and pericytes, respectively, and possibly also the recently described Ang3 and Ang4, are also critical for vessel stability and for vessel sprouting (104, 189). Ang1 makes the nascent vessels leak-resistant probably by facilitating interactions between endothelium and mural cells, while Ang2 acts as an antagonist of Ang1 in the absence of VEGF and destabilizes vessels, leading to their regression, and promotes sprouting in the presence of VEGF (104, 105, 167, 173, 200).

TGF-β1 is a multifunctional cytokine that promotes maturation of vessels by stimulating the production of extracellular matrix and inducing conversion of mesenchymal cells to mural cells (26, 132). Its effects can be both pro- and anti-angiogenic, depending on the signal cascades induced (62). Elimination of the TGF-β1 binding protein, endoglin, results in normal vasculogenesis but mice die during embryogenesis as a result of defective vascular remodeling and smooth muscle differentiation. Mutations in endoglin in man results in Telangietasia type 1, characterized by arterial-venous malformations and hemorrhages (130). The data suggest that TGF-β1/endoglin induced ALK1 signaling results in endothelial cell and fibroblast proliferation and migration and are pro-angiogenic, while TGF-β1 induced ALK5 signaling induces plasminogen activator inhibitor (PAI-1) in endothelial cells, which promotes vessel maturation by preventing degradation of the provisional matrix around nascent vessels. The ratio of TGF-β1 induced ALK1 versus ALK5 signaling determines the pro- or anti-angiogenic effects of TFG-β (62, 63).

The final or optimal pattern of the vascular network for an organ is determined by branching, remodeling, and pruning of its different segments. Some signaling pathways involved in regulating branching in the embryo have been identified (ephrins, VEGF-A, neuropilins). For example, the transmembrane molecule ephrin-B2 is highly expressed on precursors of arterial

endothelium during embryogenesis, while Eph-B4, a tyrosine receptor kinase that binds ephrin-B2, is found at much higher levels on developing veins than arteries. Bidirectional ephrin-B2 and Eph-B4 receptor signaling repels the arterial and venous sides of the developing vasculature and, thereby, guides branching (3, 192, 193). It has been recently shown that presentation of VEGF-A by heparin (or heparan sulfate proteoglycans) as opposed to soluble VEGF-A, results in enhanced vessel branching (146). In addition, studies involving zebrafish embryos suggest that VEGF-A signaling through Notch determines arterial endothelium (96). Arterial growth is then promoted by binding of a specific VEGF-A isoform , $VEGF_{164}$ (a heparin-binding form), to VEGFR2 and neuropilin signaling (120).

Organ- or tissue-specific maturation of capillary endothelium into continuous (most blood vessels), continuous with complex tight junctions (brain, retina & testis), discontinuous (liver & spleen), diaphragmed fenestrated (peri-tubular capillaries in kidney/endocrine glands) or non-diaphragmed fenestrated endothelium (glomerulus) (140, 142, 165), remains the least understood step in vessel formation. It is an aspect of vessel development that is likely to be regulated by local mediators, including both soluble factors, cell-cell and cell-matrix interactions. Heterotypic transplantation studies have shown that brain blood vessels growing into grafts of peripheral tissues acquire peripheral vascular morphology with fewer tight junction, whereas peripheral vessels growing out of grafts into brain tissue assume tight junctions characteristic of brain vessels (5, 29, 166). Further, tissue-specific vascular growth factors have recently been described, such as endocrine gland VEGF (EG-VEGF) also known as prokineticin 1 (101), and prokineticin 2 also known as Bv8, which are structurally distinct from the VEGF-family (9, 97, 106) and interact with a class of G-protein-coupled receptors (101). prokineticin 1 and 2 have been implicated in a range of physiological functions including migration, proliferation and fenestration of endothelial cells in vitro (9). The identification of such tissue-specific vascular growth factor(s) suggests the possible existence of additional tissue-specific factors that may complement the VEGF system to achieve specific, local maturation of blood vessel endothelium and also of perivascular cells (22). Alternatively, combinations of different known growth factors and their splice variants may result in a specific local cytokine signal pattern that induces local maturation of the vasculature (145).

Extracellular matrix molecules of vascular basement membranes and the subjacent interstitial matrix also provide cues for regulating proliferation, survival, migration and differentiation of both endothelial cells and mural cells, and have been implicated in several steps of vessel formation. These effects can be both direct and indirect as the extracellular matrix can accumulate, retain and present growth factors (such as VEGF and FGF-2) and proenzymes (proforms/inactive of the MMPs) involved in vessel development. In addition, cleavage products of extracellular matrix molecules can act as pro- or anti-angiogenic factors (e.g. laminin α4 G4-5, endostatin, restin). However, the role of extracellular matrix molecules in blood vessel formation remains relatively poorly studied and it is only recently that the unique and dynamic nature of the endothelial cell basement membrane has become apparent.

2. Expression of Endothelial Cell-Specific Extracellular Matrix Molecules During Endothelial Cell Development/Maturation

Basement membranes in general
Basement membranes are a highly specialized extracellular matrix that in blood vessels underlie endothelial cells, encasing associated pericytes, but also surround individual smooth muscle cells in veins and arteries. They are thin protein sheets (50 nm in thickness), the major components of which are the laminins, collagen type IV, nidogens and the heparan sulphate proteoglycan, perlecan. In addition, there are several minor components that in endothelial basement membranes include BM40 (also known as osteonectin and SPARC), fibulin 1 (or BM90) and 2, collagen types VIII, XV, XVIII, and thrombospondin 1 and 2 (176).

The basic framework of the basement membrane is thought to be created by two independent and distinct networks of type IV collagen and laminin. In vitro, it has been shown that type IV collagen and laminin self-assemble into a three-dimensional network independently of each other (204, 206). Recombinant nidogen-1 has been shown to bridge the two networks by binding to both collagen type IV and laminin in vitro (47), and in addition interacts with the heparan sulphate proteoglycan, perlecan, which in turn binds to the C-terminal portion of the laminin and to type IV collagen, thereby stabilizing the basement membrane network (175). In addition,

fibulins have been shown to bind strongly to laminins and nidogens, but also to several proteoglycans and fibronectin (152, 180). They may, therefore, contribute to crosslinking of basement membrane components and to the interaction of the basement membrane with the underlying interstitial matrix of the stroma. Recent data from nidogen knockout mice (113, 118, 154) suggest that this is a highly schematic and simplified concept of basement membrane assembly and that variations on this theme occur in vivo. Figure 2 illustrates a model for interactions between the major basement membrane components.

While the basic building blocks of basement membranes are similar, a considerable degree of complexity exists with variability in not only the relative amounts of components, but also the presence of site-specific isoforms and other unique components. It is now known that isoforms of type IV collagen (71), laminins (33, 100, 112, 186), heparan sulphate proteoglycans (12, 77) and even nidogen (88) exist, which have distinct temporal and spatial distribution patterns, resulting in basement membranes that are not only structurally but also functionally distinct (Table 1). Furthermore, these variations in basement membrane composition have significant consequences on how basement membranes interact with cellular ligands or are anchored to the surrounding interstitial matrix of the stroma.

