DISTRIBUTION AND ADHESION-PROMOTING ACTIVITY OF α4 AND α5 CHAIN LAMININS IN HUMAN ENDOTHELIA AND CARCINOMAS

Noora Vainionpää

Academic dissertation
Institute of Biomedicine/Anatomy
Faculty of Medicine
University of Helsinki
DISTRIBUTION AND ADHESION-PROMOTING ACTIVITY OF α4 AND α5 CHAIN LAMININS IN HUMAN ENDOTHELIA AND CARCINOMAS

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ACADEMIC DISSERTATION
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In memory of my beloved mum
You live in my heart.
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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following studies, which are referred to in the text by their Roman numerals:


IV. Vainionpää N, Lehto V-P, Tryggvason K, Virtanen I. Alpha4 chain laminins are widely expressed in renal cell carcinomas and have a de-adhesive function. Laboratory Investigation 87:780-791. 2007.

In addition, some unpublished results are presented.

Publication I has also been presented in conjunction with the dissertation of Dr. Zenebech Wondimu (Karolinska University, Stockholm, Sweden, 2004).
## 2. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGM-2</td>
<td>endothelial cell growth medium-2</td>
</tr>
<tr>
<td>EGM-2MV</td>
<td>endothelial cell growth medium-2 microvascular</td>
</tr>
<tr>
<td>EHS-Lm</td>
<td>laminin-111 from mouse Engelbreth-Holm-Swarm tumour</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>FESEM</td>
<td>field emission scanning electron microscopy</td>
</tr>
<tr>
<td>Fn</td>
<td>fibronectin</td>
</tr>
<tr>
<td>FVIIIR:Ag</td>
<td>factor-VIII-related antigen</td>
</tr>
<tr>
<td>HDME</td>
<td>human dermal microvascular endothelial</td>
</tr>
<tr>
<td>HPAE</td>
<td>human pulmonary artery endothelial</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>Int</td>
<td>integrin</td>
</tr>
<tr>
<td>Lm</td>
<td>laminin</td>
</tr>
<tr>
<td>Lu</td>
<td>Lutheran glycoprotein</td>
</tr>
<tr>
<td>LYVE-1</td>
<td>lymphatic vessel endothelial hyaluronan receptor-1</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>Mr</td>
<td>relative molecular mass</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>platelet endothelial cell adhesion molecule-1</td>
</tr>
<tr>
<td>RCC</td>
<td>renal cell carcinoma</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Sol-Lu</td>
<td>soluble recombinant protein corresponding to extracellular domain of Lutheran glycoprotein</td>
</tr>
<tr>
<td>SPARC</td>
<td>secreted protein, acidic and rich in cysteine</td>
</tr>
<tr>
<td>TRITC</td>
<td>tetramethylrhodamine isothiocyanate</td>
</tr>
<tr>
<td>UEA-I</td>
<td>Ulex Europaeus-I agglutinin</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>vascular endothelial growth factor receptor-3</td>
</tr>
</tbody>
</table>
3. ABSTRACT

Basement membranes are specialized sheets of extracellular matrix found in contact with epithelia, endothelia, and certain isolated cells. They support tissue architecture and regulate cell behavior. Laminins are among the main constituents of basement membranes. Due to differences between laminin isoforms, laminins confer structural and functional diversity to basement membranes. The first aim of this study was to gain insights into the potential functions of the then least characterized laminins, α4 chain laminins, by evaluating their distribution in human tissues. We thus created a monoclonal antibody specific for laminin α4 chain. By immunohistochemistry, α4 chain laminins were primarily localized to basement membranes of blood vessel endothelia, skeletal, heart, and smooth muscle cells, nerves, and adipocytes. In addition, α4 chain laminins were found in the region of certain epithelial basement membranes in the epidermis, salivary gland, pancreas, esophagus, stomach, intestine, and kidney. Because of the consistent presence of α4 chain laminins in endothelial basement membranes of blood vessels, we evaluated the potential roles of endothelial laminins in blood vessels, lymphatic vessels, and carcinomas. Human endothelial cells produced α4 and α5 chain laminins. In quantitative and morphological adhesion assays, human endothelial cells barely adhered to α4 chain-containing laminin-411. The weak interaction of endothelial cells with laminin-411 appeared to be mediated by α6β1 integrin. The α5 chain-containing laminin-511 promoted endothelial cell adhesion better than laminin-411, but it did not promote the formation of cell-extracellular matrix adhesion complexes. The adhesion of endothelial cells to laminin-511 appeared to be mediated by Lutheran glycoprotein together with β1 and αvβ3 integrins. The results suggest that these laminins may induce a migratory phenotype in endothelial cells. In lymphatic capillaries, endothelial basement membranes showed immunoreactivity for laminin α4, β1, β2, and γ1 chains, type IV and XVIII collagens, and nidogen-1. Considering the assumed inability of α4 chain laminins to polymerize and to promote basement membrane assembly, the findings may in part explain the incomplete basement membrane formation in these vessels. Lymphatic capillaries of ovarian carcinomas showed immunoreactivity also for laminin α5 chain and its receptor Lutheran glycoprotein, emphasizing a difference between normal and ovarian carcinoma lymphatic capillaries. In renal cell carcinomas, immunoreactivity for laminin α4 chain was found in stroma.
and basement membranes of blood vessels. In most tumours, immunoreactivity for laminin α4 chain was also observed in the basement membrane region of tumour cell islets. Renal carcinoma cells produced α4 chain laminins. Laminin-411 did not promote adhesion of renal carcinoma cells, but inhibited their adhesion to fibronectin. Renal carcinoma cells migrated more on laminin-411 than on fibronectin. The results suggest that α4 chain laminins have a counteradhesive function, and may thus have a role in detachment and invasion of renal carcinoma cells.
4. REVIEW OF THE LITERATURE

4.1 EXTRACELLULAR MATRIX

Tissues, and thereby the whole human body, consist of cells and extracellular spaces filled by the extracellular matrix (ECM). ECM is a dynamic structure; it is produced and undergoes constant remodelling by cells. The amount and molecular composition of ECM are specific for each tissue and affect the form, physical properties, and function of the tissues. ECM maintains tissue architecture and forms passageways for macromolecule and cell trafficking. In addition, it regulates cell behaviour via interactions with cell surface receptors and serves as a reservoir of growth factors. Many ECM constituents are essential for embryonic development, and mutations in genes of ECM constituents lie behind several human diseases (for a thorough review of the functions of ECM constituents, see Aszódi et al. 2006).

The main constituents of ECM comprise glycosaminoglycans, proteoglycans, and fibrillar and other proteins (Aumailley and Gayraud 1998). Glycosaminoglycans are large unbranched molecules consisting of repeating disaccharide units. Proteoglycans comprise glycosaminoglycans bound to a core protein. Both glycosaminoglycans and proteoglycans are negatively charged molecules that attract cations and thereby also water molecules. They function in resistance to compression, as a space filler during embryonic development, in assembly of other ECM constituents, and in regulation of cell behaviour by binding to cell surface receptors, growth factors, and other proteins (Whitelock and Iozzo 2005, Bülow and Hobert 2006).

Among the fibrillar proteins, collagens are the most abundant proteins in the human body. Collagen molecules are triple helices of collagen α chains. In humans, 42 different collagen α chains are known to form at least 27 different collagen types. Many collagen types assemble to fibrils or networks, which give tissues their tensile strength. Collagens have collagen type-specific interactions with cell surface receptors and have various effects on cell behaviour (Myllyharju and Kivirikko 2004). Elastic fibres, formed by elastin and microfibrils consisting of fibrillins and other glycoproteins, give tissues their elastic properties. They also regulate cell behaviour by binding to cell surface receptors, growth factors, and other proteins (Kielty et al. 2002).

In addition, ECM comprises a wide variety of other proteins. Fibronectins are prod-
ucts of a single gene found in many soluble and insoluble isoforms, which function for instance in blood clotting and in cell adhesion, migration, growth, and differentiation (Pankov and Yamada 2002). Matricellular proteins, such as tenasin-C and SPARC (secreted protein, acidic and rich in cysteine; also known as BM-40/osteonectin), function as modulators of cell adhesion (Bornstein and Sage 2002). Laminins are primarily constituents of basement membranes (BMs), cell-associated sheets of ECM (Miner and Yurchenco 2004).

4.2 BASEMENT MEMBRANES

In contact with epithelia and endothelia and around certain isolated cells, including muscle, adipose, and Schwann cells, ECM forms specialized sheets called BMs. They were first identified by light microscopy by Bowman in 1845 (Kefalides 1979, Merker 1994). At the ultrastructural level, the BM region has been described to comprise three layers: an electron-lucent layer located next to the plasma membranes of cells and called the lamina lucida or lamina rara, an electron-dense layer called the lamina densa, and a layer between the lamina densa and the surrounding interstitial connective tissue called the lamina or pars fibroreticularis (Kefalides et al. 1979). Later, it has been shown, however, that the lamina lucida may be an artifact caused by tissue dehydration methods (Chan et al. 1993, Chan and Inoue 1994). The lamina densa forms the BM proper. The lamina fibroreticularis is present only in certain epithelia and includes collagen type VII anchoring filaments, which link these BMs to the interstitial connective tissue (Merker et al. 1994). Some authors use the term basal lamina particularly to denote the BMs lacking a lamina fibroreticularis, but according to the prevailing practice the term BM is preferred for all BMs (Kefalides et al. 1979, Merker et al. 1994).

Based on the current model (Timpl and Brown 1996, Yurchenco et al. 2004), the core of the BM consists of two independent networks of laminins and type IV collagens (Yurchenco and Furthmayr 1984, Yurchenco et al. 1992). These networks have been suggested to be interlinked via nidogens (entactins; Fox et al. 1991, Timpl and Brown 1996), but it seems that nidogens are not essential for BM formation (Bader et al. 2005, Gersdorff et al. 2007). All BMs comprise heparan sulphate proteoglycans, among which the most abundant is perlecan (Hassell et al. 1980, Murdoch et al. 1994). BMs variably comprise other constituents such as agrin, type XV and XVIII collagens,

BM s support tissue architecture and form boundaries between tissue compartments. During the development, growth, and regeneration processes BMs provide scaffolds, guiding migrating cells and their growing processes, such as axons of neurons, to their destinations (Merker et al. 1994, Timpl and Brown 1996, Colognato and Yurchenco 2000). BMs function as filters for macromolecule and cell trafficking (Noakes et al. 1995, Sixt et al. 2001a). The constituents of BMs regulate behaviour of cells via interactions with integrins and other cell surface receptors. These interactions influence intracellular signalling and cytoskeletal organization and have further effects on cell adhesion, migration, proliferation, polarization, differentiation, and cell survival (Timpl and Brown 1996, Miner and Yurchenco 2004, Aszódi et al. 2006). Also cleavage fragments of BM constituents, such as collagen type IV fragment designated as tumstatin, have been suggested to have specific effects on cells and to inhibit angiogenesis and reduce tumour growth (Ortega and Werb 2002, Kalluri 2003). In addition, BMs regulate cell behaviour by binding certain growth factors, enzymes, and ions, and thus concentrating their activity to specific regions (Timpl and Brown 1996, Erickson and Couchman 2000, Aszódi et al. 2006).

4.3 LAMININS

4.3.1 NOMENCLATURE AND STRUCTURE OF LAMININS

The first laminin was discovered from a BM-forming tumour, mouse Engelbreth-Holm-Swarm sarcoma, in 1979 (Timpl et al. 1979). Laminin was found to consist of three chains designated A, B1, and B2 (Sasaki et al. 1988, Beck et al. 1990). Alternative laminin chains and trimers were identified and given such diverse names as merosin, s-laminin, kalinin, and nicein (Hunter et al. 1989, Ehrig et al. 1990, Marinkovich et al. 1993). To reduce terminological diversity, laminins were then renamed in the order of their description. The first identified laminin was designated as laminin-1, its constituent chains as α1, β1, and γ1, and genes of the chains as LAMA1, LAMB1, and LAMC1 (Burgeson et al. 1994; Table 1).

To date, five laminin α, three β, and three γ chains, each encoded by separate genes,
have been identified in humans and other mammals (Colognato and Yurchenco 2000). In addition, the gene of the laminin β4 chain has been described (Scherer et al. 2003; GenBank accession AF028816), but it is unknown whether this chain is expressed as a protein. Laminin chains, including their major splice variants, are known to assemble into at least 15 αβγ heterotrimeric isoforms (Miner and Yurchenco 2004). The increasing number of the isoforms presented a problem in remembering the specific chain composition of each isoform. Moreover, the nomenclature of the protein domains was variable. Therefore, a simplified laminin nomenclature was described in 2005 (Aumailley et al. 2005; Table 1). According to this nomenclature, used also in this thesis, each laminin trimer is designated with three numbers based on the numbers of the constituent chains. For instance, laminin-1 is now called laminin-111 according to its chain composition α1β1γ1.

Laminins are cruciform or T-shaped molecules with a relative molecular mass (Mr) of approximately 400,000–900,000 (for structure of laminins, see Sasaki et al. 1988, Beck et al. 1990, Colognato and Yurchenco 2000, Aumailley et al. 2005). The

<table>
<thead>
<tr>
<th>Chain composition</th>
<th>Current abbreviation</th>
<th>Previous abbreviation</th>
<th>Previous names</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1β1γ1</td>
<td>Laminin-111</td>
<td>Laminin-1</td>
<td>EHS laminin</td>
</tr>
<tr>
<td>α2β1γ1</td>
<td>Laminin-211</td>
<td>Laminin-2</td>
<td>merosin</td>
</tr>
<tr>
<td>α1β2γ1</td>
<td>Laminin-121</td>
<td>Laminin-3</td>
<td>s-laminin</td>
</tr>
<tr>
<td>α2β2γ1</td>
<td>Laminin-221</td>
<td>Laminin-4</td>
<td>s-merosin</td>
</tr>
<tr>
<td>α3Aβ3γ2</td>
<td>Laminin-332 (-3A32)</td>
<td>Laminin-5 (-5A)</td>
<td>kalinin, nicein, BM 600</td>
</tr>
<tr>
<td>α3Bβ3γ2</td>
<td>Laminin-3B32</td>
<td>Laminin-5B</td>
<td></td>
</tr>
<tr>
<td>α3Aβ1γ1</td>
<td>Laminin-311 (-3A11)</td>
<td>Laminin-6 (-6A)</td>
<td>k-laminin</td>
</tr>
<tr>
<td>α3Aβ2γ1</td>
<td>Laminin-321 (-3A21)</td>
<td>Laminin-7 (-7A)</td>
<td>ks-laminin</td>
</tr>
<tr>
<td>α4β1γ1</td>
<td>Laminin-411</td>
<td>Laminin-8</td>
<td></td>
</tr>
<tr>
<td>α4β2γ1</td>
<td>Laminin-421</td>
<td>Laminin-9</td>
<td></td>
</tr>
<tr>
<td>α5β1γ1</td>
<td>Laminin-511</td>
<td>Laminin-10</td>
<td></td>
</tr>
<tr>
<td>α5β2γ1</td>
<td>Laminin-521</td>
<td>Laminin-11</td>
<td></td>
</tr>
<tr>
<td>α2β1γ3</td>
<td>Laminin-213</td>
<td>Laminin-12</td>
<td></td>
</tr>
<tr>
<td>α4β2γ3</td>
<td>Laminin-423</td>
<td>Laminin-14</td>
<td></td>
</tr>
<tr>
<td>α5β2γ3</td>
<td>Laminin-523</td>
<td>Laminin-15</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Nomenclature of laminins.
two or three short arms are formed by the N-termini of the single constituent chains, whereas the long arm is formed by the C-termini of all three chains. In the prototype of laminins, laminin-111 (Fig. 1), each short arm begins with a laminin N-terminal globular LN domain, followed by tandems of epidermal growth factor-like LE domains. Between the LE tandems are globular domains designated as L4 domains in the α chain and γ chains, and as laminin four (LF) domain in the β chain. At the centre of the cross, all three chains are bound together by disulphide bridges. In the long arm, the three chains form an α-helical laminin coiled-coil (LCC) domain, which is interrupted by a short loop in the β chain (laminin β knob or Lβ domain). At the C-terminal end of the LCC domain, the β and γ chains terminate and are connected by a disulphide bridge. The C-terminus of the α chain terminates in five globular LG domains. The L4 domains and LE tandems are designated by letters, and the single LE and LG domains by numbers starting from the N-terminus.