Table 1 Major Basement Membrane Components and their Principal Sites of Localization

Component	Composition	Major Site/s of Expression
Laminin 1	$\alpha1\ \beta1\ \gamma1$	developing epithelia
Laminin 2	$\alpha2\ \beta1\ \gamma1$	all myogenic tissues, peripheral nerves
Laminin 3	$\alpha1\ \beta2\ \gamma1$	myotendinous junction
Laminin 4	$\alpha2\ \beta2\ \gamma1$	neuromuscular junction, mesangial matrix of glomerulus
Laminin 5	$\alpha3\ \beta3\ \gamma2$	epidermis
Laminin 6	$\alpha3\ \beta1\ \gamma1$	epidermis
Laminin 7	$\alpha3\ \beta2\ \gamma1$	epidermis
Laminin 8	$\alpha4\ \beta1\ \gamma1$	endothelium, smooth muscle, adipose tissue, peripheral nerves
Laminin 9	$\alpha4\ \beta2\ \gamma1$	endothelium, smooth muscle, neuromuscular junction, mesangial matrix of glomerulus
Laminin 10	$\alpha5\ \beta1\ \gamma1$	mature epithelium, mature endothelium, smooth muscle
Laminin 11	$\alpha5\ \beta2\ \gamma1$	mature epithelium, mature endothelium, smooth muscle, neuromuscular junction, glomerular basement membrane
*Laminin 12	$\alpha2\ \beta1\ \gamma3$	surface of ciliated epithelia
Laminin 13	$\alpha3\ \beta2\ \gamma3$	central nervous system/ retina

Laminin 14	$\alpha 4\ \beta 2\ \gamma 3$	central nervous system/ retina
Laminin 15	$\alpha 5\ \beta 2\ \gamma 3$	central nervous system/ retina
Collagen IV	$[\alpha 1(IV)_2\alpha 2(IV)]$	most embryonic & mature tissues
	$[\alpha 3(IV),\alpha 4(IV),\alpha 5(IV)]$	glomerular basement membrane
	$[\alpha 5(IV)_2\alpha 6(IV)]$	skin, oesophagus, Bowman's capsule, smooth muscle
Heparan sulfate proteoglycans	perlecan	widespread in most BMs
	agrin	widespread in most BMs
Nidogen 1		widespread in most BMs
Nidogen 2		widespread in most BMs, predominantly in endothelial cell BMs

* the existence of this isoform is controversial; BMs refers to basement membranes.

One function of basement membranes is to provide structural support for cells and to separate tissue compartments. More importantly, the individual components of basement membranes are dynamic in their interactions with cells and provide distinct spatial and molecular information that influence cell proliferation, migration and differentiation. The highly glycosylated nature of basement membrane components and the ability of molecules like heparan sulphate proteoglycans to interact with heparin domains render basement membranes high affinity and high capacity binders of growth factors, like VEGF and FGF-2 (125). In endothelium, the basement membrane is further likely to contribute to the barrier function (to both soluble molecules and migrating cells); to interactions with mural cells and thereby vessel stability; and to the transduction of mechanosensing signals from the lumen of the vessel to the vessel wall.

Basement membrane components are typically large oligomeric molecules that assemble to form supramolecular networks. Because of their functions, the proteins have low solubilities and show a tendency to aggregate. These characteristics have made them difficult to handle with traditional biochemical methods and have impeded studies on the structure and function of the specific isoforms of basement membrane components. However, modern techniques of nucleic acid analysis and manipulation, and of production of recombinant protein modules and fragments now provide possibilities for such studies. Data from structure-function studies as well as analysis of genetically manipulated mice and genetic diseases suggest that collagen type IV confers structural stability to the basement membrane, while the heparan sulphate proteoglycans act principally to crosslink the collagen type IV and laminin networks, bind soluble factors such as growth factors, and are important for the filtration properties of basement membranes. By

contrast, laminins are the major biologically active components of basement membranes, with different isoforms conveying different signals in different tissues.

Laminins

The laminins are approximately cross-shaped heterotrimeric glycoproteins, composed of an α, β and γ chain (Fig. 3). To date 5 distinct α, 4 β and 3 γ laminin chains have been identified that can combine to form 15 different isoforms (Table 1) (30, 149, 177, 186). While the existence of the majority of these laminins chains have been confirmed at both the gene and protein levels, there is little evidence to date for the existence of laminin β4 protein.

A major achievement in basement membrane research over the last 15 years has been the accumulation of a wealth of protein and cDNA sequence data, which has revealed that all components of basement membranes are multidomain and multifunctional proteins bearing distinct, independently active domains that mediate interactions with cells, other extracellular matrix molecules or growth factors (35, 68). These studies have revealed conservation of distinct structural entities both within and between different groups of extracellular matrix molecules, hence, defining distinct "domains". Laminin α, β and γ chains share homologous structures which include globular domains (domains IV and VI), rod-like domains containing EGF-like repeats known as laminin-like EGF or LE repeats (domains III and V), and domains forming the α-helical coiled-coil of the long arm of the molecule (domains I and II) (Fig. 3). In addition, all laminin α chains identified to date contain a large C-terminal globular (G) domain with five internal repeat motifs (known LG domains) (Fig. 3) (10, 181, 186). Important for the current review, is the fact that domains IV and VI, which comprise N-terminal portions of all chains and constitute the "short arms" of most laminins, are essential for self-assembly and, therefore, incorporation into the basement membrane, while the major cell binding domains are located in the C-terminal G domain of the α chains (Fig. 3).

The laminin α-chains are considered the functionally active portion of the heterotrimers as they carry the major domains that interact with the cellular receptors. Further, their tissue-specific distribution patterns siggests that different laminin isoforms have different functions in different tissues (Table 1). This has been confirmed by genetic inactivation of laminin α chains and the

analysis of genetic diseases: For example, elimination or mutation of laminin α2 in mouse and man results in a congenital muscular dystrophy (7, 114, 115, 198), elimination or mutation of laminin α3 lead to junctional epidermolysis bullosa, a severe skin blistering disease (28, 86, 147, 188, 191), elimination of laminin α4 results in an embryonic blood vessel defect (174) (discussed in detail below), and elimination of laminin α5 results in multiple defects and late gestational lethality due to placental defects (109).

Vascular endothelium expresses two laminin α chains, depending on the endothelial cell type and state of growth or activation (Figs. 3 & 4) (49, 75, 161, 162). Laminin α4 is expressed by all endothelial cells regardless of their stage of development (Fig. 4), while laminin α5 is detectable primarily in basement membranes of capillaries and some venules commencing 3-4 weeks after birth (Fig. 4). Both laminin α4 and α5 chains can combine with laminin β1 and γ1 chains in endothelial cell basement membranes to form laminins 8 and 10, respectively (Fig. 3, Table 1). In situ hybridization studies have shown that laminin α5 is expressed by endothelial cells (161); in addition unpublished data from our laboratory suggest that pericytes also contribute laminin α5 to the endothelial basement membrane. Laminin α5 is not expressed by aortic endothelium, as revealed by the isolation of laminin α4 containing isoforms only from bovine aortic endothelial cells and by in situ hybridization studies, but rather by the surrounding smooth muscle cells (161, 162). Laminin α5 is also absent from the basement membranes underlying fenestrated endothelium of some glands and of the peritubular capillaries in the kidney, suggesting a correlation between absence of laminin α5 and fenestration formation.

In vitro studies using several endothelial cell lines and primary endothelial cells have shown that laminin α4 expression is strongly upregulated by cytokines and growth factors that play a role in inflammatory events (49, 159, 183). By contrast, endothelial cell expression of laminin α5 is upregulated only by strong proinflammatory signals (such as TNFα) and angiostatic agents, such as progesterone, that are considered necessary for maintenance of the endothelial cell phenotype (159, 183) (Fig. 4).

Why different vessels express different laminins and why they are differentially regulated in endothelial cells is unknown, but suggests functional distinction. Mice lacking laminin α4 or laminin α5 have been generated and while laminin α4 null mice exhibit a blood vessel phenotype (described below) (174), laminin α5 null mice die during embryogenesis before this laminin chain is detectable in endothelial cell basement membranes (109, 183). Mice lacking laminin α5 alone or laminin α4 and α5 chains specifically in endothelial cell basement membranes are currently being generated in our laboratory and will aid in deciphering their potential functions. However, the temporal expression patterns and the structural characteristics of the laminin α and α chains, provide some clues to possible functions. Only laminin α4 is expressed in the developing endothelium from early stages of embryonic development (E8.5) (49), while laminin α5 occurs in endothelial cell basement membranes of mature vessels, predominantly in capillaries (161). This excludes the possibility that laminin α5 could have a role in the early stages of vessel development, but rather that it may be associated with maturation of the endothelium or its tissue /organ-specific characteristics.