It seems that laminin trimers assemble intracellularly first by formation of βγ chain dimers, which are not secreted until the incorporation of α chains (Utani et al. 1994, Yuchenco et al. 1997). Laminins possess glycosylation sites (Beck et al. 1990), and at

Figure 1. Scheme of the structure of laminins-111 (α1β1γ1), -411 (α4β1γ1), and -511 (α5β1γ1) (modified from Aumailley et al. 2005).
least laminin α4 chain is modified by glycosaminoglycan side chains (Sasaki et al. 2001, Kortesmaa et al. 2002). In humans, alternative splicing occurs at least in the α3 and α4 chains; their shorter A forms differ from the longer B forms in the length of the N-terminal short arms (Ryan et al. 1994, Hayashi et al. 2002). Laminin α3A and γ2 chains are proteolytically cleaved in the N-terminal short arms (Marinkovich et al. 1992, Amano et al. 2000). Relevance of the N-terminal modifications is largely unknown, but they have been suggested to be important in correct organization of BMs and in interactions with other ECM constituents (Aumailley et al. 2003). Laminin α2, α3, α4 chains, and likely also α5 chain, are modified by proteolytic cleavage between LG3 and LG4 domains. As described for laminin α2 chain, the cleavage fragments may remain attached to the main molecules in tissues. Taking into account that the binding sites of most laminin receptors are found in the LG domains, the cleavage of LG domains has been suggested to influence laminin-cell interactions (Timpl et al. 2000).

Compared with laminin-111, all α1 and α2 chain laminins are similar in domain structure and have three short arms. Also laminin α3B chain has a "full-length" short arm with LN and L4 domains and LE tandems. Laminin α3A and α4 chains, on the contrary, lack short arm domains, with the exception of a few LE domains. Thus, α3A and α4 chain laminins (Fig. 1) have only two short arms. Laminin-332 is truncated also in the β and γ chains. α5 chain laminins (Fig. 1) have an elongated short arm because of additional LE domains in the α chain (Colognato and Yurchenco 2000).

4.3.2 BIOLOGICAL FUNCTIONS OF LAMININS

All BMs contain laminins, but they differ in the combinations of laminin isoforms. Each laminin isoform has a unique developmentally and spatially regulated expression pattern. Laminins have many functions due to interactions with themselves, with other constituents of ECM, and with cells. Because of differences in molecular structure and receptor binding, laminin isoforms confer structural and functional diversity to BMs (Miner and Yurchenco 2004, Yurchenco et al. 2004).

Laminins are among the main structural constituents of BMs. Mouse laminin-111 was first demonstrated in vitro to self-assemble into polymers. The polymerization requires low laminin concentration and calcium and is reversible. In this process, the LN domains of all three short arms of laminin monomers interact to form grossly hexagonal networks (Yurchenco et al. 1985, 1992). Similar networks have been observed in
situ independently of the presence of collagen type IV (Yurchenco et al. 1992). It has been demonstrated that also laminins-211 and -221, both possessing three short arms, have the ability to form polymers as well as co-polymers with laminin-111, whereas the truncated laminins -332 and -311 have not (Cheng et al. 1997). According to the current understanding, only the laminin isoforms possessing three short arms are capable of polymerization (Cheng et al. 1997, Li et al. 2002, Yurchenco et al. 2004). The truncated laminins may, however, be incorporated into BMs because of interactions with other laminin isoforms and BM constituents. Laminin-322, for instance, has been suggested to be incorporated into BMs due to interactions with laminins-311 and -321 (Champliaud et al. 1996, Rousselle et al. 1997).

Based on mouse studies, laminins appear to be crucial for early embryonic development (Leivo et al. 1980, Yurchenco et al. 2004). Laminin γ1 chain is a constituent of most known laminin isoforms and is the only laminin γ chain in the earliest embryonic BMs (Gersdorff et al. 2005). Homozygous null mutation of laminin γ1 chain gene (LAMC1) in mice prevents the formation of early embryonic BMs, arrests blastocyst differentiation, and leads to death at 5.5 days of development (Smyth et al. 1999; Table 2). Furthermore, embryoid bodies derived from laminin γ1 chain-null mouse embryonic stem cells fail to form BMs. Adding laminin-111 to the culture medium of the laminin γ1 chain-null embryoid bodies restores BM formation, whereas inhibition of laminin polymerization or blocking of LG4-LG5 domains prevents it (Murray and Edgar 2000, Li et al. 2002). In contrast, mice lacking type IV collagens, nidogens, or perlecan are able to form BMs and survive considerably longer than mice lacking laminin γ1 chain (Costell et al. 1999, Pöschl et al. 2004, Bader et al. 2005). Embryoid bodies from integrin β1- or dystroglycan-null mouse embryonic stem cells are able to form BMs (Li et al. 2002). Increasing genetic and developmental data suggest that laminin-laminin and cell-laminin interactions are the only prerequisites for the assembly of early embryonic BMs. Collagen type IV, nidogen, perlecan, and the other BM constituents are then incorporated into the initial laminin scaffolds to provide stability for the BMs (Yurchenco et al. 2004).

In addition to influencing the mechanical properties of cell surroundings by serving as structural constituents of BMs, laminins interact with cell surface receptors (Belkin and Stepp 2000, Miner and Yurchenco 2004). These interactions have been suggested
to affect adhesion, migration, proliferation, differentiation, polarization, and survival of cells, as well as metastasis of tumour cells (Rizzino et al. 1980, Terranova et al. 1980, 1982, McCarthy et al. 1983, 1988, Klein et al. 1988, Öcalan et al. 1988). Most of the early studies were done using the first identified mouse laminin-111. Laminin-111, however, has a restricted tissue distribution after early embryonic development (Klein et al. 1990, Virtanen et al. 2000). Studies with knock-out mice have emphasized the importance of laminins in normal development and tissue function (Miner and Yurchenco 2004; Table 2). For instance, laminin α1, β1, and γ1 chains seem to be crucial for early embryonic development (Smyth et al. 1999, Miner et al. 2004a). Laminin α2 chain is suggested to affect the myelination of motor nerves and function of skeletal muscle cells (Miyagoe et al. 1997, Patton 2000). Laminin α3 chain, in turn, appears critical for formation of cell-ECM adhesion structures called hemidesmosomes, and thereby for attachment of certain epithelia, as well as for formation of kidney glomeruli (Ryan et al. 1999, Abrass et al. 2006). Laminin α5 chain seems necessary for formation of kidney glomeruli, vascularization of placenta, septation of lungs and digits, and closure of the neural tube (Miner et al. 1998, Miner and Li 2000, Nguyen et al. 2002). Laminin β2 chain is needed for maintenance of glomerular filtration barrier and organization of the neuromuscular junction (Noakes et al. 1995, Patton 2000).

Further insights into the importance of laminins come from human diseases (Table 2). Epidermolysis bullosa is a heterogeneous group of diseases characterized by blistering of skin as a result of minor trauma or friction. The epidermis is normally attached to the dermis via a hemidesmosomal complex comprising a keratin cytoskeleton, integrin α6β4, laminin-332-containing epidermal BM, and collagen type VII anchoring filaments linking the BM to interstitial connective tissue. Mutations in laminin α3, β3, or γ2 chains of laminin-332 can cause a junctional type of epidermolysis bullosa (Pulkkinen and Uitto 1999). In a recent study, keratinocyte stem cells of a patient with laminin β3 chain-deficient junctional epidermolysis bullosa were transduced with laminin β3 chain cDNA and used to generate epidermal grafts for the patient. The results suggest that laminin gene therapy can be used at least to alleviate the symptoms of the disease (Mavilio et al. 2006). Laminin-332 is also involved in two other skin diseases: anti-laminin cicatrical pemphigoid is caused by autoantibodies against laminin-332, whereas laryngo-onycho-cutaneous syndrome is caused by
<table>
<thead>
<tr>
<th>ECM protein</th>
<th>Mouse phenotype</th>
<th>Reference</th>
<th>Human disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin α1</td>
<td>EL at E7, lack of Reichert's membrane</td>
<td>Miner et al. 2004a</td>
<td></td>
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<tr>
<td>Laminin α5</td>
<td>EL at E13.5-17, defects in glomerulogenesis, digit and lung septation, neural tube closure, placental vasculature</td>
<td>Miner et al. 1998, Miner and Li 2000, Nguyen et al. 2002</td>
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<tr>
<td>Laminin β1</td>
<td>EL at E5.5, failure of BM formation</td>
<td>Miner et al. 2004a</td>
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<tr>
<td>Laminin β2</td>
<td>L, defects in glomerular filtration and neuromuscular junctions</td>
<td>Noakes et al. 1995</td>
<td>Pierson syndrome (nephrotic syndrome, eye abnormalities, neural defects)</td>
<td>Zenker et al. 2004</td>
</tr>
<tr>
<td>Laminin β3</td>
<td>L, blistering of skin and mucous membranes, defective hemidesmosomes</td>
<td>Kuster et al. 1997</td>
<td>Junctional epidermolysis bullosa</td>
<td>Pulkkinen and Uitto 1999</td>
</tr>
<tr>
<td>Laminin γ1</td>
<td>EL at E5.5, failure of BM formation</td>
<td>Smyth et al. 1999</td>
<td></td>
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<tr>
<td>Laminin γ2</td>
<td>L, blistering of skin and mucous membranes, defective hemidesmosomes</td>
<td>Meng et al. 2003</td>
<td>Junctional epidermolysis bullosa</td>
<td>Pulkkinen and Uitto 1999</td>
</tr>
<tr>
<td>Laminin γ3</td>
<td>V, no defects described</td>
<td>Dénes et al. 2007</td>
<td></td>
<td></td>
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<tr>
<td>ECM protein</td>
<td>Mouse phenotype</td>
<td>Reference</td>
<td>Human disease</td>
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<tr>
<td>Collagen IV α1 and α2</td>
<td>Double knock-out: EL at E10.5-11.5, BM defects, bleeding, rupture of Reichert’s membrane, neuronal ectopias</td>
<td>Pöschl et al. 2004</td>
<td></td>
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<tr>
<td>Collagen VII α1</td>
<td>L, blistering of skin and mucous membranes, lack of anchoring fibrils</td>
<td>Heinonen et al. 1999</td>
<td>Dystrophic epidermolysis bullosa</td>
<td>Pulkkinen and Uitto 1999</td>
</tr>
<tr>
<td>Collagen XV α1</td>
<td>V, skeletal myopathy, cardiovascular defects</td>
<td>Eklund et al. 2001</td>
<td></td>
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<tr>
<td>Nidogens-1 and -2</td>
<td>Double knock-out: L, defects in myocardium, delayed development of lungs, restricted BM defects in heart and lungs, syndactyly</td>
<td>Bader et al. 2005, Böse et al. 2006, Gersdorff et al. 2007</td>
<td></td>
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<tr>
<td>Perlecan</td>
<td>EL-L at E10.5 or perinatal period, defects in heart and brain, endochondral ossification, neuromuscular junctions</td>
<td>Arikawa-Hirasawa et al. 1999, 2002a, Costell et al. 1999</td>
<td>Dyssegmental dysplasia, Silver-Handmaker type; Schwartz-Jampel syndrome (myotonic myopathy, chondrodysplasia)</td>
<td>Arikawa-Hirasawa 2001, 2002b</td>
</tr>
<tr>
<td>Agrin</td>
<td>EL after E18, defective neuromuscular junctions, branching of peripheral nerves</td>
<td>Gautam et al. 1996</td>
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<tr>
<td>Fibronectin</td>
<td>EL at E8.5, neural tube, mesodermal and vascular defects</td>
<td>George et al. 1993</td>
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*Table 2.* Mouse phenotypes and human diseases caused by mutations in selected extracellular matrix proteins. Abbreviations: E=embryonic day; EL=embryonic lethal; L=postnatal lethal; V=viable
mutation in the shorter form of laminin α3 chain (Domloge-Hultsch et al. 1994, McLean et al. 2003). Mutations in laminin α2 chain can cause congenital muscular dystrophy type 1A. The mutations disconnect the force-transmitting link between actin-cytoskeleton and laminin α2 chain-comprising BM of skeletal muscle cells. The patients suffer from severe muscle weakness, hypotonia, contractures, white matter abnormalities, epilepsy, peripheral neuropathy, respiratory and cardiovascular complications, and failure to thrive. The disease often leads to death in early childhood (Helbling-Leclerc et al. 1995, Jones et al. 2001). Moreover, mutations of laminin β2 chain affect the neuromuscular system in a disease called Pierson syndrome, which is characterized by congenital nephrotic syndrome, eye abnormalities, and impaired neural development (Zenker et al. 2004). In addition, laminins have been associated with such common human diseases as cancer, asthma, and infectious diseases, but the roles of laminins in the pathogenesis of these diseases remain elusive (Schéele et al. 2007).

4.3.3 ALPHA4 CHAIN LAMININS

The gene of the human laminin α4 chain, LAMA4, was discovered in 1994 (Richards et al. 1994, Iivanainen et al. 1995). It consists of 39 exons in chromosome 6q21, near the laminin α2 chain gene LAMA2 in 6q22-q23 (Richards et al. 1994, 1997). Laminin α4 chain is a truncated laminin α chain with only three complete LE domains flanked by two incomplete LE domains in the N-terminus. It has the greatest sequence and domain-structure similarity with the shorter form of the laminin α3 chain (Iivanainen et al. 1995, Richards et al. 1996).

Laminin α4 chain is a constituent of at least three laminin trimers, including laminins-411 (α4β1γ1), -421 (α4β2γ1), and -423 (α4β2γ3), previously designated as laminins-8, -9, and -14 (Miner et al. 1997, Libby et al. 2000). In rotary shadowing electron microscopy, laminin-411 appears as a T-shaped molecule (Frieser et al. 1997, Kortesmaa et al. 2000). Two laminin α4 chain splice variants have been described that differ from each other by 21 nucleotides near the N-terminus (Hayashi et al. 2002). Laminin α4 chain also undergoes proteolytic cleavage between the LG3 and LG4 domains, resulting in release of LG4 and LG5 domains. The cleaved form seems to predominate in tissues (Talts et al. 2000). Compared with other laminins, the laminin α4 chain is unique in having a chondroitin sulphate modification in the N-terminal short arm (Sasaki et al.

At the onset of this study, information about the localization of α4 chain laminins in human tissues relied largely on data for mRNA expression. Northern blotting of adult human tissues showed that laminin α4 chain mRNA is highly expressed in the heart, lung, skin, liver, ovary, and placenta, weakly expressed in the intestine, kidney, pancreas, testis, prostate, and skeletal muscle, and negligibly expressed in the brain (Richards et al. 1994, 1996, Iivanainen et al. 1995). In fetal human tissues, Northern blotting detected laminin α4 chain mRNA in the lung and kidney, but not in the brain or liver. In situ hybridization of fetal tissues detected laminin α4 chain mRNA in mesenchymal perialveolar cells of the lungs, vascular smooth muscle cells, skin dermis, adipose tissue, ependymal cells lining the brain ventricles, but not in other areas of the brain, heart, liver, alveolar epithelium of lungs, endothelial cells, or skeletal muscle fibres (Iivanainen et al. 1995). At the protein level, immunohistochemistry of human tissues with rabbit antisera detected laminin α4 chain in the vasculature of bone marrow, skeletal muscle, and brain (Gu et al. 1999, Patton et al. 1999, Ljubimova et al. 2001).

In adult mouse tissues, on the other hand, Northern blotting and in situ hybridization showed that laminin α4 chain mRNA was strongly expressed in the heart, endothelia, lungs, and skeletal muscle, and weakly expressed in the brain, spleen, liver, kidney, and testis. In developing mouse tissues, in situ hybridization revealed laminin α4 chain mRNA in mesenchymal tissues of the lungs, head, and kidney, dermis of skin, villi and submucosa of intestine, external root sheath of vibrissae, fat, peripheral nerves, dorsal root ganglia, cardiac, skeletal muscle, and smooth muscle cells, and endothelia (Frieser et al. 1997, Iivanainen et al. 1997, Miner et al. 1997, Sorokin et al. 1997a, Lefebvre et al. 1999). Immunohistochemistry of mouse tissues with rabbit antisera detected laminin α4 chain in Schwann cell sheaths, perineurium, neuromuscular junctions, developing skeletal muscle cells, heart muscle cells, smooth muscle cells, adipocytes, endothelial BMs of capillaries, interstitium of lung septa, and developing kidney glomeruli, but not in epithelia or liver (Iivanainen et al. 1997, Miner et al. 1997,
α4 chain laminins are produced by rodent fibroblasts, adipogenic, Schwannoma, and endothelial cells, human glioblastoma, erythromegakaryocytic, monocytic, and lymphoid cells, and human umbilical vein endothelial cells (Frieser et al. 1997, Niimi et al. 1997, Pierce et al. 1998, Geberhiwot et al. 2000, 2001, Pedraza et al. 2000, Talts et al. 2000, Fujiiwara et al. 2001). In mouse/chick interspecies hybrid intestines, laminin α4 chain was found to be a product of smooth muscle cells, but not of epithelial cells (Lefebvre et al. 1999).