Laminin α4 is also unusual among the laminins in lacking almost all N-terminal domains, including domains VI, V, IVa, IVb and IIIb (49, 75, 186) (Fig. 3). The N-terminal domains of laminin α, β and γ chains, which comprise three short arms of most laminins, have been shown to be important for laminin self-assembly (178, 204). Further, early studies on the globular domains within these short arms hypothesized a role in cross-linking the laminin network to the collagen type IV network (178). Although the latter has not been confirmed, it is possible that the ability of laminin α4 containing isoforms to both self-assemble and to be incorporated into the basement membrane may be compromised, due to the N-terminal truncation of the laminin α4 chain, resulting in a "looser" network that may be easier to penetrate by migrating lymphocytes or tumour cells (see below). In contrast, laminin α5 has an exceptionally long N-terminus, due to the presence of a larger than usual IVb domain, and additional EGF repeats in domains V, IIIb and IIIa (Fig. 3) (110, 186). Further, laminin α5 is the only laminin α chain that carries exposed arginine-glutamine-aspartic acid (RGD)-cell binding sites in the N-terminal portion of the chain. Although such RGD sequences occur in other laminins, they are normally exposed only after proteolytic cleavage and do not represent major cell binding sites. Two RGD binding sites occur

in domain IVa and adjacent EGF-like repeats of the mouse laminin α5 chain, which support integrin αvβ3, αvβ5 and α5β1 mediated binding of endothelial cells, as well as binding of several other cell types (153). Integrins of the αv series have been recently proposed to be negative regulators of angiogenesis and to be required for the stabilization of vessels upon maturation (72). In view of the exclusive expression of laminin α5 in quiescent, mature vessels, it cannot be excluded that some of the αv integrin effects are mediated by interactions with laminin α5 in the endothelial basement membrane.

3. Differential Expression of Laminin Receptors on Developing and Mature Endothelium

Integrins

Endothelial cells are anchored to their basement membrane by several receptor types, the principal ones being β1 and β3 integrins (Fig. 5). Integrins α6β1 and α3β1 are the major laminin α4 and α5-binding integrins that have been reported to be expressed by both developing and mature endothelial cells (Fig. 6) (34, 50, 51, 90). Integrins αvβ1, αvβ3, αvβ5 and α5β1 are also highly expressed on endothelium and have been implicated in RGD-mediated binding to domain IVa of laminin α5 (Fig. 6) (153), in addition to fibronectin and, in the case of the αv integrins, also to vitronectin, osteopontin, thrombospondin 1 and tenascin-C (190). Interestingly, integrin α6β4, which is best known as a receptor for laminin 5 (composed of α3 β3 γ2 chains, see Table 1) and is the major component of hemidesmosomes in the skin (91, 121, 160), can also bind to laminin α5 (84) and has been reported to occur on the growing tips of sprouting blood vessels (36, 55, 76). However, laminin α5 does not occur in association with developing vessels and to date laminin 5 has not been reported to occur at this site. There is also no evidence that integrin α6β4 on endothelial cells can bind to the laminin α4 chain. Hence, the significance of this laminin receptor on sprouting vessels is not clear.

Lutheran blood group glycoprotein

More recently, the Lutheran blood group glycoprotein has been described as a specific receptor that binds to laminin α5 (Fig. 5). The Lutheran blood group glycoprotein represents a new

member of the immunoglobulin superfamily with five extracellular Ig-like domains, a transmembrane domain and a cytoplasmic tail with three potential phosphorylation sites and a proline-rich consensus sequence for binding of Src homology 3 (SH3) domains, suggesting possible involvement in intracellular signaling pathways (127, 128) (Fig. 5). The extracellular portion of the Lutheran is highly glycosylated and contains two consensus integrin binding motifs and an RGD-site. In endothelium, it is expressed only post-natally where its appearance coincides precisely with that of laminin α5 in the endothelial cell basement membrane (117, 138). Solid phase binding studies utilizing recombinant laminin α5 domains have identified the LG3 domain as the Lutheran binding site (Fig. 5 & 6) (83). Lutheran has been shown to be upregulated on sickle red cells compared to normal red blood cells, and sickle red cells show increased adhesion to laminin α5 in in vitro assays (42, 98, 187). In sickle cell disease, detachment of endothelial cells leads to exposure of the subendothelial basement membrane (164) containing high levels of laminin α5. Hence, the involvement of Lutheran-mediated sickle cell binding to laminin α5 is likely to be an important factor in causing vaso-occlusion and painful crisis in sickle-cell disease patients (98). However, the functional significance of Lutheran-laminin α5 interactions in non-pathological situations remains to be investigated.

Dystroglycan

Dystroglycan exists as an extracellular α-subunit and a transmembrane β-subunit that are the products of the same gene and result from post-translation processing of the molecule (37, 181) (Fig. 5). The α-dystroglycan subunit acts as an extracellular receptor for several basement membrane components and represents the major non-integrin laminin receptor (37). Dystroglycan constitutes the central part of a larger complex know as the dystrophin-glycoprotein complex (DGC), best known from skeletal muscle where deletions or mutations in several of its components result in muscular dystrophies in both man and in mice (37, 107, 181). The major components of the DGC of skeletal muscle are shown in Fig. 5. However, the expression of α-dystroglycan on endothelium in vivo remains controversial and the data suggest a differential expression pattern depending on endothelial cell type or possibly tissue type. In vitro studies have demonstrated the expression of dystroglycan on bovine aortic endothelial cells which supports binding to laminin 1 (a non-endothelial cell laminin isoform - see Table 1) (156), and there has been suggestion that α-dystroglycan is upregulated on endothelium in vivo during tumour

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angiogenesis (69). However, in non-pathological situations, α-dystroglycan is principally associated with vascular smooth muscle cells (37, 38). Data from our laboratory has also shown the absence of dystroglycan from the surface of brain capillaries and post-capillary venules in a murine inflammation model, where endothelial and subjacent astrocyte endfeet are separated by a perivascular cuff of infiltrating leukocytes, allowing clear identification of the vascular endothelium (4) (see below). Studies utilizing recombinant laminin G-domains have clearly shown the absence of any interaction between α-dystroglycan and laminin α4 but high affinity binding to laminin α5 (Fig. 6) (73, 170, 203). It is difficult to define whether α-dystroglycan contributes to endothelial cell adhesion to the basement membrane due to the early lethality of dystroglycan null mice (E5.5), which die before blood vessels form (66, 67). Conditional knockout mouse studies will be necessary to define a function for dystroglycan in the vasculature.

Syndecans

Apart from the above described *bona fide* laminin receptors, endothelium expresses cell surface heparan sulphate proteoglycans that can also act as extracellular matrix receptors. Cell surface heparan sulphate proteoglycans all contain a core protein covalently linked to heparan sulphate-type glycosaminoglycan (GAG) side chains. The two main categories are the syndecans, which are transmembrane molecules (31), and the glypicans, which are anchored to the surface via a glycosyl phosphatidyl inositol (GPI) linkage (11). These cell surface heparan sulphate proteoglycans may function as independent receptors binding to heparin-binding domains in extracellular matrix molecules and growth factors, or they can act as cofactors in integrin signaling. For example, integrin α5β1 and syndecan 4 bind to fibronectin, but both interactions are necessary for formation of focal contacts and intracellular signaling (78, 148). Endothelial cells have been reported to express syndecans 1 and 4 and glypicans 1 and 4 (76, 108). At present there is no data on the role of glypicans on vessel formation, but syndecan 1 and 4 have been reported to be induced during neovascularization during wound healing. In addition, both syndecan 1 and syndecan 4 null mice show abnormalities in capillary formation in a skin wound healing model (40, 76), consistent with a role in pathological angiogenesis.

The fact that several different receptors can mediate binding to laminin α4 and α5 chains substantiates the hypothesis that laminin isoforms containing these chains convey several

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different signals to endothelial cells, but may also indicate some redundancy, with different receptors conveying the same signal depending on the organ, developmental stage or pathophysiological situation. At present there is little evidence that laminin interactions with any of the above described receptors are involved in early endothelial cell development or tube formation. Even though β1 integrins are clearly required for endothelial tube formation and branching, these effects appear to be mediated by interactions with fibronectin surrounding the nascent vessels via integrin α5β1 (48, 54). This is evident from the elimination of integrin α5 subunit in mice, which does not affect early blood vessel formation, but results in failure of dorsal aorta closure and structural abnormalities and leakiness of the yolk sac vessels, resulting in death at E10-E11 (201).

Interestingly, only the phenotype of integrin αv null mice is consistent with a potential role as a laminin α5 receptor in blood vessels, although this requires further investigation. In vitro studies had implicated the integrin αv series of receptors in blood vessel formation. This was based on the observation that αvβ3 is upregulated on certain tumour vessels (18), and the ability of cyclic RGD peptides (specific for αv-mediated interactions) and a specific integrin αv antibody (LM609) to block angiogenesis in response to growth factors in tumours and in retinal angiogenesis (19, 20, 124). However, elimination of the integrin αv subunit (8) and several of its β chain partners, including β3, β5, and β6 (70, 139, 172), in mice have failed to show a defect in the development of endothelium, its migration or differentiation. Rather, current data suggest that the αv integrins are likely to be negative regulators of blood vessel development and that in their absence vessel growth and branching is enhanced (72). Upon vessel maturation, integrin αv-mediated interactions appear to be required for vessel stability, possibly by mediating interactions with mural cells, a function that is consistent with the appearance of laminin α5 in endothelial basement membranes upon maturation and the fact that several murine endothelial cell lines can bind to laminin α5 in a RGD-dependent manner.

4. Function of Laminin-Endothelial Cell Interactions

Vessel Formation

In vitro assays have suggested several potential roles for laminin α4 in angiogenesis. In general, in in vitro adhesion assays both native and recombinant laminin 8 are poor substrates for endothelial cell adhesion, when compared to other laminin isoforms such as laminins 1, 2, and 10/11 (34, 90). Binding tends to be of low affinity and is mediated principally by integrins α6β1 and α3β1 (90). This low affinity binding to laminin 8 has been interpreted as conducive for cell migration and has been substantiated by in vitro transwell migration assays (50, 51), although this data should be viewed with some reservation due to the over simplification of migration processes in such in vitro assays. Recently, several laminin peptides (from laminin α1 and γ1 chains) (134, 135) or laminin fragments produced in bacterial systems (laminin α4) (32, 60, 61) have been described that show inhibitory effects on endothelial tube formation in in vitro assays. However, these peptides and bacterial fragments are not glycosylated and have not been shown to exhibit the correct and complex folding of the native protein, which may be necessary for receptor binding. The endothelial cell binding peptides/fragments also represent very different structural domains and yet have very similar effects. It is, therefore, not clear whether these inhibitory effects reflect a role of laminins in in vivo angiogenesis processes, or whether they influence other protein-protein interactions not related to laminin function.

The possibility exists, however, that not only intact laminin molecules are biologically relevant, but that laminin fragments generated by in vivo protease activity during pathological situations can induce signals different to those induced by the intact molecule. In vitro studies have shown that the C-terminal G-domain of laminin α4 is proteolytically cleaved by as yet undefined proteases, resulting in the release of the last two globular domains (LG4-LG5) (170) and potentially also of peptides such as those mentioned above. Recombinant laminin α4 LG1-3 and LG4-5 fragments have been produced in an eukaryotic cell system and have been shown to exhibit correct folding in rotary shadowing experiments (170). The use of these recombinant proteins in in vitro assays has shown that laminin α4 shows lower binding to sulfatides and to heparin than laminin α1 and α2 chains and, therefore, low affinity interaction with heparan sulphate proteoglycans (either in the basement membrane or on the cell surface) (168-170, 182). These recombinant G-domains also do not bind to nidogens 1 or 2, BM40, collagen types I or IV, or to the cell surface receptor α-dystroglycan. Neither laminin α4 LG1-3 nor LG4-5 was found to support high levels of adhesion of several different cell types, including endothelial cells, in in

vitro assays (as compared to similar domains from laminin α1 or α2 chains). However, laminin α4 LG4-5 but not LG1-3 was shown to inhibit endothelial cell migration in an in vitro wound healing assay, and to perturb tube formation in an in vitro model (170). Although the mode of action of laminin α4 LG4-5 is unclear, it has been proposed to involve interference with integrin α6β1 and α3β1-mediated interactions.

These latter data are consistent with the phenotype of the laminin α4 knockout mouse, which exhibits hemorrhages during the late embryonic and neonatal periods (174). The phenotype is rescued by laminin α5 expression, which occurs earlier than in wild type mice, at around birth rather than 3-4 weeks postnatally. Interestingly, neonatal laminin α4 null mice show reduced immunofluorescent staining for collagen type IV, laminin β1 and γ1 chains and nidogen 1 in endothelial cell basement membranes, but no change in perlecan staining. Electron microscopy also shows clear defects in the endothelial cell basement membrane, with discontinuities in the lamina densa. This suggests that blood vessels can exist even in the absence of laminin and collagen type IV in the basement membrane, and that perlecan is sufficient for early vessel formation. This is substantiated by data from the collagen type IV null mouse (137) and the endothelial cell specific perlecan null mouse (64) (see below). However, the integrity of vessels and their proper maturation requires the presence of laminin 8, since severe defects are apparent in a cornea angiogenesis model in the laminin α4 null mice (174, 208). Blood vessel formation is enhanced in this model, with dilation of vessels, aberrant branching and excessive edema. This suggests that laminin α4 may have two roles: 1) an inhibitory role in early tube formation (at least in the case of pathological angiogenesis), and 2) an instructive role in branching and maturation of vessels in both embryonic and pathological angiogenesis. Whether development of mural cells and their interactions with endothelial cell are normal in the laminin α4 null mice has not been studied to date, and further analysis of the blood vessels of this mouse is required.

Vessel Stability

There is little data to suggest that endothelial cell derived laminin α5 is involved in angiogenesis, as its expression occurs late in the formation of blood vessels (161). Rather, the data argue for a role in the stability/maturation of vessels and their barrier function (see below).