Shortly after the beginning of this project, laminin α4 chain knock-out mice were described (Table 2). The mice show slightly increased mortality rates and decreased growth, impaired microvessel maturation, haemorrhages, anaemia, abnormal structure of capillary BMs, and mild locomotion defects (Thyboll et al. 2002). In another study, laminin α4 chain knock-out mice had improperly localized synaptic specializations (Patton et al. 2001). In synapses, laminin-421 is suggested to be linked to presynaptic voltage-gated calcium channels and spectrin cytoskeleton (Sunderland et al. 2000, Nishimune et al. 2004). Microarray studies have reported an association between overexpression of laminin α4 chain mRNA and human glial tumours and arterial occlusive disease (Ljubimova et al. 2001, Armstrong et al. 2002), but the role of
α4 chain laminins in the pathogenesis of these diseases remains obscure.

### 4.3.4 ALPHA5 CHAIN LAMININS

Laminin α5 chain is the most recently identified laminin α chain. The laminin α5 chain gene, LAMA5, was first discovered in the mouse in 1995 (Miner et al. 1995), and two years later in humans (Durkin et al. 1997). The human gene comprises 80 exons in chromosome 20q13.2-q13.3 (Durkin et al. 1997, Doi et al. 2002). Laminin α5 chain is a constituent of at least three laminin trimers, including laminins-511 (α5β1γ1), -521 (α5β2γ1), and -523 (α5β2γ3), previously designated as laminins-10, -11, and -15 (Miner et al. 1997, Libby et al. 2000). In rotary shadowing electron microscopy, laminin-511 appears as a cruciform molecule (Doi et al. 2002). The laminin α5 chain seems to be glycosylated and proteolytically cleaved in the C-terminus, likely between the LG3 and LG4 domains, resulting in two forms of M_r of ~350 000-400 000 (Tani et al. 1999, Doi et al. 2002).

Concerning the distributions of laminin α5 and α1 chains in human tissues, controversy arose because of an erroneous interpretation of the specificity of the widely used monoclonal antibody (MAb) 4C7. This MAb was raised against human placental laminins before the current knowledge about the diversity of laminin α chains, and it was thus considered to detect laminin α1 chain (Engvall et al. 1986). Discrepancies in tissue distribution of the laminin α1 chain between mouse and human tissues, as well as between laminin α1 chain mRNA expression and immunoreactivity pattern in human tissues, called the specificity of MAb 4C7 into question (Miner et al. 1997). Further studies indicated that MAb 4C7 is specific for the laminin α5 chain, and thus, all results on the distribution of the laminin α1 chain acquired with this antibody should be re-interpreted as distribution of the laminin α5 chain (Tiger et al. 1997). According to current knowledge, α5 chain laminins are the most widely expressed laminins and are found for instance in BMs of most epithelia, BMs of endothelia and smooth muscle cells, and BMs of developing skeletal muscle cells (Miner et al. 1995, 1997, 1998, Sorokin el. 1997a, 1997b, Ekblom et al. 1998, Määttä et al. 2001). Laminin-521 has a more restricted distribution pattern, being present, for example, in the glomerular BM of the kidney, neuromuscular junctions, perineurium of peripheral nerves, and BMs of arterial smooth muscle cells (Miner and Patton 1999).

The crucial role of α5 chain laminins in vivo has been revealed by laminin α5 chain-deficient mice (Table 2). The mice die between embryonic days 13.5 and 17 and show multiple developmental defects, including exencephaly, syndactyly, and defects in lung septation, glomerulogenesis, and placental vascularization (Miner et al. 1998, Miner and Li 2000, Nguyen et al. 2002). While α5 chain laminins are widely expressed in human carcinomas (Määttä et al. 2001), their role in the pathogenesis of human diseases remains elusive.

4.3.5 LAMININ RECEPTORS

Nearly a decade of intensive searching for receptors linking the actin cytoskeleton to ECM proteins, particularly to fibronectin fibrils, led to the discovery of integrins in 1985 (Pytela et al. 1985a, Hynes 2004). The first integrins described, later named integrins α5β1, αvβ3, and αIIbβ3, were isolated from human osteosarcoma cells and platelets and shown to bind to arginine-glycine-aspartic acid (RGD) sequences in fibronectin, vitronectin, and fibrinogen, respectively (Pytela et al. 1985a, 1985b, 1986, Hynes 1987, Hemler 1990). The integrins are heterodimeric transmembrane receptors that consist of α and β subunits. Currently, 18 α and 8 β subunits are known to combine into at least 24 isoforms in mammals. Alternative splicing and post-translational modifications further increase the diversity. In addition, genes of six more α subunits and one more β subunit have been described in the human genome, but the existence
of functional subunits has not been confirmed (van der Flier and Sonnenberg 2001, Hynes 2002a).

Most of the integrins mediate binding to ECM constituents such as fibronectin, vitronectin, collagens, and laminins. Some integrins, especially those of leukocytes, bind to counter-receptors on other cells. Other ligands of integrins include plasma proteins, complement factors, integrin inhibitors, and many human microbial pathogens. The ligand specificity of integrin is determined by both α and β subunits. Each integrin can typically bind to several ligands, and many ligands are recognized by several integrins (van der Flier and Sonnenberg 2001, Hynes 2002a). The ligand-binding affinity of integrins is allosterically regulated by conformational states. Interactions of the intracellular domains of integrin subunits affect the conformation and ligand affinity of the extracellular domains (inside-out signalling). Ligand binding to the extracellular domains leads to conformational changes, which influence intracellular signalling and cytoskeletal organization (outside-in signalling). The ligand-binding affinity is also affected by the presence of divalent cations; Mg$^{2+}$ and especially Mn$^{2+}$ increase the affinity, whereas Ca$^{2+}$ decreases it. Clustering of integrins affects ligand binding by increasing avidity (Hynes 2002a, Mould and Humphries 2004). Furthermore, integrin expression and activity is affected by other transmembrane and membrane-associated proteins, as well as by other integrins and other types of adhesion molecules (van der Flier and Sonnenberg 2001, Schwartz and Ginsberg 2002). Thus, the interactions are determined by the expression, affinity, avidity, and activation state of the integrins and the availability of the ligands in vivo.

Ligand occupation of integrins regulates signalling cascades, which intersect with signalling cascades of growth factor receptors and other receptors. Many cellular responses to soluble growth factors depend on ligand occupation of integrins. In contrast to receptors of soluble ligands, integrins form mechanical links between the cytoskeleton and ECM. The mechanical stresses mediated by integrins also influence intracellular signalling. These interactions regulate gene expression and cell growth, proliferation, differentiation, and survival. Ligand occupation of integrins has effects on the organization of the actin cytoskeleton and thereby on the formation of cell-ECM adhesion structures, membrane extensions, and cell adhesion and migration (Schwartz and Ginsberg 2002, Danen 2005).
Integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_7\beta_1$ are generally considered laminin receptors (Belkin and Stepp 2000, Hynes 2002a, Danen 2005). They bind to LG domains of laminin $\alpha$ chains. Integrin $\alpha_3\beta_1$ binds primarily to laminins -332 and -511/521. Integrin $\alpha_6\beta_1$ binds to all laminin isoforms, with a preference for laminins-111, -332, and -511/521. Integrin $\alpha_6\beta_4$ binds to all laminins, with the exception of laminin-332. In contrast to other integrins, which bind via linker proteins to the actin cytoskeleton, integrin $\alpha_6\beta_4$ is a constituent of cell-ECM adhesion complexes termed hemidesmosomes, which are intracellularly linked to intermediate filaments and extracellularly to laminin-332. Integrin $\alpha_6\beta_4$ has been suggested to also have an affinity for laminin-511/521 (von der Mark 2002, Miner and Yurchenco 2004, Nishiuchi et al. 2006).

In addition to these principal laminin receptors, collagen-binding integrins $\alpha_1\beta_1$ and $\alpha_6\beta_1$ bind to LN-domains of short arms of laminin $\alpha$1 and $\alpha$2 chains. Integrin $\alpha_2\beta_1$ can also bind to the laminin $\alpha$5 chain, as well as to short arms of laminin $\beta$1 and $\gamma$1 chains (Belkin and Stepp 2000, Pouliot et al. 2000, Doi et al. 2002, Miner and Yurchenco 2004). Integrin $\alpha_6\beta_1$ has been suggested to interact with laminin-111 (Forsberg et al. 1994). Laminin $\alpha$5 chain has two RGD sequences in the short-arm L4b domain (former IVa domain; Doi et al. 2002), which can interact with the RGD-binding integrin $\alpha_4\beta_3$ (Sasaki and Timpl 2001, Genersch et al. 2003). Laminin $\alpha$3 and $\alpha$4 chains have RGD sequences in the long arm (Richards et al. 1996). Laminin $\alpha$4 chain has, however, been suggested to interact with integrin $\alpha_v\beta_3$ via its LG domain (Gonzalez et al. 2001, Gonzalez et al. 2002).

Cells can interact with laminins also with non-integrin receptors such as dystroglycan (for reviews, see Durbeej et al. 1998, Barresi and Campbell 2006). Dystroglycan consists of two non-covalently bound subunits, $\alpha$- and $\beta$-dystroglycan, which are products of a single gene. $\beta$-dystroglycan is a transmembrane protein, whereas $\alpha$-dystroglycan is extracellular and highly glycosylated. Dystroglycan was first isolated from skeletal muscle cells as a constituent of the dystrophin-glycoprotein complex. In this complex, dystroglycan is intracellularly linked to dystrophin and further to actin, and extracellularly to laminin-211, forming a force-transmitting connection between cytoskeleton and ECM. Similar to mutations of the laminin $\alpha$2 chain, mutations of other constituents of this complex can lead to human muscle diseases. Dystroglycan is also found in many other tissues and appears to have a role in early mouse development,
structure and function of the central nervous system, peripheral nerves, and neuromuscular junctions, signal transduction, branching morphogenesis, cell adhesion, and as a receptor of human microbial pathogens. Dystroglycan binds to LG domains of laminin α1 and α2 chains and with relatively low affinity also to laminin α4 and α5 chains (Talts et al. 1999, 2000, Yu and Talts 2003). In addition, it binds to other ECM constituents such as perlecan and agrin (Durbeej et al. 1998, Barresi and Campbell 2006).

Another non-integrin laminin receptor is Lutheran glycoprotein (Lu). It was discovered in independent studies as a basal cell adhesion molecule expressed in carcinoma cells and as Lutheran erythrocyte blood group antigens (Campbell et al. 1994, Garin-Chesa et al. 1994, Parsons et al. 1995), which were later found to be alternatively spliced products of a single gene (Rahuel et al. 1996). Lu is a transmembrane glycoprotein belonging to the immunoglobulin superfamily (Campbell et al. 1994, Parsons et al. 1995). The smaller form of Mr 78 000 differs from the larger form of Mr 85 000 in lacking part of the cytoplasmic tail (Rahuel et al. 1996). Lu is widely expressed in human tissues (Campbell et al. 1994, Garin-Chesa et al. 1994, Parsons et al. 1995, Rahuel et al. 1996). It was first shown to mediate binding of erythrocytes, especially those of sickle cell disease patients, Lu-transfected human erythroleukemia cells, and Lu-transfected mouse fibroblasts to laminin (El Nemer et al. 1998, Udani et al. 1998, Zen et al. 1999). The binding of Lu to laminin was later demonstrated to be specific for laminin α5 chain (Parsons et al. 2001, Kikkawa et al. 2002). Lu appears to mediate adhesion of mesangial cells to laminin-511/521 together with integrin α3β1 (Kikkawa et al. 2003).

Less well characterized laminin receptors include heparan sulphate side chains of cell surface proteoglycans (e.g. syndecans), and sulphated glycolipids (e.g. sulphatides) (Hoffman et al. 1998, Talts et al. 1999, 2000, Utani et al. 2001, Lin and Kurpakus-Wheater 2002, Okazaki et al. 2002, Matsuura et al. 2004, Li et al. 2005). A voltage-gated calcium channel might also function as a receptor for the laminin β2 chain (Nishimune et al. 2004).

4.4 OTHER BASEMENT MEMBRANE CONSTITUENTS

Together with the network of laminins, a network of type IV collagens forms the core of BMs. All collagen molecules are triple helices of three α chains (for structure and
polymerization of type IV collagens, see Prockop and Kivirikko 1995). The collagen α chains are characterized by repeating Gly-X-Y sequences in which every third amino acid is glycine, essential for triple helix formation. The X and Y positions can have any amino acid other than glycine, often proline and 4-hydroxyproline, the latter being important for stability of the triple helix. The type IV collagen α chains consist of three domains: an N-terminal 7S domain, middle triple-helical domain, and a C-terminal globular non-collagenous (NC) 1 domain. The triple-helical domains have many interruptions to make the molecule more flexible. The type IV collagen molecules self-assemble into a covalently stabilized network. The C-terminal NC1 domains bind two triple-helical collagen molecules into dimers, and the N-terminal 7S domains bind four collagen IV molecules into tetramers. In addition to the end-to-end associations, the triple-helical portions can have lateral associations and form supercoiled structures. Six type IV collagen α chains, α1(IV)-α6(IV), have been identified. They seem to form three types of collagen molecules with chain compositions of α1.α1.α2(IV), α3.α4.α5(IV), and α5.α5.α6(IV). These collagen molecules form three types of networks. The network of α1.α1.α2(IV) is found in virtually all BMs. The network of α3.α4.α5(IV) is restricted to BMs of glomeruli, some tubuli of the kidney, lung, testis, cochlea, and eye. Collagen α5.α5.α6(IV) is found in the skin, esophagus, Bowman’s capsule of kidney, and smooth muscle. Due to heterodimerization via NC1 domains, α5.α5.α6(IV) forms a combined network with α1.α1.α2(IV) (Boutaud et al. 2000, Borza et al. 2001, 2002, Hudson et al. 2003).

Type IV collagens were previously considered to provide scaffolds for the assembly of BMs (Timpl et al. 1981, Yurchenco and Furthmayer 1984, Timpl and Brown 1996). Knock-out mice lacking both collagen α1(IV) and α2(IV) chains developed, however, for 10-11 days and deposited other BM constituents despite the absence of type IV collagen trimers (Table 2). Their embryonic lethality was likely caused by local structural deficiencies in BMs, bleeding in the heart and arteries, and rupture of the Reichert’s membrane. They also presented neuronal ectopias caused by defects in the BM separating the neuroectoderm from the mesenchyme (Pöschl et al. 2004). Thus, type IV collagens are apparently not essential for assembly of early embryonic BMs, but are needed later in development for stability and function of BMs (Pöschl et al. 2004, Yurchenco et al. 2004). Type IV collagens interact with cell surface receptors, such as
α₁β₁, α₂β₁, α₃β₁, and α₁₀β₁ integrins, and influence adhesion, proliferation, migration, and invasion of cells (Kalluri 2003, White et al. 2004, Pasco et al. 2005). Also the proteolytic fragments of type IV collagens have effects on cell behaviour. For instance, the NC1 domain of the α3(IV) chain, designated as tumstatin, interacts with αvβ₃ integrin and seems to induce apoptosis of proliferating endothelial cells, inhibit angiogenesis, and suppress tumour growth (Ortega and Werb 2002, Kalluri 2003). Type IV collagens are also involved in the pathogenesis of human diseases (Table 2). Alport’s syndrome, characterized by progressive renal failure and sensorineural deafness, is caused by a mutation in any of the chains of collagen α3α4α5(IV). Goodpasture’s syndrome, presenting with glomerulonephritis and lung haemorrhage, is caused by autoantibodies against the collagen α3(IV) chain (Hudson et al. 2003).

In addition to the network-forming type IV collagens, the BM region comprises many other collagens, such as collagen types XVIII, XV, and VII. Collagen XVIII is found in most endothelial and epithelial BMs (Saarela et al. 1998a). It is considered to be a homotrimer of three collagen α1(XVIII) chains and consists of ten triple-helical collagenous domains interrupted and flanked by non-collagenous domains (Oh et al. 1994, Rehn and Pihlajaniemi 1994, Saarela et al. 1998b, Kalluri 2003). Collagen XVIII is modified by heparan sulphate side chains, and can thus be considered a proteoglycan (Halfter et al. 1998). A cleavage fragment of its C-terminal domain, designated as endostatin, seems to inhibit endothelial cell proliferation, angiogenesis, and tumour growth (O’Reilly et al. 1997, Ortega and Werb 2002). The C-terminus of collagen type XVIII is located in the BM proper, whereas the N-terminus projects to interstitial ECM, suggesting a role for this collagen in attaching BM to the interstitial ECM (Fukai et al. 2002, Marneros et al. 2004). Lack of collagen XVIII in mice leads to delayed regression of hyaloid vessels and delayed and abnormal outgrowth of retinal vessels in developing eyes, formation of BM protein deposits under retinal pigment epithelium, hydrocephalus, abnormal cell morphology in the choroid plexus, thickening of BMs in the choroid plexus, skin, and kidney tubules, abnormal mesangial matrix in the kidney, and elevated serum creatinine levels (Table 2; Fukai et al. 2002, Marneros et al. 2004, Utirainen et al. 2004). In humans, lack of collagen XVIII can lead to Knobloch syndrome, which is characterized by advanced myopia, vitreoretinal degeneration, macular abnormalities, and occipital encephalocele (Sertié et al. 2000, Suzuki et al. 2002).
Collagen XV is structurally homologous to type XVIII collagen (Myers et al. 1992, Muragaki et al. 1994, Rehn et al. 1994). It is a proteoglycan modified by chondroitin sulphate side chains (Li et al. 2000). Its C-terminal domain, homologous to endostatin, has been proposed to inhibit angiogenesis (Ramchandran et al. 1999, Sasaki et al. 2000). Collagen XV is widely distributed in BMs of endothelia and certain epithelia as well as in the interstitial connective tissue (Myers et al. 1996, Hägg et al. 1997). Its functions are largely unknown. Mice lacking collagen XV show collapsed capillaries and endothelial cell degeneration in heart and skeletal muscles, progressive skeletal myopathy, and susceptibility to exercise-induced damage of skeletal and heart muscles (Table 2). Thus, collagen XV has been suggested to maintain the linkage between cells and ECM in muscles and capillaries. The BMs of mice lacking collagen XV appear, however, normal (Eklund et al. 2001, Aszódi et al. 2006).

Collagen VII is a major constituent of anchoring fibrils (Sakai et al. 1986a, Keene et al. 1987). Anchoring fibrils are found in the epidermis and in certain other epithelial BMs. They function in attaching the BM to the surrounding interstitial ECM. Mutations in type VII collagen can lead to the dystrophic form of the human skin blistering disease epidermolysis bullosa (Table 2; Pulkkinen and Uitto 1999).

The nidogen (entactin) family includes two glycoproteins, nidogen-1 and -2, which are ubiquitously found in BMs (Kohfeldt et al. 1998, Miosge et al. 2001, Salmivirta et al. 2002). They consist of three globular domains, G1-G3, connected by thread-like and rod-like regions (Fox et al. 1991, Kohfeldt 1998). The C-terminal G3 domain of nidogen-1 binds to the LEb3 (previous III4) -domain of the short arm of laminin γ1 chain, type IV collagens, and fibulins, whereas the more middle G2-domain binds to type IV collagens, perlecan, and fibulins (Fox et al. 1991, Mayer et al. 1993, Ries et al. 2001). Nidogen-2 has a weaker affinity for the laminin γ1 chain (Kohfeldt et al. 1998, Salmivirta et al. 2002). Because of their ability to link various BM constituents, especially laminins and type IV collagens, together, nidogens were previously thought to function as central organizers of BMs (Timpl and Brown 1996, Yurchenco et al. 2004). Mice lacking both nidogens die within 24 h after birth and have a thinner and less organized myocardium, bleeding within the heart wall, delayed lung development, and restricted defects in BMs of the heart and lungs (Table 2; Bader et al. 2005, Böse et al. 2006, Gersdorff et al. 2007). Thus, nidogens are not essential for formation BMs, but are required to
maintain the integrity of certain BMs (Yurchenco et al. 2004). Nidogens have been suggested to have a role in cell adhesion, growth, and migration, angiogenesis, axon guidance, formation of synapses and neuromuscular junctions, and localization of growth factors. These interactions with cells seem to be mediated by $\alpha_3\beta_1$, $\alpha_6\beta_1$, and RGD-binding integrins (Senior et al. 1992, Salmivirta et al. 2002, Ackley et al. 2003, 2005, Böse et al. 2006).

Perlecan is a proteoglycan found in virtually all BMs and certain other ECMs, e.g. in cartilage (Hassell et al. 1980, Murdoch et al. 1994, SundarRaj et al. 1995). The large ($M_r \sim 470\,000$) core protein comprises five domains and has a "beads-in-a-string" appearance (Paulsson et al. 1987, Yurchenco et al. 1987, Noonan et al. 1991, Kallunki and Tryggvason 1992). The core protein is modified with heparan sulphate side chains and to a lesser extent with other glycosaminoglycans (Paulsson et al. 1987, Brown et al. 1997, Costell et al. 1997). Perlecan has the ability to form oligomers and to bind other ECM constituents such as nidogens, fibulins, and fibronectin (Yurchenco et al. 1987, Reinhardt et al. 1993, Hopf et al. 1999). Perlecan was therefore considered to participate in the assembly of BMs (Iozzo et al. 1994, Yurchenco et al. 2004). Mice lacking the core protein die between embryonic day 10.5 and the perinatal period (Table 2). They present local defects in BM integrity in the developing heart and brain, leading to disrupted integrity of the myocardium, haemorrhages to the pericardium, exencephaly, defective endochondral ossification, and lack of acetylcholinesterase from neuromuscular junctions (Arikawa-Hirasawa et al. 1999, 2002a, Costell et al. 1999). Thus, perlecan is not essential for BM formation, but is essential for integrity of certain BMs and localization of acetylcholinesterase to neuromuscular junctions (Aszödi et al. 2006). In humans, mutations in the perlecan gene can lead to skeletal dysplasias, including dyssegmental dysplasia of Silverman-Handmaker type and Schwarz-Jampel syndrome (Table 2; Arikawa-Hirasawa et al. 2001, 2002b). Perlecan seems to interact with cell surface receptors, including $\beta_1$ and $\beta_3$ integrins, and with growth factors, such as basic fibroblastic growth factor, and has been associated with many cellular events (Hayashi et al. 1992, Aviezer et al. 1994, Iozzo et al. 1994, Knox and Whitelock 2006).

In addition to the main constituents, BMs variably contain other macromolecules. Among these, agrin is another heparan sulphate proteoglycan found in the BMs of neuromuscular junctions, the lung, kidney, and capillaries, and in the central nervous
system (Tsen et al. 1995, Barber and Lieth 1997, Groffen et al. 1998a, 1998b). It is best characterized as an organizer of neuromuscular junctions and other synapses. It has also been suggested to have effects on organization of the actin cytoskeleton, stability of myotube BMs in laminin α2 chain-deficient muscular dystrophy, and activation of T-lymphocytes (Bezakova and Ruegg 2003).

Fibronectin is a glycoprotein found in soluble form in plasma and other body fluids (plasma fibronectin) and in insoluble form in tissues (cellular fibronectin). In tissues, it is primarily considered to be a constituent of interstitial ECM, but it is also found in BMs (Stenman and Vaheri 1978, Sanes 1982, Ruoslahti 1988). Fibronectin consists of three types (I-III) of repeating units, all of which are also present in other molecules (Kornblihtt et al. 1985, Ruoslahti 1988, Pankov and Yamada 2002). It is usually found as a dimer, in which two fibronectin monomers are bound together near their C-termini by disulphide bonds; however in certain tissues it also manifests as a monomer (Ruoslahti 1988, Burton-Wurster et al. 1999, Pankov and Yamada 2002). Fibronectin is a product of a single gene, but it has as many as 20 alternatively spliced variants in man. Alternative splicing occurs at three sites: extracellular domain A (EDA, also called EIIIA or EDI) and extracellular domain B (EDB, also called EIIIB or EDII) can be included or excluded from fibronectin mRNA by exon skipping, whereas more complicated splicing of the V region (for variable, also called type III connecting segment or IIICS) can produce five variants (ffrench-Constant 1995). Plasma fibronectin, primarily produced by liver hepatocytes, almost completely lacks EDA and EDB domains. Plasma fibronectin has been proposed to account for a large proportion of the fibronectin located in the ECM of other tissues (ffrench-Constant 1995, Moretti et al. 2007). Fibronectin found in tissues comprises variable proportions of EDA and EDB domains. The expression of both forms declines during development (ffrench-Constant 1995). Among the five V variants, one seems to be rare, whereas the other four are produced by many cell types (Hershberger and Culp 1990, ffrench-Constant 1995). Further, fetal and tumour tissues express a differently glycosylated form of fibronectin, termed oncofetal fibronectin (Matsuura and Hakomori 1985, Matsuura et al. 1989).

Fibronectin binds to a dozen integrins, including the first identified fibronectin receptor α₅β₁, as well as to cell surface proteoglycans, e.g. syndecans (Pytela et al. 1985a, Plow et al. 2000, Woods et al. 2000). Fibronectin is generally considered to promote
cell adhesion and migration, and to have important roles in various biological processes, such as embryogenesis, wound healing, haemostasis, and malignancy (Wartiovaara et al. 1978, Hynes and Yamada 1982, Grinnell 1984, Ruoslahti 1999). Mice lacking fibronectin die by embryonic day 8.5 likely due to defects in mesodermal differentiation (Table 2; George et al. 1993). Experiments with mice having inducible deletion of fibronectin suggest that fibronectin supports neuronal survival and reduces brain injury after ischaemia, promotes thrombus formation, reduces virulence of human pathogen Streptococcus pyogenes, and is needed for the function of anti-angiogenic fragments of ECM proteins (Sakai et al. 2001, Ni et al. 2003, Yi et al. 2003, Nyberg et al. 2004). The exact roles of the various fibronectin variants are unclear. The V region comprises a binding site for integrin α₄β₁, whereas the EDA domain comprises binding sites for α₄β₁ and α₉β₁, and possibly also for α₅β₁ and other RGD-binding integrins (ffrench-Constant 1995, Manabe et al. 1997, Liao et al. 2002). Mice lacking the EDA domain of fibronectin show a shorter life span, abnormal wound healing, and impaired motor coordination, whereas mice constitutively expressing the EDA domain show a shorter life span, decreased fibronectin levels due to a reduced amount of plasma fibronectin in all tissues, and decreased locomotory activity (Muro et al. 2003, Chauhan et al. 2005, Moretti et al. 2007). Mice lacking the EDB domain develop normally, but their fibroblasts grow slowly and deposit a decreased amount of fibronectin in vitro (Fukuda et al. 2002).

4.5 INTERACTIONS OF CELLS WITH THE EXTRACELLULAR MATRIX

4.5.1 CELL ADHESION

Adhesion of cells to the ECM is essential for various fundamental processes such as cell migration and maintenance of tissue integrity. It is involved in transmembrane signalling, thereby regulating cell behaviour and fate and assembly of ECM (Gumbiner 1996, Hynes 1999). Current knowledge on mechanisms of cell adhesion is largely based on two-dimensional in vitro experiments (Adams 2002, Cukierman et al. 2002). The earliest steps in cell attachment are suggested to be mediated by stereospecific chemical interactions of cell-surface-associated hyaluronan. The hyaluronan-mediated attachment is transient and replaced by binding of more specific cell surface receptors, such
as integrins, to their ligands (Zimmerman et al. 2002, Cohen et al. 2004, Zaidel-Bar et al. 2004). After the initial attachment, the cells form protrusions, including thin, needle-shaped filopodias and broader, sheet-like lamellipodias. The formation of these protrusions involves actin polymerization and has been associated with sensory and exploratory functions, cell motility, organization of membrane domains, phagocytosis, and formation of cell-matrix adhesion complexes (Adams et al. 2002, Small et al. 2002, Faix and Rottner 2006).

In cell-matrix adhesion complexes, transmembrane receptors, primarily integrins, link ECM constituents to intracellular protein complexes and thereby to the cytoskeleton and intracellular signalling cascades (Gumbiner 1996, Hynes 1999, Zamir and Geiger 2001). Largely based on experiments performed with fibroblasts and endothelial cells in vitro, a model for the maturation of certain cell-matrix adhesion complexes has been proposed (Geiger et al. 2001, Zamir and Geiger et al. 2001, Cukierman et al. 2002, Zaidel-Bar et al. 2004). When the cells spread, small dot-like structures, designated as focal complexes, appear at the edges of lamellipodia. The formation of focal complexes is induced by Rho family guanine triphosphatase Rac. The constituents of focal complexes assemble gradually, eventually comprising, for instance, αvβ3 integrin, talin, paxillin, vinculin, and focal adhesion kinase embedded in a diffuse meshwork of actin. The focal complexes have been associated with cell motility. When the lamellipodia continue protruding, new focal complexes appear on the leading front and old ones disappear. When the lamellipodia stops protruding or retracts, local mechanical forces induce some of the focal complexes to mature into focal adhesions in a Rho- and actomyosin-contractility-dependent manner (Rottner et al. 1999, Ballestrem et al. 2001, Riveline et al. 2001, Zaidel-Bar et al. 2003, 2004).

Focal adhesions (also termed focal contacts) are larger, streak-like structures usually located at the cell periphery. They are considered to reflect close and tight adhesion to the ECM. They seem to act as sensors of force and as sites at which force is applied to both the cytoskeleton and ECM, affecting their organization. They have been reported to comprise over 50 proteins. αvβ3 integrin, talin, paxillin, vinculin, and focal adhesion kinase are found in both focal complexes and focal adhesions, but focal adhesions can be distinguished from focal complexes, for instance, in the presence of zyxin and by their linkage to actin stress fibres (Zamir et al. 1999, Ballestrem et al. 2001, Geiger et al.
In the presence of Rho-activity and actomyosin-contractility, α₅β₁ integrin and tensin seem to move from focal adhesions towards the centre of the cell to form another type of cell-matrix adhesion, termed fibrillar adhesion. Fibrillar adhesions (also called ECM contacts) are usually elongated structures, which seem to be associated with fibronectin fibrillogenesis. Focal adhesions, in contrast, are usually devoid of α₅β₁ integrin, tensin, and fibronectin (Avnur and Geiger 1981, Chen and Singer 1982, Zamir et al. 1999, 2000, Katz et al. 2000, Pankov et al. 2000). Further, in three-dimensional ECM preparations, the cells form cell-matrix adhesions, in which constituents of focal adhesions and fibrillar adhesions, such as paxillin, vinculin, α₅β₁ integrin, tensin, and fibronectin, co-localize. These three-dimensional adhesions have been suggested to be the end-points of the maturation process from focal complexes to focal adhesions and further to fibrillar adhesions. A similar maturation process has been proposed to exist in vivo (Cukierman et al. 2001, 2002).

In addition to the aforementioned adhesions, characterized primarily in fibroblasts and endothelial cells, other types of cell-matrix adhesions have been identified in certain other cell types. Briefly, podosomes are integrin-linked cell-matrix adhesions found in macrophages, osteoclasts, dendritic cells, carcinoma cells, and transformed fibroblasts. They are primarily present in migratory cells and cells crossing tissue boundaries. Podosomes consist of a core of filamentous actin, surrounded by a ring of other proteins, e.g. vinculin and talin. They also contain and secrete proteins associated with matrix degradation, including matrix metalloproteinases. Thus, podosomes have been associated with cell invasion and ECM remodelling (Linder and Aepfelbacher 2003). Hemidesmosomes are cell-matrix adhesions found in stratified and certain other epithelia, e.g. epidermis, cornea, parts of gastrointestinal and respiratory tracts, and amnion. The core of hemidesmosome comprises α₆β₄ integrin; this is intracellularly linked to a protein plaque that is further linked to keratin intermediate filaments, and extracellularly to laminin-332, which appears to be further linked to collagen type VII anchoring filaments. Hemidesmosomes have a key role in attaching epithelia to the underlying BMs and also participate in signalling functions (Borradori and Sonnenberg 1999, Nievers et al. 1999).
4.5.2 CELL MIGRATION

Cell migration is a fundamental process involved in embryogenesis, function, renewal and repair of tissues, as well as in various pathological conditions. The current model on cell migration is largely based on two-dimensional in vitro experiments with various cell types. Considering slow moving cells, such as fibroblasts, cell migration can be viewed as a cyclic process (Lauffenburger and Horwitz 1996, Ridley et al. 2003, Even-Ram and Yamada 2005). In response of extracellular stimuli, including concentration gradients of chemokines, growth factors, or ECM molecules, cell migration begins by polarization of the cell into distinct leading and trailing edges. Polarization seems to be regulated primarily by Rho family guanidine triphosphatase Cdc42. Polarization involves defining the sites of actin polymerization and formation of protrusions, directing distinct molecules to different subcompartments of the cell, and in many cells localizing the microtubule-organizing centre and the Golgi complex in front of the nucleus on the leading edge side (Etienne-Manneville and Hall 2001, Ridley et al. 2003). Formation of filopodias and lamellipodias is driven by actin polymerization and regulated by Cdc42 and Rac, respectively. In filopodias, actin filaments are organized to parallel bundles, whereas in lamellipodias actin-associated proteins, such as Arp2/3, enable actin filaments to form a branching network (Nobes and Hall 1995, Small et al. 2002, Pollard and Borisy 2003). The lamellipodias are stabilized by focal complexes, which appear at the leading edge. The focal complexes seem to be used as primary sites of traction to move the cell forward. The force needed for traction derives from interaction of actin with myosin II. The migration cycle is completed by disassembly of cell-matrix adhesions and retraction at the trailing edge of the cell. Disassembly of cell-matrix adhesions seems to involve calcium-dependent protease calpain and microtubule dynamics. Retraction of the trailing edge is apparently dependent on Rho and interaction of actin with myosin II (Ballestrem et al. 2000, 2001, Beningo et al. 2001, Bhatt et al. 2002, Ridley et al. 2003, Small and Resch 2005).
4.6 ENDOTHELIAL BASEMENT MEMBRANES

4.6.1 BLOOD VESSELS

In general, blood vessels consist of three layers. The innermost layer, the tunica intima, comprises a single layer of endothelial cells surrounded by endothelial BM and subendothelial connective tissue. The medial layer, the tunica media, is characterized by layers of smooth muscle cells and elastin, whereas the outermost layer, the tunica adventitia, consists of connective tissue. The thickness and structural complexity of each layer depend on the size and function of the vessel. In capillaries, i.e. vessels with the smallest diameter, the endothelial BM is only surrounded by smooth muscle-like cells called pericytes (Gallagher 1997, Veinot et al. 2001).