The laminin α5 chain has been eliminated in mice resulting in a late embryonic lethality, before laminin α5 appears in endothelial cell basement membranes (109). However, there is evidence from this mouse that glomerular capillaries require cues from laminin α5 present in the glomerular basement membrane for migration into the glomerulus, for capillary looping, and for vessel stability. In the developing kidney, laminin α5 replaces laminin α1 in the glomerular basement membrane at the capillary loop stage, a transition required for glomerulogenesis (111, 163). Mice lacking laminin α5 exhibit avascular glomeruli associated with breakdown of the glomerular basement membrane (111). Using transgenic mice, expressing a chimeric laminin composed of laminin α5 domains VI to I fused to the human laminin α1 G domains, bred onto the laminin α5 null background (referred to here as Mr51 x lama5 -/-), glomerular basement membrane breakdown was prevented but glomerular capillaries remained defective (85). Capillary loops did not form and vessels were distended, exhibiting a ballooned appearance. A similar phenotype is seen in mice lacking mesangial cells in the glomerulus due to absence of PDGFB/ PDGF receptor β signaling (102). Although mesangial cells were present in the Mr51 x lama5 -/- mouse strain, their adhesion to the glomerular basement membrane at the bases of capillary loops was defective (85), suggesting a mechanism whereby mesangial cells organize the glomerular capillaries by adhering to the G domain of laminin α5 in the glomerular basement membrane (via integrin α3β1 and Lutheran blood group glycoprotein). The similarity between mesangial cells and pericytes suggests that a similar situation may occur in other capillary types, with pericytes binding to laminin α5 (either produced by the endothelium or by the pericytes themselves) organizing and stabilizing the blood vessel.

Barrier Function

The endothelial basement membrane is likely to contribute to the barrier function of endothelium by impeding the movement of large, charged molecules and the migration of cells, such as leukocytes and tumour cells. There has been little investigation of the former, but there is accumulating evidence that the endothelial cell basement membrane may influence the movement of leukocytes into inflamed tissues. The best evidence for such a role comes from studies on murine autoimmune encephalomyelitis (EAE) (159). EAE can be actively induced in susceptible mouse strains by immunizing with myelin proteins, myelin fragments, or by adoptive transfer of myelin reactive CD4$^+$ T cell blasts (43) and shares some similarities with the human disease multiple sclerosis (43). The extravasation of autoaggressive T cells from postcapillary venules into the central nervous system (CNS) parenchyma is critical in this inflammation and is a multistep process, due to the specialized structure of blood vessels in the CNS.

Apart from the endothelial cell monolayer, blood vessels in the CNS are bordered by astrocyte endfeet (Fig. 7). Ultrastructurally, at least two basement membranes can be identified in association with larger blood vessels in the brain, the endothelial and an astroglial basement membrane (196). In addition, the epithelium of the meninges coinvaginates with blood vessels from the surface of the brain and contributes to the astroglial basement membrane (6, 196, 207). Collectively, the astroglial basement membrane and the meningeal epithelial basement membrane are known as the parenchymal basement membrane, as they delineate the border to the brain parenchyma (Fig. 7).

The biochemical composition of the endothelial and parenchymal basement membranes differ; the endothelial cell basement membrane is characterized by the presence of laminin $\alpha4$ and $\alpha5$ chains, while the parenchymal basement membrane contains laminin $\alpha1$ and $\alpha2$ chains (159) (Fig. 7). In the course of EAE, leukocytes first penetrate the endothelial basement membrane and, subsequently, accumulate in the perivascular space, defined by the endothelial cell basement membrane and the parenchymal basement membrane, before they penetrate the outer barrier and enter the brain parenchyma to induce disease symptoms. Leukocyte penetration of these two basement membranes have been shown to be distinct steps that are independent of one another (89, 159, 185). T cell transmigration of the endothelial cell basement membrane occurs

exclusively at sites defined by the presence of laminin α4 and the absence of laminin α5, suggesting that laminin α4 is permissive for transmigration and laminin α5 is inhibitory (Fig. 7) (4, 159). When EAE is actively induced in laminin α4 knockout mice, which show a compensatory ubiquitous expression of laminin α5 in all blood vessels (174) and no regulatory expression of this chain in response to proinflammatory cytokines, the onset of clinical symptoms of the disease is significantly delayed (Sixt et al., manuscript in preparation) (Fig. 7). Histological analysis of the brains of these mice after disease induction show accumulation of leukocytes in the lumen of the postcapillary venules, suggesting impaired transendothelial migration. Current data from intravital microscopy studies suggest that postcapillary venules are mosaic in their content of laminin α4 and α5, with areas containing little or no laminin α5. Transmigrating T cells appear to seek sites containing exclusively laminin α4 to transmigrate and avoid those that also contain laminin α5. At present the data suggest that the barrier function conferred by the presence of laminin α5 in basement membranes of postcapillary venules is due to both direct effects on leukocyte adhesion (159), as well as indirect effects on endothelium and leukocytes.

Recently, Patarroyo and coworkers have demonstrated that extravasation of polymorphonuclear granulocyte (PMN) is also influenced by the laminin composition of endothelial cell basement membranes (197). Using a murine peritonitis model, significantly less PMN infiltration into the peritoneal cavity was measured in laminin α4 knockout mice as compared to wildtype littermates, suggesting deficiencies in the transmigration across peritoneal postcapillary venules. However, these authors suggest an additional effect due to PMN secretion of laminin 8 onto their surfaces, which they hypothesize facilitates PMN interaction with the basement membrane and, thereby, its penetration. In order to distinguish between the direct barrier effects of the endothelial basement membrane and potential additional effects due to secretion of laminin 8 onto the PMN surfaces it will be necessary to demonstrate impaired migration of laminin α4 null PMNs as compared to PMNs from wildtype littermates in in vitro assays, or to induce peritonitis in chimeric mice carrying laminin α4 null bone marrow on a wildtype background (studies that are currently underway in our laboratory). Leukocyte secretion of laminins and their potential role in extravasation is an interesting possibility that, however, requires further investigation (52, 53, 131).

The endothelial cell basement membrane not only presents a barrier to the movement of leukocytes but also to tumour cells in metastasis. However, few physiologically relevant studies exist in this area, in particular with respect to the endothelial laminins. The only study to date involves the use of the laminin α4 knockout mouse in a lung metastasis model, revealing increased metastases, probably due to the enhanced angiogenesis and pronounced leakiness of newly formed vessels within the primary tumour (208). In addition, the primary tumour showed reduced apoptosis and, therefore, enhanced growth in the laminin α4 knockout mice. Since newly formed blood vessels lack laminin α5 in both wildtype and the laminin α4 knockout mice, presumedly the tumour cells can more readily migrate out of the tumour in the laminin α4 knockout mice due to increased leakiness of vessels, and enhanced growth may result in larger numbers of emigrating cells. However, whether this is sufficient to explain the increased metastasis to the lung remains unclear, where all blood vessels show a compensatory aberrant expression of laminin α5 which, based on the leukocyte studies, one may expect to reduce penetrability of these vessels. The data suggest that the mechanism of tumour cell transmigration of endothelial cell basement membranes differs from that utilized by leukocytes, and that tumour cells may be able to overcome the barrier conferred by the presence of laminin α5.

Endothelial laminins as signal transducers to mural cells/ mechanosensing

Endothelial cells line the luminal surfaces of all blood vessels and are, therefore, in an ideal position to act as mechanosensers of changes in blood flow and, consequently, shear stress. The endothelium communicates changes in shear stress to other compartments of the vascular wall, which is important for vascular homeostasis. Nitric oxide (NO) produced by endothelial-derived nitric oxide synthase (eNOS) is considered an important factor in such mechanosensing events, leading to cellular responses aimed at adaptions of vessel diameter (57). According to a widely accepted hypothesis, shear stress is sensed by endothelial cells via "focal adhesions", which occur abluminally and consist of aggregations of integrin receptors and linker molecules that interconnect the cytoplasmic tails of the integrins to the actin cytoskeleton (158). Most important, ligand occupation of the integrins is necessary for focal adhesion formation. Increases in shear stress result in the formation of such focal adhesions and intracellular activation of kinases (in particular, focal adhesion kinases (FAK) and mitogen-activated protein kinases (MAPK)), which

in turn initiate signaling cascades leading to altered gene expression, including upregulation of eNOS (158).