Interest in the constituents of endothelial BM and subendothelial connective tissue has recently focused on their role in the formation and growth of new blood vessels, i.e. in neovascularization (Patarroyo et al. 2002, Kalluri 2003, Davis and Senger 2005, Hallmann et al. 2005, Rhodes and Simons 2007). Neovascularization comprises the processes of vasculogenesis, arteriogenesis and angiogenesis. Vasculogenesis denotes de novo formation of immature blood vessels by angioblasts during development or remodelling of pre-existing vessels by circulating endothelial progenitor cells. Arteriogenesis denotes maturation of newly formed arteries or remodelling, e.g. growth or enlargement, of pre-existing arteries. Angiogenesis was previously used to describe formation of new capillaries from post-capillary venules, but is nowadays more generally applied to describe growth and remodelling of simple vascular networks into more complex networks (Carmeliet 2000). Inappropriate angiogenesis has been associated with as many as 70 disorders, the best-known being cancer, ocular, and inflammatory diseases (Carmeliet 2005). Blood vessels of tumours differ from those of normal tissues in gene expression of endothelial cells and pericytes, as well as in the thinner, leakier, and more irregular structure of the vessel wall (Pasqualini et al. 2002).

Adhesion of endothelial cells to ECM constituents regulates migration, proliferation, differentiation, and survival of endothelial cells, response of endothelial cells to growth factors, and maintenance of endothelial integrity. These interactions are central in vessel formation, blood clotting, and atherosclerosis (Dejana et al. 1993,
Patarroyo et al. 2002, Kalluri 2003, Katsuda and Kaji 2003, Sottile 2004, Davis and Senger 2005, Hallmann et al. 2005, Rhodes and Simons 2007). Endothelial cells appear quiescent when bound to endothelial BM. Thus, some authors have suggested that endothelial BMs inhibit angiogenesis-related functions and angiogenesis is induced by degradation of endothelial BMs by matrix-digesting enzymes such as matrix metalloproteinases, followed by liberation of growth factors sequestered to endothelial BMs, exposure of cryptic sites from BMs constituents, and exposure of constituents of interstitial ECM (Kalluri 2003, Rhodes and Simons 2007). The exact roles of constituents of endothelial BMs in the formation, maintenance, and function of blood vessels remain, however, largely unclear.

In mouse tissues, laminin α4 and α5 chains have been localized to blood vessel endothelia by immunohistochemistry (Iivanainen et al. 1997, Miner et al. 1997, 1998, Patton et al. 1997, Sorokin et al. 1997a, 1997b, Talts et al. 2000, Sixt et al. 2001a). Cultured mouse and bovine endothelial cells produce laminin α4, α5, β1, and γ1 chains, constituents of laminins-411 and -511 (Tokida et al. 1990, Frieser et al. 1997, Sorokin et al. 1997b). Sorokin and co-workers have shown that the production of laminin α4 chain mRNA in certain mouse endothelial cell lines in vitro is increased by interleukin-1, lipopolysaccharide, and tumour necrosis factor α, and decreased by hydroxymethylprogesterone. The production of laminin α5 chain mRNA is increased by interleukin-1 and tumour necrosis factor α, but also increased by hydroxymethylprogesterone (Frieser et al. 1997, Sixt et al. 2001a). With immunohistochemistry and in situ hybridization, laminin α4 chain has been detected in mouse blood vessels after embryonic day 11 (Frieser et al. 1997, Iivanainen et al. 1997, Patton et al. 1997). With immunohistochemistry, laminin α5 chain has been observed in mice in placental capillaries by embryonic day 9.5 and in larger blood vessels by embryonic day 13 (Sorokin et al. 1997b, Miner et al. 1998), but with in situ hybridization laminin α5 chain mRNA was seen in capillaries 3–4 weeks after birth (Sorokin et al. 1997b). Based on this data, Sorokin and co-workers concluded that blood vessel endothelia comprise two laminin isoforms: laminin-411 is expressed by all endothelia at all developmental stages and is strongly upregulated by inflammatory cytokines and growth factors, whereas laminin-511 is only found in certain blood vessels 3–4 weeks after birth and is upregulated by strong inflammatory signals or anti-angiogenic agents (Hallmann et al. 2005). The phenotypes of laminin α4 chain- and laminin α5 chain-deficient mice suggest important roles for these lam-
inins in development of microvessels (Miner et al. 1998, Miner and Li 2000, Thyboll et al. 2002). In a mouse model of autoimmune encephalomyelitis, transmigration of mononuclear cells through endothelial BM was detected only at sites where laminin α4 chain was present but laminin α5 chain was absent, implying a role for laminins in regulation of endothelial transmigration (Sixt et al. 2001a).

Laminin α2 chain has also been detected in endothelia of skeletal muscle and the thymus in mice (Ringelmann et al. 1999, Talts et al. 2000). Laminin β2 chain, in turn, has been observed in endothelia of skeletal muscle and in the glomerular BM of the kidney (Sanes et al. 1990, Patton et al. 1997). Laminin α3 and γ2 chains may be present in pulmonary vasculature of rats (Wang et al. 2004). Laminin α3 chain-deficient mice have bleeding in the limbs (Ryan et al. 1999), whereas laminin β2 chain-deficient mice show proteinuria (Noakes et al. 1995). No other vascular defects have been reported in mice lacking laminin α2, α3, or β2 chains (Noakes et al. 1995, Miyagoe et al. 1997, Ryan et al. 1999, Aszódi et al. 2006).

Information about the presence of laminins in blood vessel endothelial BMs in human tissues is scattered. Laminin α4 chain has been detected in blood vessels of skeletal muscle, bone marrow, and brain (Gu et al. 1999, Patton et al. 1999, Ljubimova et al. 2001). Laminin α5, β2, β1, and γ1 chains have been found in, for instance, blood vessels of skeletal muscle and normal tissue structures of various tumour specimens (Patton et al. 1999, Määttä et al. 2001). Laminin α2, α3, and β3 chains have been suggested to be present in capillaries of lymphatic tissues (Määttä et al. 2004).

4.6.2 LYMPHATIC VESSELS

Lymphatic vessels return excess fluid and macromolecules from extracellular spaces of tissues back into blood circulation. They serve as passageways for cells of the immune system. Impairment of these functions leads to lymphoedema and disturbances of immune responses (Oliver and Alitalo 2005, Randolph et al. 2005). Lymphatic vessels are among the main routes for dissemination of cancer cells. Lymph node metastasis is a prognostic factor for clinical outcome in many carcinomas. It is still unclear, however, whether the intravasation of cancer cells occurs into pre-existing lymphatic vessels or whether it requires formation of new lymphatic vessels, i.e. lymphangiogenesis (Oliver and Alitalo 2005, Wilting et al. 2005).

Lymphatic capillaries are fluid conduits lined by endothelial cells, but they differ from blood capillaries in many structural aspects: initial lymphatic capillaries are blind-ended structures. Lumina of lymphatic capillaries are more irregular and wider than those of blood capillaries. Lymphatic endothelial cells may overlap each other, form interdigitations, and frequently lack tight junctions. The cytoplasm of lymphatic endothelial cells is attenuated, with the exception of the perinuclear region and nucleus, which protrude into the vessel lumen. The lymphatic capillaries are not enveloped by smooth muscle-like cells (Leak 1970, Schmid-Schönbein 1990, Ji 2006). The larger lymphatics, i.e. precollectors and collecting vessels, differ from lymphatic capillaries in the presence of smooth muscle-like cells in their intima (Schmid-Schönbein 1990, Sacchi et al. 1997, 1999). In even larger lymphatics, i.e. lymphatic trunks and right lymphatic and thoracic ducts, the structure of the vessel wall is the three-layered one typical of most vascular channels: tunica intima comprising a layer of endothelial cells and a narrow layer of subendothelial connective tissue, tunica media comprising mainly smooth muscle cells, and adventitia comprising connective tissue (Boggon and Palfrey 1973, Gallagher 1997). Many but not all tumour types lack intratumoural lymphatic vessels (Jackson et al. 2001, Mouta Carreira et al. 2001, Williams et al. 2003, Koukourakis et al. 2005). Despite the many differences described between normal and tumour blood vessels (Pasqualini et al. 2002), little is known about differences between normal and tumour lymphatic vessels (Cao 2005).

As in the case of blood vessels, ECM constituents have been suggested to have important roles in the development and function of lymphatic vessels (Pepper and Skobe
Lymphatic endothelia seem to be attached to fibrillin anchoring filaments, which have been suggested to function in resisting collapse of vessels and in propulsion of lymph within the vessels (Leak 1970, Solito 1997). In lymphatic capillaries, precollectors, and collecting vessels, endothelial BMs appear discontinuous in electron microscopy (Leak 1970, Sacchi 1997, 1999). A general lack of immunoreactivity for BM constituents, especially laminin, has been described in lymphatic vessels (Barsky et al. 1983, Hultberg and Svanholm 1989, Wigle et al. 2002). Microarray analyses have shown that the expression of genes encoding BM constituents is lower in lymphatic endothelial cells than in endothelial cells of blood vessels (Petrova et al. 2002, Podgrabinska et al. 2002, Hirakawa et al. 2003). These results have been interpreted as a general absence of BM constituents in lymphatic vessels. Sauter et al. (1998), however, localized collagen type IV to endothelial BMs of lymphatic capillaries. The role of BM constituents in lymphatic vessels thus remains elusive.

Despite the aforementioned structural differences between blood and lymphatic vessels, studies on lymphatic vessels were until recently largely hindered by difficulties identifying lymphatic capillaries in routine histological preparations. Lymphatic endothelial cells express blood vessel endothelial markers, such as platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), factor-VIII-related antigen (FVIII:Ag), and Ulex Europaeus-I agglutinin (UEA-I) binding sites, to some extent (Gnepp 1987, Sauter et al. 1998, Banerji et al. 1999, Sleeman et al. 2001). In the past decade, more specific markers of lymphatic endothelia, including lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), podoplanin, and vascular endothelial growth factor receptor-3 (VEGFR-3/flt4), have been identified (Kaipainen et al. 1995, Banerji et al. 1999, Breiteneder-Geleff et al. 1999), providing tools for lymph vessel research. None of these markers is, however, exclusively found in lymph vessel endothelial cells; immunoreactivity for LYVE-1 has been found in liver and spleen sinusoids and tissue macrophages, immunoreactivity for podoplanin in kidney podocytes, osteoblastic cells, type I pneumocytes, and cells of choroid plexus, and immunoreactivity for VEGFR-3 in the cells of the haematopoietic system, developing blood vessels, fenestrated capillaries, and blood capillaries of tumours and chronic wounds (Sleeman et al. 2001).
4.7 LAMININS IN RENAL CELL CARCINOMAS

Interactions of cells with neighbouring cells and ECM regulate proliferative and invasive capacity of cells and maintain normal tissue architecture. Changes in cell-ECM interactions have been suggested to contribute to malignant transformation of epithelial cells (Liotta and Kohn 2001, Ingber 2002). For carcinoma cells to metastasize, they must invade through epithelial BM, connective tissue stroma, and blood or lymph vessel walls to reach the circulation. In the target tissue, the carcinoma cells must extravasate through the vessel wall and invade into the tissue parenchyma to form the metastasis. According to current view, BMs are degraded by proteases, such as matrix metalloproteinases, to enable invasion of carcinoma cells (Liotta and Kohn 2001, Gupta and Massagué 2006). Discontinuities in epithelial BMs have been observed in many but not all carcinomas (Bosman 1994, Flug and Köpf-Maier 1995). It remains questionable whether degradation of BM by proteases is a prerequisite for invasion of carcinoma cells, or whether there are alternative mechanisms, such as changes in BM composition, which render the BM permeable for invasion (Flug and Köpf-Maier 1995). Further, BM constituents have been suggested to have roles in tumour cell migration, extravasation, and proliferation, as well as in tumour-associated angiogenesis (Engbring and Kleinman 2003).

Kidney cancer accounts for approximately 3% of new cancer cases and cancer deaths in Finland (Finnish Cancer Registry 2005). According to the most recent classification of renal cell tumours, which in addition to morphology also takes into account genetic alterations, the vast majority (~70%) of malignant renal cell tumours are conventional (clear cell) renal cell carcinomas (RCCs). Other malignant renal cell tumour types are papillary RCCs, chromophobe RCCs, collecting duct carcinomas, and unclassified RCCs. Benign renal cell tumours include renal oncocytomas, papillary adenomas, and metanephric adenomas (Kovacs et al. 1997, Störkel et al. 1997, Delahunt et al. 2001, Eble et al. 2004).

In conventional and papillary RCCs, laminin α1 chain has been found in BMs of tumour cell islets and in tumours with a high malignancy grade also more diffusely in stroma (Rissanen et al. 2003). Laminin α2 chain, by contrast, is not present in RCCs or oncocytomas. Laminin α3 and β3 chains, constituents of laminin-332, seem to be restricted to BMs of tumour cell islets of papillary RCCs and some oncocytomas.
Laminin α5, β1, and γ1 chains have been observed in all RCCs and oncocytomas in both BMs of tumour cell islets and endothelial BMs of blood vessels. Laminin β2 chain has been found in BMs of tumour cells islets and blood vessel endothelia in conventional RCCs and oncocytomas, but not in papillary RCCs (Lohi et al. 1996). In several microarray studies comparing mRNA expression of renal tumours with that of normal kidneys, laminin α4 chain appeared to be the only laminin α chain overexpressed in renal tumours (Boer et al. 2001, Young et al. 2001, Gieseg et al. 2002, Lenburg et al. 2003).

Laminins promote adhesion, migration, and invasion of renal carcinoma cells in vitro (Grossi et al. 1992, Price et al. 1996, Brenner et al. 2000, Rissanen et al. 2003). RCC cells adhere to laminin-511/521 more strongly than to laminin-111. In RCC cell adhesion to laminin-111, a shift from integrin α6β1 to integrin α3β1 appears to be associated with increasing malignancy (Rissanen et al. 2003). The role of laminins in the pathogenesis of RCC remains, however, to be elucidated.
5. AIMS OF THE STUDY

Laminins are among the main constituents of BMs and have various effects on cell behaviour. Mutations in laminin genes lie behind several human diseases. At the onset of this study, much less was known about the then least characterized laminins, α4 chain laminins. The first aim of this study was thus to create a MAb specific for the human laminin α4 chain to examine the localization of this protein in human tissues. The finding that α4 chain laminins are prominent in endothelial BMs of blood vessels elicited our interest in the distribution and functions of laminins in vasculature and carcinomas. The aims of this study were as follows:

1. To create a MAb against laminin α4 chain.
2. To evaluate the distribution of α4 chain laminins in normal human tissues.
3. To examine the production of α4 chain laminins by human endothelial cells.
4. To evaluate the role of laminins of blood vessel endothelia, primarily α4 chain and α5 chain laminins, in the adhesion of human endothelial cells, as well as the cell surface receptors mediating these interactions.
5. To assess the distribution of laminins and other main BM constituents in lymphatic vessels of normal tissues and carcinomas. Ovarian carcinomas were used as an example of carcinomas possessing intratumoural lymphatic capillaries.
6. To evaluate the distribution and production of α4 chain laminins in carcinomas, as well as their effect on adhesion and migration of carcinoma cells. RCC tissues and cells were used as examples.
6. MATERIALS AND METHODS

6.1 CELL CULTURE (I, II, IV)

Human pulmonary artery endothelial (HPAE) cells (PromoCell, Heidelberg, Germany) and human dermal microvascular endothelial (HDME) cells (PromoCell; Cambrex Bioscience, Walkersville, MD) were cultured in endothelial cell growth medium-2 (EGM-2; PromoCell; Cambrex) and endothelial cell growth medium-2 microvascular (EGM-2MV; PromoCell), respectively.