Interactions between the vascular endothelium and laminins in its basement membrane have been implicated in the mechanosensing of changes in shear stress in vessels. Although these laminins have not been shown to be the endothelial cell laminins discussed in this review, this is suggested by the data. In vitro studies have shown shear stress induced upregulation of eNOS only when porcine aortic endothelial cells are plated on laminin 1 (58), while shear stress-induced focal adhesion formation occurs only when human umbilical vein endothelial are plated on a human laminin substrate (80). Laminin 1 does not occur in endothelial cell basement membranes but represents a prototype laminin (Fig. 3) that is readily prepared in large quantities from a mouse Engelbreth-Holm-Swarm (EHS) tumour (129, 179) and, therefore, widely used in in vitro cell adhesion and migration assays. However, the experiments performed with laminin 1 report an RGD-dependent adhesion of the porcine aortic endothelial cells to laminin 1 (58). As laminin 1 does not contain an exposed RGD site, this implies that the observed shear stress-induced upregulation of eNOS may stem from adhesion to a substrate produced by the cells themselves and which is either induced by or binds well to the plated laminin 1 substrate. In contrast, commercially available human laminin is typically a laminin 10/11 preparation (see Table 1), isolated from trypsin-treated placenta. It, therefore, reflects one of the endothelial laminins, albeit in a proteolytically processed form. Further, the proposed involvement of integrin $\alpha v \beta 3$ in endothelial cell mechanosensing (80), which as discussed above can act a receptor for the laminin $\alpha 5$ chain of laminin 10 in endothelium, substantiates a potential involvement of endothelial cell laminin 10 in mechanosensing of changes in shear stress. It is also noteworthy in this context that the other major ligands of $\alpha v \beta 3$, vitronectin and fibronectin, are likely to occur in close contact with endothelium only in the provisional matrix of nascent vessels but not in mature and undamaged vessels, as they represent interstitial matrix molecules that occur subjacent to the basement membrane.

Recent evidence suggests that shear force or other pathophysiological stimuli also leads to release of growth factors from vascular endothelial cells, in particular FGF-2, which acts to induce endothelial proliferation and differentiation (resulting in neovascularization that occurs in many

pathological situations) (59). This growth factor release is dependent on specific integrin αvβ3-mediated interactions of the endothelial cells with the extracellular matrix and can be blocked by RGD peptides. The αvβ3 ligand in the vascular endothelial cell basement membrane has not yet been identified, but here too it is noteworthy that αvβ3 binds laminin α5 in an RGD-dependent manner in endothelial cells.

Clearly the role of endothelial laminins in the physiology of blood vessels is an area that requires further investigation with the physiologically relevant laminin isoforms. The laminin α4 knockout mouse and mice lacking laminin α5 specifically in the endothelial cell basement membranes will be vital to such studies.

5. Other Endothelial Cell-Associated Extracellular Matrix Molecules

It is important to note that apart from laminins α4 and α5, several other extracellular matrix molecules are differentially expressed by endothelium depending on developmental stage, endothelium type and/or physiological state. These include SPARC (also known as BM40 and osteonectin), nidogens 1 and 2, thrombospondins 1 and 2, fibronectin, and collagen types VIII, XV and XVIII (Table 2).

Table 2: Minor Endothelial Cell-Associated Extracellular Matrix Molecules

Molecule	*Mol. Mass (kDa)	In vivo Localization	Potential Interactions	Potential Functions	Ref
SPARC/BM40/ osteonectin	40	Matricellular	Collagen I, vitronectin	Inhibits endothelial cell proliferation & regulates activity of growth factors	(17,199)
Fibulin 1/ BM90 Fibulin 2	90 (monomer) 200 (homodimer)	Some endothelial basement membranes & elastic fibres	Nidogen 1, fibronectin, endostatin, laminin 1 & perlecan in the case of fibulin 2	May represent an alternative to nidogen 1 for cross-linking basement membrane components	(180)

Thrombospondin I & II	145 (homotrimer)	Matricellular/ endothelial cell surfaces; upregulated in pathological situations; thrombospondin II occurs in association with developing vessels	Syndecans, CD36, fibrinogen, fibrin, collagen V, fibronectin, laminin-1	Anti-angiogenic	(2, 14)
Fibronectin	250 (homodimer)	Provisional matrix of developing vessels, or interstitial matrix of mature vessels	Perlecan, fibulins, laminin 1	Pro-angiogenic	(54, 116)
Collagen VIII	60 (heterotrimer)	Human endothelial BMs; some mouse endothelial BMs	Not known	Confers resistance to compression & maintains an open porous & translucent structure	(157)
Collagen XV, C-terminal fragment is restin	116-139 (homotrimer) 22-25	Most capillaries	Fibulin 2, nidogen 2	# Anti-angiogenic	(133, 150, 151)
Collagen XVIII, C-terminal fragment is endostatin	180-200 (homotrimer) 22	Most capillaries & fenestrated endothelium of glomerulus, discontinous endothelium of liver sinusoids, splenic sinusoids & capillaries of lung alveoli	Fibulin 2, nidogen 2, nidogen 1 laminin 1/ nidogen1 complex, weak fibulin 1 binding	# Anti-angiogenic	

* kDa refers to the monomer/ fragment; # the function of collagens type XV and XVIII are largely undefined, the anti-angiogenic effects of the C-terminal fragments remain controversial.

Many of these molecules have been eliminated in mice but do not show defects in blood vessel formation during embryogenesis. Nevertheless, the data suggests that they may have specific functions in defined pathological situations (inflammation, metastasis), which requires further investigation. For example, nidogen 2 is highly expressed in endothelial cell basement membranes, as compared to nidogen 1, regardless of the age of the mouse (88), but there is no developmental blood vessel phenotype either in single knockouts (118, 154) or in double nidogen 1 and 2 knockout mice (R. Nischt and N. Smyth personal communication). Similarly, collagen type XV and XVIII are both localized in the basement membrane of most capillaries, with collagen type XVIII but not collagen type XV occurring in association with the fenestrated endothelium of the glomerulus, the discontinous endothelium of liver sinusoids and splenic

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sinusoids, and capillaries of lung alveoli (184). Collagen types XVIII and XV are best known for their reported anti-angiogenic activities of their C-terminal domains, endostatin and restin, respectively, which are released by proteolytic cleavage (123, 150, 151). However, this issue remains highly debatable. Endostatin is present at concentrations in the serum that would efficiently inhibit endothelial cell proliferation in vitro, leading to the suggestion that this circulating form may be involved in the homeostatic control of angiogenesis. However, elimination of one or both of these collagens does not affect angiogenesis in the embryo, and the only blood vessel related defect identified to date is a delay in the postnatal regression of hyaloid capillaries in the retina due to loss of collagen type XVIII (202). It can also not be excluded that the C-terminal fragments of collagen type XV and XVIII play a role in pathological angiogenesis (13).

Thrombospondins and SPARC (also known as BM40 or osteonectin) belong to a group of proteins that have been defined as "matricellular", to denote a subset of extracellular matrix molecules whose properties could be distinguished from structural macromolecules on the one hand, and more bioactive proteins such as cytokines, growth factors and proteases on the other hand (14, 16). They are not true basement membrane molecules as they occur at the cell surface but interact with basement membrane components. Thrombospondin 2 occurs primarily in association with developing vessels (14, 15), while both thrombospondin 1 and 2 are associated with endothelial cells in pathological conditions (wound healing and tumours) (15, 94, 95). Both thrombospondin 1 and 2 are potent inhibitors of angiogenesis (1, 94). Thrombospondin 1 can activate TGFβ and act as a negative regulator of MMP9 activation (143). TGFβ in turn can upregulate tissue inhibitors of metalloproteinases (TIMPs) and MMP 9 is a known activator of angiogenesis by its ability to release VEGF from the extracellular matrix. Thrombospondin 1 also binds CD36 on endothelial cells and activates apoptotic pathways (81). Multiple adhesive interactions have been identified for the thromobospondins including several plasma proteins such as fibrinogen, thrombin, and urokinase, and the extracellular matrix molecules fibronectin, collagen V and heparan sulphate proteoglycans (mainly syndecans). SPARC is associated with developing vessels and with vessels of tumours (17, 93). Unlike the thrombospondins, it is not an adhesive molecule but alters cell adhesion to other substrates and cell shape, it inhibits endothelial cell proliferation and regulates the activity of growth factors, such as FGF-2, PDGF

and VEGF. Elimination of SPARC, thrombopondins 1 or 2 in mice does not have any effect on angiogenesis in the embryo (56, 122, 199). However, increased vascular density has been reported in tumour angiogenesis models in both thrombospondin 1 and 2 null mice, consistent with a role as negative regulators of pathological vessel growth (27, 194).