RCC cell lines, including Caki-2 isolated from a clear cell RCC, ACHN isolated from malignant pleural effusion of a patient with metastatic renal adenocarcinoma, and Caki-1 isolated from a cutaneous metastasis of clear cell RCC, were obtained from the American Type Culture Collection (Manassas, VA). Previous studies have shown that when injected subcutaneously in nude athymic mice Caki-2 cells form well-differentiated clear cell grade 1 tumours, whereas ACHN and Caki-1 cells form poorly differentiated grade 3 tumours (Korhonen et al. 1994). The RCC cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Cambrex) with 10% fetal bovine serum and antibiotics.

6.2 TISSUES (I, III, IV)

The following human tissue specimens were retrieved from the files of Institute of Biomedicine/Anatomy: adult colon (n=4), endometrium (n=2), esophagus (n=4), kidney (n=8), lung (n=3), pancreas (n=9), salivary gland (n=1), skin (n=8), small intestine (n=4), stomach (n=6), testis (n=2), and thyroid gland (n=2), which were obtained from surgical operations at Jorvi Hospital (Espoo, Finland) and the Second Department of Surgery, Helsinki University Central Hospital (Helsinki, Finland); adult brain (n=1) and spinal cord (n=1) obtained from autopsies at the Department of Pathology, University of Oulu (Oulu, Finland); specimens (n=1-4) of 16- to 23-week fetal brain, esophagus, kidney, lung, pancreas, peripheral nerve, skin, skeletal muscle, small intestine, stomach, testis, and thyroid gland from fetuses legally aborted due to several maternal complications or from spontaneous abortions resulting from the rupture of fetal membranes obtained from the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, and Jorvi Hospital; specimens of 9- to 40-week placenta
(n=7) obtained from Jorvi Hospital; specimens of adult ovaries (n=5) and ovarian carcinomas (n=15) obtained from surgical operations at the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital; RCCs (n=33), RCC metastases (n=2), and oncocytomas (n=2) obtained from nephrectomies at Jorvi Hospital and Oulu University Central Hospital (Oulu, Finland). In addition, adult skeletal muscle (n=3), heart (n=3), blood vessels (n=6), fetal heart (n=3), and fetal salivary gland (n=1) were obtained from Umeå University Hospital (Umeå, Sweden). The Ethics Committee at Umeå University approved the specimen collection protocol.

The tissues were frozen in liquid nitrogen and stored at −80°C until use. Histopathological evaluation of the specimens was done by a pathologist on sections stained with haematoxylin-eosin. Normal tissue specimens were found to be free of all pathological processes. The ovarian carcinoma specimens were classified and graded according to the World Health Organization classification (Tavassoli and Devilee 2003), and included 10 endometrioid carcinomas (6 grade 1, 2 grade 2, 2 grade 3), 3 serous carcinomas (2 grade 1, 1 grade 2), and 2 mucinous carcinomas (1 grade 1, 1 grade 2). The renal tumours were initially classified and graded according to the World Health Organization criteria (Thoenes et al. 1986, Mostofi and Davis 1998). For the purposes of this study, however, a reclassification was performed according to the Heidelberg/Rochester classification (Kovacs et al. 1997, Störkel et al. 1997, Delahunt et al. 2001, Eble et al. 2004). The renal tumours included 31 conventional (clear cell) RCCs (10 grade 1, 15 grade 2, and 6 grade 3), 2 metastases of conventional RCCs (1 grade 1, and 1 grade 3), 2 papillary RCCs (2 grade 1), and 2 oncocytomas.

**6.3 EXTRACELLULAR MATRIX PROTEINS (I, II, IV)**

Native human laminin (Lm)-411 was immunopurified from the culture medium of T98G glioblastoma cells (Fujiwara et al. 2001). Recombinant hybrid Lm-411, comprising human Lm α4 and γ1 chains and mouse Lm β1 chain (Kortesmaa et al. 2000), as well as recombinant human Lm-411 (Kortesmaa et al. 2002) were produced in mammalian expression systems. Native human Lm-511 was immunopurified from the culture medium of PANC-1 pancreatic adenocarcinoma cells as previously described (Tani et al. 1999). Human placental Lm-511/521 preparations were obtained from Sigma (St. Louis, MO) and Life Technologies (Grand Island, NY). Native human Lm-332 was immu-
nopurified from the culture medium of SCC25 tongue squamous cell carcinoma cells (Vivinus-Nebot et al. 2004). Mouse Lm-111 from an Engelbreth-Holm-Swarm tumour (EHS-Lm) was obtained from Sigma. In enzyme immunoassay (EIA), recombinant human Lm β1- (Pikkarainen et al. 1992) and Lm γ1-chains (kindly provided by Dr. Sirpa Salo, University of Oulu, Finland; unpublished data) were used. Fibronectin (Fn) was purified from outdated human plasma (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) using Gelatin Sepharose™ 4B affinity chromatography (Amersham Biosciences, Uppsala, Sweden) according to Engvall and Ruoslahti (1977).

6.4 ANTIBODIES

6.4.1 MONOCLONAL ANTIBODY TO HUMAN LAMININ α4 CHAIN (I)

The study protocol for generation of a MAb against the Lm α4 chain was approved by the Animal Experimentation Committee of the University of Helsinki (decision no. HY-85/99). Female Balb/c mice were first immunized using Freund’s complete adjuvant (Sigma) and native human Lm-411. Further, three immunizations were administered using Freund’s incomplete adjuvant (Sigma) and recombinant hybrid Lm-411. Hybridomas were generated according to standard methods (Köhler and Milstein 1975). Spleen of immunized mouse was minced, cells were collected in RPMI 1640 medium (Cambrex), and mixed with P3X63Ag 8.653 mouse myeloma cells (American Type Culture Collection). The mixed cells were centrifuged, the cell pellet was resuspended in 1 ml of polyethyleneglycol (PEG 4000; Life Technologies), prewarmed to 37°C, and RPMI 1640 medium was added to the suspension. The cells were centrifuged, resuspended in HAT selection medium (Biological Industries, Kibbutz Bait Maemek, Israel), and transferred to 96-well plates, onto which macrophages had been plated the day before. The macrophages were collected from Balb/c mice by rinsing the peritoneal cavity with RPMI 1640 medium. For screening of the hybridomas, sections of fetal and adult human skeletal muscle and kidney were immunostained with the hybridoma supernatants. Selected hybridomas were cloned manually by picking single cells from the cultures. Further characterization of the MAbs was performed using EIA and immunoprecipitation techniques.
6.4.2 OTHER ANTIBODIES (I, II, III, IV)

Antibodies used in this study are listed in Table 3.

6.5 IMMUNOHISTOCHEMISTRY AND MICROSCOPY (I, II, III, IV)

For indirect immunolabelling techniques, tissues frozen with liquid nitrogen were cut into 6- to 7-µm sections, and fixed in acetone at –20°C for 10 min. Cells grown on glass cover slips were fixed in methanol at –20°C for 15 min. In certain experiments, the cells were exposed to 5 µM monensin (Sigma) for 16 h before fixation to inhibit the secretion of proteins (Tartakoff 1983).

For indirect immunofluorescence microscopy, the specimens were first exposed to mouse MAbS, followed by either fluorescein isothiocyanate-coupled goat anti-mouse immunoglobulin G (IgG; Jackson Immunoresearch, West Grove, PA), Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes, Eugene, OR), or Alexa Fluor 568 goat anti-mouse IgG (Molecular Probes). For double-labelling, the specimens were subsequently exposed to either rat MAbS or rabbit polyclonal antibodies, followed by either tetramethylrhodamine isothiocyanate (TRITC)-coupled goat anti-rat IgG (Jackson Immunoresearch), TRITC-coupled goat anti-rabbit IgG (Jackson Immunoresearch), Alexa Fluor 568 goat anti-rabbit IgG (Molecular Probes), Alexa Fluor 594 goat anti-rabbit IgG (Molecular Probes), or Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). TRITC-coupled UEA-I (Vector Laboratories, Burlingame, CA) was used in double-labelling to identify blood vessel endothelial cells (Holthöfer et al. 1982). The specimens were embedded in sodium veronal-glycerol buffer (pH 8.4) or polyvinylalcohol mounting medium, and examined under a Leitz Aristoplan microscope (Leica Microsystems, Wetzlar, Germany) equipped with appropriate filters or laser-scanning confocal microscopy. For laser scanning confocal microscopy, a Leica TCS SP2 system (Leica Microsystems) and argon and krypton laser excitation lines of 488 nm and 568 nm were used. Image stacks were collected using sequential scanning through the sections applying standardized 120-nm or 366-nm z-sampling densities for 63x and 20x objectives, respectively. The results are shown as maximum intensity projections of four section stacks.

For light microscopy, the specimens were exposed to MAbS, and the bound anti-
<table>
<thead>
<tr>
<th>MAb clone</th>
<th>Specificity</th>
<th>Reference</th>
<th>Method</th>
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<td>161EB7</td>
<td>Lm α1 chain</td>
<td>Virtanen et al. 2000</td>
<td>IHC</td>
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<td>5H2</td>
<td>Lm α2 chain</td>
<td>Leivo and Engvall 1988</td>
<td>IHC</td>
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<td>4H8-2</td>
<td>Lm α2 chain</td>
<td>Schuler and Sorokin 1995</td>
<td>IHC</td>
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<td>BM-2</td>
<td>Lm α3 chain</td>
<td>Rousselle et al. 1991</td>
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<td>168FC10</td>
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<td>Petäjäniemi et al. 2002</td>
<td>IHC, IP, EIA</td>
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<td>Engvall et al. 1997, Tiger et al. 1997</td>
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<td>Lm B1 chain</td>
<td>Virtanen et al. 1997</td>
<td>IHC, IP, WB</td>
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<td>Lm B2 chain</td>
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<td>Marinkovich et al. 1992</td>
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<td>Lm γ1 chain</td>
<td>Määttä et al. 2001</td>
<td>IHC, IP</td>
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<td>22</td>
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<td>Lm γ3 chain</td>
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<td>collagen VII</td>
<td>Sakai et al. 1986a</td>
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<td>collagen XVII</td>
<td>Valtola et al. 1999</td>
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<td>Katz et al. 1991</td>
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<td>201</td>
<td>fibrilllin-1</td>
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<td>TS2/7</td>
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<td>IHC</td>
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<td>PIE6</td>
<td>Int α2 subunit</td>
<td>Wayner and Carter 1987; Chemicon, Temecula, CA</td>
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<td>Int α3 subunit</td>
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<td>Wayner and Carter 1987; Chemicon</td>
<td>QCA</td>
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<td>Int α3 subunit</td>
<td>Kikkawa et al. 2000</td>
<td>QCA</td>
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<td>Werb et al. 1989</td>
<td>IHC</td>
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<td>GoH3</td>
<td>Int α4 subunit</td>
<td>Sonnenberg et al. 1987; Chemicon</td>
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<td>LM142.69</td>
<td>Int α5 subunit</td>
<td>Cheresh and Spiro 1987</td>
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<td>LM609</td>
<td>Int αβ1 subunit</td>
<td>Cheresh 1987; Chemicon</td>
<td>QCA</td>
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<tr>
<td>102DF5</td>
<td>Int β1 subunit</td>
<td>Yläne and Virtanen 1989</td>
<td>IHC</td>
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<td>13</td>
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<td>Yamada et al. 1990</td>
<td>QCA</td>
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<td>30</td>
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<td>Hessle et al. 1984; Chemicon</td>
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<td>BRIC221</td>
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<td>Parsons et al. 1997; Serotec, Oxford, UK</td>
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<td>Porter et al. 1992; Serotec</td>
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<td>FB11</td>
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<td>Biohit, Helsinki, Finland</td>
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<td>52DH1</td>
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<td>Vartio et al. 1987</td>
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<td>EC-1</td>
<td>PECAM-1</td>
<td>Tani et al. 1996</td>
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<td>C8</td>
<td>FVIII-R:Ag</td>
<td>Meyer et al. 1984</td>
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<td>anti-VEGFR-3</td>
<td>VEGFR-3</td>
<td>Ludwig Institute for Cancer Research, and Licentia, Zürich,</td>
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<td>hSM-V</td>
<td>smooth muscle myosin</td>
<td>Frid et al. 1992; Sigma</td>
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<td>CD163</td>
<td>Novocastra, Newcastle upon Tyne, UK</td>
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<td>KP1</td>
<td>CD68</td>
<td>NeoMarkers, Fremont, CA</td>
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<td>EHS-Lm</td>
<td>Sigma</td>
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<td>Lm α1 chain (immunoaffinity-purified)</td>
<td>Tiger et al. 1997</td>
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<td>Lm α4 chain (immunoaffinity-purified)</td>
<td>Iivanainen et al. 1997</td>
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<td>fibronectin</td>
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<td>LYVE-1</td>
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<td>podoplanin</td>
<td>Acris Antibodies, Hiddenhausen, Germany</td>
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<td>Breiteneder-Geleff et al. 1999</td>
<td>IHC</td>
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**Table 3.** Antibodies, their specificities, and references.

Abbreviations: EIA=enzyme immunoassay; FVIII-R:Ag=factor-VIII-related antigen; IHC=immunohistochemistry; Int=integrin; IP=immunoprecipitation; Lm=laminin; LYVE-1=lymphatic vessel endothelial hyaluronan receptor-1; MAb=monoclonal antibody; PECAM-1=platelet endothelial cell adhesion molecule-1; QCA=quantitative cell adhesion assay, function-blocking antibody; VEGFR-3=vascular endothelial growth factor receptor-3; WB=Western blot analysis
bodies were visualized with the alkaline phosphatase-anti-alkaline phosphatase method (APAAP mouse monoclonal, DAKO) and a substrate solution containing NaNO₂ (Merck, Darmstadt, Germany), New Fuchsin (Sigma), levamisole (Sigma), naphthol AS-BI phosphate (Sigma), and dimethylformamide (Merck). The sections were counterstained with Mayer’s haematoxylin (Merck), mounted in Eukitt® (O. Kindler, Freiburg, Germany), and examined using a Leitz Dialux 20 microscope (Leica Microsystems) or an Olympus AX70 microscope (Olympus, Tokyo, Japan). In indirect immunolabelling techniques, negative controls were prepared by omitting the primary antibodies.

For phase contrast microscopy and field emission scanning electron microscopy (FESEM), the cells cultured on ECM protein-coated glass cover slips were fixed in 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) at room temperature (RT) for 45 min. For phase contrast microscopy, the specimens were then embedded in sodium veronal-glycerol buffer (pH 8.4) and examined under an Olympus AX70 microscope. For FESEM, the specimens were then coated with 20 nm of chromium with Emitech K575X sputter (Emitech, Ashford, Kent, UK) and examined with a Jeol JSM 6335F microscope (Jeol, Tokyo, Japan) at 5 kV operating voltage and 40° inclination.

6.6 ENZYME IMMUNOASSAY (I)

The specificity of MAb 168FC10 to the Lm α4 chain was assessed by reactivity against recombinant hybrid Lm-411, human recombinant Lm β1- and Lm γ1-chains, and placental Lm-511/521. Briefly, 96-well plates were coated overnight with either Lms (1 µg/ml) or chains (0.4 µg/ml). After blocking with human serum albumin (10 mg/ml) (Sigma), undiluted 168FC10 hybridoma supernatant was added and allowed to interact with the proteins for 1 h at 4°C. After three washes with 0.1% Tween® 20 in phosphate-buffered saline (PBS), bound antibodies were detected using horseradish peroxidase-coupled goat anti-mouse immunoglobulins (DAKO). The enzyme activity was measured using orthophenylenediamine (Sigma).

6.7 IMMUNOPRECIPITATION, FLUOROGRAPHY, AND WESTERN BLOT ANALYSIS (I, II, IV)

To test the ability of MAb 168FC10 to detect α4 chain laminins in Western blot analysis,
immunoprecipitation of laminin from platelet lysates with MAb 114DG10 was performed as previously described (Geberhiwot et al. 1999). To test the ability of MAb 168Fc10 to detect α4 chain laminins in immunoprecipitation, platelet lysates were immunoprecipitated with undiluted 168Fc10 hybridoma supernatant using a method described elsewhere (Geberhiwot et al. 1999). In negative controls, the platelet lysates were immunoprecipitated with mouse IgG. The samples were run in 6% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970) under reducing conditions and transferred onto filters. Filters were blocked with 0.1% Tween® 20 and 5% dry milk in PBS. MAb 168Fc10, 114DG10, and 22, as well as affinity-purified rabbit antibodies to the Lm α4 chain were used in Western blotting. Mouse IgG was used also as a negative control in Western blotting. Peroxidase-coupled anti-mouse and anti-rabbit immunoglobulins were used as secondary antibodies (DAKO), and bound antibodies were visualized using enhanced chemiluminescence (Amersham Biosciences).