It is worth mentioning collagen type VIII in this review as it is often used as a marker of blood vessels in vivo. Collagen type VIII is a short non-fibrillar collagen produced by endothelial cells and is localized in or close to most endothelial cell basement membranes in human but not in mouse tissues, where only few vessels contain collagen type VIII (157). In all species, collagen type VIII occurs in the thick corneal endothelial basement membrane, Descemet's membrane, where it forms a regular hexagonal array. It has been suggested that the hexagonal lattice structure of Descement's membrane represents a structural solution to the problem of creating a matrix that can resist compression, yet maintain an open porous and translucent structure. How collagen type VIII interacts with other basement membrane components is not known and its functional significance in endothelial cell basement membranes remains to be determined.

Only for some basement membrane molecules has clear evidence been provided by targeted gene elimination in mice for a role in blood vessel formation or stability, in particular fibronectin, fibulin-1, perlecan and collagen type IV. The best evidence exists for fibronectin and its receptor $\alpha5\beta1$ (48, 54, 201): Although endothelial cells form in the absence of fibronectin or $\alpha5\beta1$, studies involving embryoid bodies derived from integrin $\alpha5$ or fibronectin null embryonic stem cells have shown that the defect lies primarily in branching and stability of the primary vessel plexus (171), which is consistent with a role for fibronectin in the provisional matrix surrounding sprouting nascent vessels during embryogenesis.

Elimination of the collagen type IV [$\alpha1(IV)_2\alpha2(IV)$] in mice been recently shown to develop up to E10.5-E11.5 of gestation, whereupon ruptures in Reichert's membrane result in death (137). Interestingly, blood vessels form in these mice, but both large and small vessels show aberrant branching and are leaky and instable, even though pericyte recruitment appears normal. In view of the hypothesis that collagen type IV provides the scaffold integrating other basement membrane components, such as the laminins, nidogen and perlecan, it is interesting that although

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these molecules are secreted and incorporated into networks in the collagen type IV knockout mouse, a gradual reduction in staining for these molecules was apparent, suggesting that they were not retained within the basement membrane in the absence of collagen type IV. The data suggest a role for collagen type IV in vessel integrity under conditions of increasing mechanical demands.

Mice lacking perlecan specifically in endothelial cell basement membranes (64) and fibulin 1 knockout mice (92) show a surprisingly similar blood vessel phenotype, even though neither of these molecules occur exclusively in endothelial cell basement membranes. In both cases, dilations and bleeding occur in microvessels but not larger vessels, appearing at mid-gestation and leading to perinatal death. Vessels affected occur predominantly in skin, heart, gut and brain, however, endothelial cell development and recruitment of pericytes and smooth muscle cells appear normal. Although the basement membrane of affected vessels appears abnormal at the ultrastructural level in the endothelial cell specific perlecan null mouse (64), this is not the case in the fibulin 1 null mouse. The data suggest that the fibulin 1 phenotype may in part be due to loss of interactions with perlecan, one of its main binding partners and, consequently, reduced crosslinking to other endothelial cell basement membrane components, resulting in basement membrane instability without apparent ultrastructural changes (180). In vitro differentiation of perlecan null embryonic stem cells in embryoid bodies has revealed a reduced efficiency to form blood vessels, which can be rescued by addition of FGF-2 (64). This suggests that perlecan may have direct effects as an adhesive molecule on vessel formation, but also modulates angiogenesis by presenting growth factors to endothelial cells.

Taken together the data suggests that endothelial cells can form in the absence of any one of the major basement membrane components, including collagen type IV, perlecan, and nidogens, or in the absence of fibronectin as a major component of the provisional matrix surround forming vessels. Whether or not blood vessels can form without any laminin is not yet clear and would require generation of a mouse lacking the ubiquitously expressed laminin γ1 chain specifically in endothelium. However, the laminin α4 null mice provide some insight to the role of laminins in vessel formation and suggest that also laminins are not essential for endothelial cell formation. Rather, the loss of any one of the major endothelial cell basement membrane components has

clear consequences on vessel branching and/or stability and differentiation of specialized vessels. Much of this data has come from mice lacking basement membrane components at all sites, which results in various defects and complicates the analysis of blood vessel phenotypes. It is now necessary to generate mice that selectively lack basement membrane components in endothelial basement membranes, in order to better define their precise role on vessel stability or physiology.

Conclusion:

Although considerable advances have been made on deciphering the genes and soluble factors involved in vessel formation both in the embryo and in pathological situations, relatively little is known about cell-extracellular matrix interactions that may either directly or indirectly contribute to these processes. We are only just starting to understand the complexity of the endothelial cell basement membrane and its dynamic nature. This is certainly related to the difficult nature of basement membrane molecules, which are typically large, highly glycosylated, oligomeric molecules with the tendency to aggregate. As a consequence, isolation of these molecules without tissue digestion and denaturation of proteins is difficult and their use in in vitro structure-function assays has been limited to those few molecules that can be isolated in sufficient quantity and quality, e.g. laminin 1, fibronectin, vitronectin, thrombospondin 1. Therefore, the establishment of modified *p*CEP4 (136) or *p*Cis vectors (205) and the use of HEK293 cells (human kidney epithelial cell line) for production of basement membrane proteins or fragments thereof has been a significant achievement in basement membrane research. Both are widely used for production of various extracellular matrix and receptor molecules and are equally effective for both mouse and human proteins. To date molecules that have been recombinantly produced using this mammalian system include intact laminins 1, 8 and 10 (34, 90, 205) and various laminin-1 fragments, BM40, nidogen-1, fibulins, perlecan fragments, endostatin, integrins α6 and α7, and α-dystroglycan, to name just a few.

With sufficient amounts of purified recombinant protein and the advent of sophisticated protein cystallography and NMR techniques, the three dimensional structure of basement membrane proteins or specific domains thereof can now be determined. This information is crucial to the

development of models of how such molecules interact either with themselves and other proteins in basement membrane formation, or with their ligands on the cell surface or in the interstitium. Furthermore, co-crystallography techniques will allow definition of protein-protein interactions important for structure-function relationships. These techniques will also facilitate a more detailed analysis of growth factor binding to extracellular matrix molecules, or structural domains thereof, and their presentation to cell surface receptors. Such studies will provide insight into the different signals that can be induced by growth factors. An example of this is the recent discovery that VEGF-A signal different information to blood vessel endothelium when it is perceived by the endothelium bound to heparin as opposed to in the soluble form (146). Only heparin-bound VEGF-A, which probably reflects VEGF-A bound to basement membrane components or cell surface heparan sulfate proteoglycans in vivo, induces enhanced branching of the endothelium.