For other immunoprecipitation experiments, culture media of overnight cultures of HPAE, HDME, Caki-2, ACHN, and Caki-1 cells were collected. For radioactive metabolic labelling to study the effect of cycloheximide on protein synthesis, HPAE and HDME were first incubated in a methionine-deficient culture medium with or without cycloheximide (10 µg/ml; Sigma) for 1 h. Then, 25µCi/ml S35-labelled methionine (Amersham Biosciences) was added to the culture medium, and the cells were incubated for 2–3 h before collection of culture medium.

In immunoprecipitation, the culture media were first cleared by centrifugation, and supplemented with Triton X-100 (0.5%) and normal mouse serum. The supernatants were preabsorbed with GammaBind™ Plus Sepharose™ beads (Amersham Biosciences) and subsequently applied onto GammaBind™ Plus Sepharose™ beads precoupled with MAb 114DG10, 113BC7, 168Fc10, 4C7, and 52DH1. In negative controls, the MAb was omitted. For SDS-PAGE, the bound proteins were eluted with Laemmli’s reducing sample buffer.

For SDS-PAGE and Western blotting, we also used adult human kidney and lung tissues to produce cell-free ECMs according to Hedman et al. (1979). The tissues were first exposed to 0.5% sodium deoxycholate (Sigma) in 10 mM Tris-HCl, 150 mM NaCl, and 1 mM PMSF (pH 8.0) three times for 10 min at 0°C, then washed three times
in 2 mM Tris-HCl, 150 mM NaCl, and 1 mM PMSF (pH 8.0) at 0°C, and suspended in Laemmli’s reducing sample buffer. For comparison, recombinant human Lm-411 (Kortesmaa et al. 2002) was suspended in Laemmli’s reducing sample buffer. To examine the production of Lu, samples of HPAE and HDME cells were made by boiling the detached cells in Laemmli’s non-reducing sample buffer.

For detection of laminins, the proteins were separated in SDS-PAGE in 5% and 6.5% gels, and for detection of Lu in 8% gels. Radioactive proteins were detected by fluorography using Hyperfilm MP (Amersham Biosciences) according to standard methods. For Western blot analysis, the proteins were transferred onto nitrocellulose filters. The filters were blocked using 5% dry milk in PBS, and exposed to either MAb BRIC221 or rabbit antiserum for the Lm α4 chain. Immunoreactive bands were visualized either using an avidin-biotin-peroxidase bridge method (Vectastain Elite® ABC-kit; Vector Laboratories) with goat anti-mouse or goat anti-rabbit IgG, nickel intensification, and diaminobenzidine as a substrate (Sigma), or horseradish peroxidase-coupled anti-rabbit immunoglobulins (DAKO) with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). As size markers, we used Standard Mixture for Molecular Weights 30 000–200 000 for SDS-PAGE (Sigma).

6.8 NORTHERN BLOT ANALYSIS (II, IV)

For Northern blotting, total RNA was isolated from HDME, HPAE, Caki-2, ACHN, and Caki-1 cells by acid phenol-guanidinium thiocyanate-chloroform extraction (Chomczynski and Sacchi 1987) or by using Eurozol (EuroClone, Milano, Italy). Poly(A)+ RNAs were enriched by using Dynabeads Oligo (dT)25 beads (Dynal, Oslo, Norway). The RNAs were separated according to size in a denaturing 1.2% agarose gel and transferred onto Hybond membranes (Amersham Biosciences) or Nylon Membranes, Positively Charged (Roche, Mannheim, Germany) by upward capillary transfer.

To study the expression of Lu, a cDNA expression plasmid containing the full-length human Lu coding region was purchased from Invitrogen (Carlsbad, CA) and modified as previously described (Kikkawa et al. 2002). A 700-bp DNA probe was created by restriction with SmaI (Promega, Madison, WI). The Northern hybridization was performed by using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche).
according to the manufacturer’s instructions. Prehybridization was carried out at 55°C for 30 min and hybridization at 55°C for 24 h. The blots were exposed to Hyperfilm MP (Amersham Biosciences). For re-use of the blots, the probes were erased by pouring boiling 0.1% sodium dodecyl sulphate (SDS) on the filter according to the instructions by Roche. Hybridization with a DNA probe to glutaraldehyde-3-phosphate-dehydrogenase was used as a loading control. As size markers, 0.24-0.5 kb RNA ladder (Invitrogen) and 0.28-6.58 kb RNA marker (Promega) were used.

To examine the expression of Lm α4 chain, a pBluescript plasmid vector (Stratagene, La Jolla, CA), comprising 2714 nucleotides of human Lm α4 chain, was created as described earlier (HL4-5'; Kortesmaa et al. 2000). An antisense RNA probe was created first by linearizing with EcoRI (Sigma), and then incorporating a digoxigenin label simultaneously with in vitro transcription using a DIG RNA Labelling Kit (SP6/T7) (Roche) and T7 RNA polymerase (Roche). The Northern hybridization was performed by using DIG Easy Hyb Granules (Roche) according to the manufacturer’s instructions. Prehybridization was carried out at 68°C for 30 min, and hybridization at 68°C for 16 h. The hybridized probes were detected with Anti-Digoxigenin-AP Conjugate (Roche) and ready-to-use CSPD (Roche). The blots were then exposed to Hyperfilm MP (Amersham Biosciences). For re-use of the blots, the probes were erased by pouring boiling 0.1% SDS on the filter according to the instructions by Roche. Hybridization with an antisense RNA probe to glutaraldehyde-3-phosphate-dehydrogenase was used as a loading control, and hybridization with a sense RNA probe to Lm α4 chain was used as a negative control. As a size marker, RNA molecular weight marker I, digoxigenin labelled (0.3-6.9 kb) (Roche) was used.

6.9 CELL ADHESION ASSAYS (II, IV)

Quantitative cell adhesion assays were performed using a method based on intracellular acid phosphatase (Prater et al. 1991). Ninety-six-well plates were coated with laminins or Fn (1-20 µg/ml) at RT for 1 h. In certain experiments, some of the wells were exposed to soluble recombinant protein corresponding to the extracellular domain of Lu (Sol-Lu; 10 µg/ml in PBS; Kikkawa et al. 2002) at RT for 1 h, while other wells were exposed only to PBS. In all experiments, the wells were treated with 3% bovine serum albumin (BSA) in PBS at RT for 1 h. In some experiments, to avoid the interference of
protein secretion during the experiment (Clark et al. 1986), cycloheximide was added (10 µg/ml; Sigma) to the culture medium of the cells 1 h before the cells were plated, as well as to washing and adhesion media (serum-free EGM-2, EGM-2MV, or RPMI 1640). The cells were detached with trypsin and EDTA, exposed to trypsin-neutralizing solution (PromoCell), and washed with the adhesion medium. Thereafter, the function-blocking MAbs (MAb 3G8 6 µg/ml, others 2 µg/ml) were added to the cell suspensions. The cells were plated at 2x10⁴ cells/well, and the plates were incubated at 37°C in 5% CO₂ for 1 h. The wells were carefully washed to remove non-adherent cells. The control wells showing the amount of cells originally plated were not washed. The plate was centrifuged with a Hermle Z 400 K centrifuge (Hermle Labortechnik GmbH, Germany) at 500 rpm for 5 min to minimize loss of the cells from the control wells, and the adhesion media of these wells were carefully removed. Substrate solution (Sigma 104 phosphatase substrate 6 mg/ml in 50 mM sodium acetate buffer with 0.1% Triton-X100, pH 5) was added to each well, and the plates were incubated at 37°C for 1 h. The reaction was stopped with 1 M NaOH, and the absorbances were measured at 405 nm. BSA-coated wells were used as controls. Experiments were performed in triplicate. The results were expressed either as percentage of adhered cells ± SD of three wells or as absorbances ±SD of three wells. The difference between two variables was tested with a two-sided, unpaired t-test with a significance level of α=0.05.

For visualization of the morphology and adhesion structure formation of adhering cells, we also performed morphological cell adhesion assays. For this purpose, cell culture dishes with glass cover slips were coated with laminins or Fn (4 µg/ml) at RT for 1 h. The dishes were subsequently coated with 3% BSA in PBS at RT for 1 h. BSA-coated dishes were used as controls. For certain experiments, cycloheximide (10 µg/ml) was added to the culture medium 1 h before the cells were plated, as well as to the washing and adhesion media (serum-free EGM-2 or EGM-2MV). The cells were detached with trypsin and EDTA, exposed to trypsin neutralizing solution (PromoCell), washed, plated onto cover slips, and incubated at 37°C in 5% CO₂ for 2 h. The non-adherent cells were removed by washing the samples carefully with PBS, and the morphology of the cells was visualized with either immunofluorescence microscopy, phase contrast microscopy, or FESEM. For comparison of the cell morphology on different adhesion substrata, the adhered cells were counted in FESEM in three independent visual fields,
and divided according to their morphology into three groups of not spread (roundish), moderately spread (protrusion-forming), and well-spread (lamellipodia-forming) cells. The results of each group were expressed as a percentage of all adhered cells (±SD of 3 visual fields).

6.10 CELL MIGRATION ASSAY (IV)

Undirected random migration of RCC cells on ECM proteins was examined by fluorescence live cell imaging (method modified from Fujiwara et al. 2004). Glass Bottom Culture Dishes (MatTek Corporation, Ashland, MA) were coated with recombinant human Lm-411 or Fn (5 µg/ml) in PBS at RT for 1 h, and treated with 3% BSA in PBS at RT for 1 h. The cells were labelled with 20 µM of CellTracker™ Green CMFDA (Molecular Probes) for 30 min according to the manufacturer’s instructions. The cells were detached with trypsin and EDTA, exposed to a trypsin-neutralizing solution (PromoCell), washed with experiment medium (phenol red-free DMEM, Gibco, Invitrogen, Paisley, UK), and plated at 10^5 cells in a 2-ml medium onto coated dishes. The cells were allowed to attach at 37°C in 5% CO₂ for 15 min before imaging. Fluorescence live cell imaging was performed with T.I.L.L. Photonics Multi-Color Ratio Imaging System (TILL Photonics GmbH, Munich, Germany) based on an Olympus IX-70 inverted microscope and TILLvisION software v. 4.01 (TILL Photonics) by exposing the cells for 15 ms to 492-nm monochromatic light at 30-s intervals for 2 h under cell culture conditions (37°C, 5% CO₂), and observed with a 10x objective and emission filter passing a wavelength of over 520nm. The trajectories of the cells were analysed with ImageJ 1.37c (Rasband WS: Image J. National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997–2004) and a MTrack2 plugin (Klopfenstein and Vale 2004). The experiments were performed in triplicate. Migration on each coat was expressed as average, median, and ±SD of total length of the migration trajectories of all cells found in all images of a microscope field (on average 65 cells/field) of a representative experiment. The difference between two variables was evaluated with a two-tailed Mann-Whitney U-test with significance level set at α=0.05.
7. RESULTS

7.1 SPECIFICITY OF MONOCLONAL ANTIBODY TO LAMININ α4 CHAIN (I)

The first aim of this study was to create a MAb specific for the human Lm α4 chain to enable evaluation of the localization of this protein in human tissues. Mice were first immunized with native human Lm-411 and then with recombinant hybrid Lm-411 (comprising human α4 and γ1 chains and mouse β1 chain). Hybridomas producing MAbs were generated, and screened by indirect immunohistochemistry on sections of fetal and adult human skeletal muscle and kidney based on previous reports on the distribution of α4 chain laminins in these tissues (Iivanainen et al. 1995, 1997, Miner et al. 1997, Sorokin et al. 1997a, Patton et al. 1999). The hybridomas potentially producing MAbs specific for human Lm α4 chain were cloned manually, and their specificity was further evaluated by EIA, immunoprecipitation, and Western blotting.

In EIA, MAb 168FC10 reacted with recombinant hybrid Lm-411, but not with placental Lm-511/521 or recombinant human Lm β1 or γ1 chains. In Western blots, MAb 168FC10 was unable to detect either Lm-411, immunoprecipitated from platelet lysates with MAb 114DG10 to the Lm β1 chain, or recombinant hybrid Lm-411. When the platelet lysates were immunoprecipitated with MAb 168FC10, the characteristic (Geberhiwot et al. 1999, Fujiwara et al. 2001) Lm α4 (Mr 180 000 and 200 000), β1 (Mr 230 000), and γ1 (Mr 220 000) chains were detected by Western blotting with rabbit antiserum to the Lm α4 chain, MAb 114DG10 to the β1 chain, and MAb 22 to the γ1 chain, respectively. The bands were not detected when control mouse IgG was used in either immunoprecipitation or Western blotting. The results indicated that MAb 168FC10 specifically reacts with Lm-411 in EIA and immunoprecipitation.

7.2 LOCALIZATION OF α4 CHAIN LAMININS IN FETAL AND ADULT HUMAN TISSUES (I)

As the localization of α4 chain laminins in human tissues had remained largely unresolved (Iivanainen et al. 1995, Gu et al. 1999, Patton et al. 1999), we used the MAb 168FC10 to study their presence in fetal and adult human tissues by immunohistochemistry. In double-labelling, TRITC-UEA-I was used to localize blood vessel en-
dothelia (Holthöfer et al. 1982), rabbit antiserum to EHS-Lm was used to visualize all BMs (Erickson and Couchman 2000), rat MAb 4H8-2 to the Lm α2 chain was used to localize BMs of striated muscle cells, Schwann cells, trophoblast of placenta, and gastric glands (Leivo and Engvall 1988, Virtanen et al. 1995), and affinity-purified rabbit antiserum to the Lm α1 chain was used to localize parenchymal BMs in the central nervous system (Sixt et al. 2001a). The results are summarized in Table 1 of Study I.

Immunoreactivity for the Lm α4 chain was found in contact with all TRITC-UEA-I-reactive endothelia of blood vessels and capillaries in all fetal and adult tissues, with the exception of intraglomerular capillaries in the kidney. Immunoreactivity for the Lm α4 chain was also observed around vascular smooth muscle cells in fetal tissues. In adult elastic arteries, some scattered immunoreactivity was seen among vascular smooth muscle cells in intima in the vicinity of the endothelium. In other adult blood vessels, vascular smooth muscle cells generally lacked immunoreactivity for the Lm α4 chain. Immunoreactivity was also found in adventitia of blood vessels in BMs of adipocytes and nerves.

In 16-week fetal skeletal muscle, BMs of muscle cells showed immunoreactivity for the Lm α4 chain in a striated pattern in co-localization with immunoreactivity for the Lm α2 chain. In 39-week skeletal muscle, BMs of muscle cells showed traces of immunoreactivity for the Lm α4 chain, whereas in adult skeletal muscle BMs of muscle cells lacked immunoreactivity for this Lm chain. In 14- and 20-week fetal heart, BMs of muscle cells revealed immunoreactivity for the Lm α4 chain. Immunoreactivity for the Lm α4 chain was weaker, but detectable, in BMs of muscle cells in the adult heart. In fetal and adult visceral smooth muscle from various sites, BMs of muscle cells showed immunoreactivity for the Lm α4 chain. In all fetal and adult muscle tissues, immunoreactivity for the Lm α4 chain was also found in co-localization with TRITC-UEA-I-reactive endothelia of blood vessels.

In the fetal kidney (16-week metanephros), traces of immunoreactivity for the Lm α4 chain were detected in the BM region of developing epithelial aggregates. BM of ureter bud, localized by double-labelling with rabbit antiserum to EHS-Lm, lacked immunoreactivity for the Lm α4 chain. At the comma-shaped body stage, prominent immunoreactivity for the Lm α4 chain was detected in the glomerular cleft around the invading capillaries. At the S-shaped body stage, immunoreactivity for the Lm α4 chain was also detected in co-localization with TRITC-UEA-I-reactive endothelia of blood vessels.
α4 chain was found in the glomerular cleft as well as in the BM region of the developing proximal tubule. In capillary loop-stage glomeruli, prominent immunoreactivity was detected in the intraglomerular mesangium. Glomerular BM, identified by double-labelling with rabbit antiserum to EHS-Lm, lacked immunoreactivity for the Lm α4 chain. Traces of immunoreactivity for this chain were found in the BM region of Bowman's capsule. Immunoreactivity was also detected in the BM region of some medullary tubules, in the kidney capsule, and in co-localization with TRITC-UEA-I-reactive endothelia of non-glomerular blood vessels.