Precise information on the expression of basement membrane molecules and their ligands in particular tissues or at particular stages of development provide the necessary information for "targeted" elimination of expression, and molecular techniques now allow the design of precise genetic modifications in the mouse. Not only can defined nucleotide changes be engineered into the genome of the mouse, but genetic switches can be designed to target expression or ablation of any gene (for which molecular information is available) to any tissue at any defined time. The structure-function relationships defined by the in vitro methods discussed above will further permit identification of target sites in extracellular matrix molecules and/or growth factors for mutation and, subsequent, testing in mouse models for their effects on the endothelium.

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Figure Legends

Fig. 1 Schematic representation of the steps involved in endothelial cell differentiation, including endothelial cell tube formation, sprouting angiogenesis and pericyte recruitment; endothelial cell maturation into morphologically distinct capillary types; and summary of the temporal and spatial expression patterns of laminins in endothelial cell basement membranes in vivo.

Fig. 2 The major basement membrane components and their proposed interactions. Laminin and collagen type IV form independent networks that are linked by nidogens and the heparan sulphate proteoglycan, perlecan. Endothelial cell basement membranes consist principally of the laminin 8 and 10 isoforms, collagen type IV [$\alpha1(IV)_2\alpha2(IV)$], nidogen 1 and 2.

Fig. 3 Structure and domain description of prototype and endothelial cell laminins. The laminin 1 isoform is characterized by the presence of the laminin $\alpha1$ chain, which combines with laminin $\beta1$ and $\gamma1$ chains. By contrast, endothelial cell laminins are characterized by the presence of laminin $\alpha4$ and $\alpha5$ chains, which combine with laminin $\beta1$ and $\gamma1$ chains to form laminin 8 and laminin 10, respectively. All laminin chains share structural similarities defined as domains, shown in Roman numerals. Note the N-terminal short arms composed of laminin α, β and γ chain sequences, and the C-terminal globular (G) domains composed solely of laminin α chain sequences. The central long arm of the all laminins is a coiled-coiled structure composed of all three chains.

Fig. 4 Differential expression of endothelial cell laminin isoforms. All endothelial cells can produce the 240 kDa laminin $\alpha4$ and the 400 kDa laminin $\alpha5$ chain. However, the use of different endothelial cell lines derived from different tissues (bEND.3 from brain; mlEND.1 from mesenteric lymph nodes; SVEC from peripheral lymph nodes; eEND.2 from embryonic hemangiomas; sEND.1 from skin hemangiomas) (162) revealed that some endothelial cells express predominantly laminin $\alpha4$ and others laminin $\alpha5$, and that the expression pattern can be altered by the growth state or activation state of the cells. In general, proinflammatory cytokines induced laminin $\alpha4$ synthesis, while angiostatic agents, like progesterone, induce laminin $\alpha5$ synthesis.

Fig. 5 Three major transmembrane receptor types are responsible for cell binding to laminins: the integrins (principally β1 and β3 integrins), α-dystroglycan of the dystrophin-glycoprotein complex (DGC), and the Lutheran blood group glycoprotein. The DGC is connected to the cytoskeleton via dystrophin in myogenic tissues and by utrophin in all other tissues, including endothelium. Potential phorphorylation sites involved in mediating intracellular signals are marked by (P).

Fig. 6 Potential interactions between laminins 8 and 10 and cellular receptors present on endothelial cells. Interactions with intact laminins 8 and 10 and with defined domains of the laminin α4 and α5 chains are shown. * Denotes interactions that have been demonstrated using primary endothelial cells or endothelial cell lines in vitro assays; RGD-dependent binding of murine endothelial cells to domain IVa of laminin α5 is unpublished data from our laboratory.

Fig. 7 Illustration of the cell layers, basement membranes and their laminin composition of CNS blood vessels with and without an inflammatory cuff. Larger blood vessels consist of an inner endothelial cell layer with basement membrane (containing laminins α4 and α5), bordered by the meningeal epithelium and its basement membrane (containing laminin α1), and an outer astroglial basement membrane (containing laminin α2) and astrocyte endfeet. The meningeal and astroglial basement membranes are collectively termed the parenchymal basement membranes as they delineate the border to the brain parenchyma. Only at sites of local inflammation are the endothelial and parenchymal basement membranes distinguishable and define the inner and outer limits of the perivascular space where leukocytes accumulate before infiltrating the brain parenchyma. Examination of such sites demonstrates that mononuclear infiltration occurs across endothelial basement membrane containing only the laminin α4 and bordered by a parenchymal basement membrane containing laminin α1 and α2. The basement membrane of microvessels where no epithelial meningeal contribution occurs appear to have a composite basement membrane containing the endothelial cell laminins, laminin α4 and α5, and laminin α2 produce by the astrocytes and deposited at their endfeet. Reproduced from The Journal of Cell Biology, 2001, vol. 153, pp. 933-946, by copyright permission of The Rockefeller University Press.

Differentiation
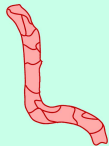
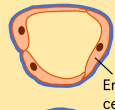Tube Formation
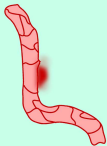
Sprouting 1
MMP activity

Sprouting 2
primitive matrix
EC proliferation
EC migration

Recruitment of
pericytes &
smooth muscle

Maturation

continuous

Endothelial
cell

fenestrated,
diaphragmed
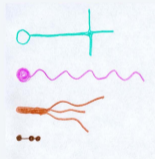
basement
membrane

fenestrated,
non-diaphragmed

discontinuous

Laminin Expression in Endothelium

Isoform	Developmental Stage	Blood Vessel Type
Laminin $\alpha 4$	E8.5 → adult	all vessels
Laminin $\alpha 5$	postnatal	capillaries some veins & venules not arteries
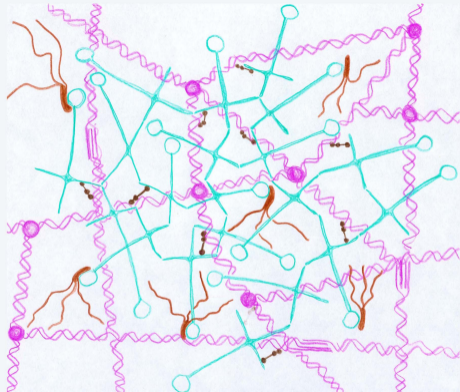
	laminin
	collagen IV
	perlecan
	nidogen

Laminin 1

Prototype Laminin

Laminin 8

Laminin 10

Endothelial Cell Laminins

α5

α4

β1   γ1

β1   γ1

subconfluence/ IL1

confluence/angiostatic

Laminin 8
sEND.1, eEND.2

Laminin 10
SVEC, mlEND.1, bEND.3

Laminin 8

Laminin α4
probably α4 LG4-5 → Integrin α3β1
Integrin α6β1

Laminin 10

Laminin α5 → Integrin α6β4
Integrin α6β1*
Integrin α3β1*

α5 LG1-3 → Integrin α3β1
Integrin α6β1

α5 VI → Integrin α3β1

α5 IVa (RGD) → Integrin α5β1
Integrin αvβ5*
Integrin αvβ3*

α5 LG3 → Lutheran blood group antigen*

α5 LG4-5 → α-dystroglycan*

Arachnoidea

Meningeal Epithelium
(laminin $\alpha 1$)

Parenchymal BM
(laminin $\alpha 2$ & $\alpha 1$)

Astrocyte Endfeet
(laminin $\alpha 2$)

Endothelial BM
(laminin $\alpha 4$ & $\alpha 5$)

Meningeal
epithelium

Parenchymal BM
(laminin $\alpha 1$ & $\alpha 2$)

Capillary

Endothelium

Composite BM
(laminin $\alpha 4$, $\alpha 5$ & $\alpha 2$)

Endothelial BM
(laminin $\alpha 4$ only)

LOCAL
INFLAMMATION

Parenchymal BM
(laminin $\alpha 1$ & $\alpha 2$)