In the adult kidney, immunoreactivity for the Lm α4 chain was detected in the intraglomerular mesangium, the BM region of some medullary tubules, and the kidney capsule. Traces of immunoreactivity for Lm α4 chain were found in the BM region of Bowman's capsule. Glomerular BM lacked immunoreactivity for the Lm α4 chain, whereas immunoreactivity was observed in co-localization with TRITC-UEA-I-reactivity in non-glomerular capillaries.

In the fetal esophagus (16-week), immunoreactivity for the Lm α4 chain was found in the BM region of developing esophageal submucous glands. In the fetal stomach (16-week), traces of immunoreactivity for the Lm α4 chain were observed in the BM region of developing gastric glands. In the small intestine (19-week), neither BM of surface epithelium nor intestinal glands were immunoreactive. In the fetal salivary gland (22-week), immunoreactivity for Lm α4 chain was seen in the BM region of acini. In the fetal pancreas (19-week), traces of immunoreactivity for the Lm α4 chain were present in the BM region of acini in co-localization with immunoreactivity for the Lm α2 chain. In all parts of the fetal gastrointestinal tract, BMs of visceral smooth muscle cells and BMs of blood vessel endothelia, latter identified by double-labelling for TRITC-UEA-I, showed prominent immunoreactivity for the Lm α4 chain.

In the adult esophagus, immunoreactivity for the Lm α4 chain was observed in the BM region of submucosal glands. In the antrum of adult stomach, immunoreactivity for the Lm α4 chain was present in the BM region of gastric glands. In the corpus of the stomach, immunoreactivity for Lm α4 chain was found in the BM region of lower parts of gastric glands in partial co-localization with immunoreactivity for the Lm α2 chain. In the adult small intestine, immunoreactivity for the Lm α4 chain was localized to the BM region of intestinal glands, but not in BMs of Brunner's glands. In
the adult colon, immunoreactivity for the Lm α4 chain was seen in the BM region of intestinal glands. In the adult salivary gland, immunoreactivity for Lm α4 chain was observed in the BM region of acini, but not in the BMs of ducts. In the adult pancreas, immunoreactivity for the Lm α4 chain was found in the BM region of acini, in co-localization with immunoreactivity for the Lm α2 chain, as well as in connective tissue of septa. In all parts of the adult gastrointestinal tract, BMs of smooth muscle cells and BMs of TRITC-UEA-I-reactive blood vessel endothelia showed prominent immunoreactivity for the Lm α4 chain.

In the fetal lung at pseudoglandular (19-week) and early canalicular (22-week) stages immunoreactivity for the Lm α4 chain was found in the endothelia of capillaries in co-localization with reactivity for TRITC-UEA-I. BM of the bronchial epithelium lacked immunoreactivity for the Lm α4 chain, whereas BMs of smooth muscle cells in the bronchial wall showed immunoreactivity. In the adult lung, immunoreactivity for the Lm α4 chain was seen in the BMs of capillaries. BM of the bronchial epithelium lacked immunoreactivity for the Lm α4 chain, whereas in the bronchial wall BMs of smooth muscle cells and adipocytes showed immunoreactivity.

In the fetal brain (16-week), adult brain, and adult spinal cord, BMs of blood vessels showed immunoreactivity for the Lm α4 chain. In larger vessels, in adults, immunoreactivity for the Lm α1 chain surrounded that for the Lm α4 chain. Smaller Lm α4 chain-immunoreactive capillaries lacked immunoreactivity for the Lm α1 chain.

In the fetal sciatic nerve (16-week), the endoneurial BMs around axons showed immunoreactivity for the Lm α4 chain in co-localization with immunoreactivity for the Lm α2 chain. In addition, immunoreactivity for the Lm α4 chain was also found in the perineurium and epineurium. In adult peripheral nerves from various sites, endoneurial BMs around the axons and epineurium revealed prominent immunoreactivity for the Lm α4 chain. The perineurium was not distinguishable with certainty in these peripheral nerves.

In fetal and adult skin, patchy immunoreactivity for the Lm α4 chain was found in contact with epidermal BM. In dermis, immunoreactivity was present in the BMs of capillaries, nerves, and adipocytes, but not in connective tissue.

In fetal (18-week) and adult thyroid glands, immunoreactivity was chiefly confined to the TRITC-UEA-I-reactive endothelia of capillaries, but in adults some scattered
immunoreactivity was also found in connective tissue between the follicles and capillaries.

In the fetal (16-week) testis, traces of immunoreactivity for the Lm α4 chain were observed in the myoid cell layer around seminiferous tubules. Scattered immunoreactivity was also found in the connective tissue between the seminiferous tubules. In the adult testis, prominent immunoreactivity for the Lm α4 chain was present in the myoid cell layer. Immunoreactivity for the Lm α4 chain was also found in the BMs of capillaries.

In the adult endometrium, immunoreactivity for the Lm α4 chain was observed in connective tissue and in co-localization with TRITC-UEA-I-reactive BMs of capillaries, but not in epithelial BMs of glands at various phases of the proliferation cycle. BMs of decidual cells showed immunoreactivity for the Lm α4 chain. In placental villi (9–40-week), immunoreactivity for the Lm α4 chain was found in BMs of capillaries, but not in the Lm α2 chain-immunoreactive BM of trophoblasts.

Thus, the results showed that the Lm α4 chain is principally localized to BMs of blood vessel endothelia, muscle tissues and other contractile cells, adipocytes, nerves, and certain epithelia of the gastrointestinal tract, salivary glands, pancreas, and skin. Its expression appeared to be developmentally regulated, especially in skeletal, heart, and vascular smooth muscles.

7.3 PRODUCTION OF α4 CHAIN LAMININS BY HUMAN ENDOTHELIAL CELLS (I)

As the findings revealed that α4 chain laminins are prominent in endothelial BMs of blood vessels, we set out to investigate whether human endothelial cells produce α4 chain laminins.

Characterization of MAb 168FC10 showed that it does not function in Western blotting. In radioactive immunoprecipitation of HPAE cells, a broad band of Mr 200 000 was unspecifically bound also in controls immunoprecipitated without a MAb. Thus, culture medium of HPAE cells was immunoprecipitated with MAb 168FC10, and analysed by Western blotting with affinity-purified rabbit antiserum to the Lm α4 chain. Two bands of Mr 180 000 and 200 000 were detected. The size of these bands corresponds to the previously published sizes of Lm α4 chains isolated from various human
blood cells and from the T98G glioma cell line (Geberhiwot et al. 1999, 2001, Fujiwara et al. 2001). When the culture medium of HPAE cells was immunoprecipitated with MAb 114DG10 to the Lm β1 chain and MAb 113BC7 to the Lm γ1 chain, the Mr 180 000 and 200 000 could similarly be detected by Western blotting with affinity-purified rabbit antiserum to the Lm α4 chain, suggesting that these cells produce Lm-411.

To compare the size of the Lm α4 chain produced by HPAE cells with the Lm α4 chain found in tissues, also cell-free ECMs of adult human kidney and lung were analysed by Western blotting with affinity-purified rabbit antiserum to Lm α4 chain. In both tissues, a single band of Mr 180 000 was detected. In comparison, in recombinant human Lm-411, a band of Mr 200 000 was detected.

We also studied whether HPAE cells deposit the Lm α4 chain into ECM. The cells were cultured on glass cover slips under normal culture conditions and exposed to indirect immunofluorescence microscopy. Immunoreactivity for the Lm α4 chain was found in fibrillar structures, indicating deposition of α4 chain laminins.

7.4 ADHESION OF HUMAN ENDOTHELIAL CELLS TO LAMININ-411 (I, UNPUBLISHED DATA)

The findings that α4 chain laminins are present in humans in endothelial BMs of blood vessels and produced by human endothelial cells piqued our interest in interactions between endothelial cells and laminins of endothelial BMs. As laminins are considered to be important in cell adhesion (Timpl and Brown 1994, Colognato and Yurchenco 2000), we set out to investigate whether human endothelial cells adhere to α4 chain laminins.

Adhesion of HPAE and HDME cells to recombinant human Lm-411 was first evaluated by quantitative cell adhesion assay. In this assay, less than 10% of the plated endothelial cells adhered to this substratum (Fig. 2). The spreading of endothelial cells was then evaluated by morphological adhesion assay and visualized by phase contrast microscopy. On recombinant human Lm-411, endothelial cells did not spread but showed a roundish morphology (Fig. 3). The formation of cell-ECM adhesion complexes was evaluated by an indirect immunofluorescence technique with antibodies to talin and vinculin. On recombinant human Lm-411, immunoreactivity for talin (Fig. 3) and vinculin (not shown) was found diffusely in the cells, suggesting that the cells do
not form any specific cell-ECM adhesion complexes on this substratum (unpublished data).

Among integrins (Int), immunoreactivity for Int α₂, α₃, α₆, and β₁ subunits, but not for Int α₁ or β₄ subunits, was present on the surface of HPAE cells. In double-labeling, fibrillar immunoreactivity for the Lm α₄ chain was found in partial co-localization with the immunoreactivity for the Int α₆ subunit, suggesting that Int α₆β₁ could be among the receptors mediating interaction of HPAE cells with α₄ chain laminins (I). In the quantitative cell adhesion assay, the adhesion of HPAE and HDME (Fig. 4) cells was significantly (p<0.04) inhibited with function-blocking MAbs to Int α₆ and β₁ subunits, but not with function-blocking MAbs to Int α₂ or α₃ (unpublished data).

7.5 ADHESION OF HUMAN ENDOTHELIAL CELLS TO LAMININS-511 AND -511/521 (II)

In addition to α₄ chain laminins, α₅ chain laminins are produced by mouse endothelial cell lines and are localized to BMs of blood vessel endothelia (Miner et al. 1997, Sorokin et al. 1997b, Määttä et al. 2001). As the results showed that recombinant hu-
man Lm-411 is a poor adhesion substratum for endothelial cells, we evaluated whether α5 chain laminins better promote adhesion of endothelial cells.

The adhesion of endothelial cells to native human Lm-511 and to commercially available mixture of Lms-511 and -521 (Lm-511/521, placental laminin) was first evaluated by quantitative cell adhesion assay. In this assay, 50% of the plated cells adhered to Lm-511 and 35% of the cells adhered to Lm-511/521. In comparison, 70% of the plated cells adhered to Fn. In morphological adhesion assay, visualized by FESEM, 15% of the adhered cells did not spread and showed a roundish morphology on recombinant human Lm-411 (A). In comparison, the cells spread moderately and formed some filopodia on native human Lm-511 (B), and spread well and showed some ruffles and lamellipodia on Fn (C). In HPAE cells adhering to Lm-411, immunoreactivity for talin was found diffusely in the cells (D), whereas in HPAE cells adhering to Fn, immunoreactivity for talin was found at the cell periphery in a streak-like pattern (E). Scale bars 10 µm.

Figure 3. Cell morphology and formation of cell-ECM adhesion complexes in adhesion of human endothelial cell to Lm-411. In morphological adhesion assay (in the presence of cycloheximide), HPAE cells did not spread, but showed a roundish morphology on recombinant human Lm-411 (A). In comparison, the cells spread moderately and formed some filopodia on native human Lm-511 (B), and spread well and showed some ruffles and lamellipodia on Fn (C). In HPAE cells adhering to Lm-411, immunoreactivity for talin was found diffusely in the cells (D), whereas in HPAE cells adhering to Fn, immunoreactivity for talin was found at the cell periphery in a streak-like pattern (E). Scale bars 10 µm.
sions, and 30% of the cells spread well and formed lamellipodia.

Focal adhesions are considered to be indicative of firm adhesion (Zamir et al. 1999, Geiger et al. 2001, Murphy-Ullrich 2001). The formation of these cell-ECM adhesion structures in endothelial cells was investigated using an indirect immunofluorescence technique. In endothelial cells cultured under normal cell culture conditions overnight, immunoreactivity for vinculin was found in the periphery of the cells in nail-like plaques, suggesting formation of focal adhesions. Immunoreactivity for Fn and Int α₅ was seen in co-localization in the centre of the cells in fibrillar structures, indicating formation of fibrillar adhesions. In a morphological adhesion assay, in a 2-h adhesion to Lm-511, endothelial cells showed diffuse cytoplasmic immunoreactivity for vinculin. Immunoreactivity for talin was found in co-localization with immunoreactivity for Fn in fibrillar structures in the cells.

Detection of these Fn-immunoreactive adhesion structures in cells adhering to Lm-511 under Fn-free experiment conditions (Fn-free medium without fetal bovine serum) suggested that endothelial cells secrete proteins during the adhesion assay.
We therefore evaluated whether protein secretion could have effects on the results of the adhesion assays. Synthesis and secretion of ECM proteins by the cells within 2-3 h were evaluated by radioactive metabolic labelling and immunoprecipitation. Immunoprecipitation with MAb 52DH1 to Fn showed that within 2 h the cells synthesise and secrete a polypeptide of M_r 220 000, corresponding to the previously published size of Fn (Vartio et al. 1987). Immunoprecipitation with MAb 4C7 to the Lm α5 chain showed that within 2 h the cells also produce two polypeptides of M_r 380 000 and 390 000, corresponding to the size of Lm α5 chain (Champliaud et al. 2000), but the production is much more prominent within 3 h. Pre-exposure of the cells to cycloheximide for 1 h prevented the production of these proteins.

To avoid possible effects of protein synthesis on the results of adhesion assays, the adhesion of endothelial cells to Lm-511 and Lm-511/521 was then studied in the presence of cycloheximide. In quantitative cell adhesion assay, 30% of the plated cells adhered to Lm-511 and 25% to Lm-511/521. By comparison, 55% of the plated cells adhered to Fn. In morphological adhesion assay, visualized by FESEM, 5% of the cells did not spread, but showed a roundish morphology, 90% spread moderately well and formed multiple protrusions, and 5% spread well and formed some lamellipodia on Lm-511. On Fn, 10% of the cells showed roundish morphology, 75% spread moderately well and formed multiple protrusions, and 15% spread well and formed some lamellipodia. On Lm-511, the cells acquired a more spindle-shaped form and their protrusions were thinner and longer than in adhesion to Fn. In 2-h adhesion to Lm-511, immunoreactivity for both vinculin and talin was found diffusely within the cytoplasm of these cells. When the cover slips were coated with the combination of Lm-511 and Fn, immunoreactivity for both vinculin and talin was observed in nail-like plaques in the cells. Thus, the results revealed that human endothelial cells show moderate adhesion and spreading on Lm-511. The endothelial cells do not form cell-ECM adhesion complexes on Lm-511, although they preserve the capacity to form focal adhesion-like structures in the presence of cycloheximide.

We next set out to study the receptors mediating adhesion of endothelial cells to α5 chain laminins. Among potential receptors of α5 chain laminins, endothelial cells are known to express Int α3β1, α5β1, α6β1, and αvβ3 (Dejana et al. 1993, Doi et al. 2002). In addition to integrins, adhesion to α5 chain laminins can also be mediated by Lu
(Parsons et al. 2001, Kikkawa et al. 2002). We therefore first examined whether HPAE and HDME cells express Lu. In Northern blot analysis of RNA extracted from HPAE and HDME cells, a 700-bp DNA probe to human Lu detected two transcripts, 2.5 kb and 4.0 kb, the smaller one being more pronounced. Western blot analysis of endothelial cell lysates with MAb BRIC221 to Lu showed two polypeptides of Mr 78,000 and 85,000; the larger one was more pronounced. In indirect immunofluorescence microscopy of endothelial cells cultured under normal cell culture conditions overnight, Lu was found uniformly on the cells in a unique, punctate distribution.

The receptors mediating adhesion of endothelial cells to α5 chain laminins were then evaluated by quantitative cell adhesion assay. Sol-Lu was used to inhibit the function of Lu by saturation of Lu binding sites on the Lm α5 chain. The specificity of the inhibitory effect of Sol-Lu was tested using Fn, Lm-332, and EHS-Lm as adhesion substrata. The endothelial cells adhered to these proteins, but the adhesion was not substantially inhibited by preincubation of the proteins with Sol-Lu.

The quantitative cell adhesion assay was then performed with HPAE cells and native human Lm-511 as an adhesion substratum. Of the plated cells, 45% adhered to this laminin. The adhesion was diminished to 20% with Sol-Lu. With function-blocking MAb to Int β1, the adhesion diminished to 15%. With the combination of Sol-Lu and MAb to β1, the adhesion was less than 10%. With the combination of Sol-Lu and function-blocking MAb to Int αvβ3, the adhesion was less than 5%. In the adhesion of HDME cells to native human Lm-511, on the other hand, 30% of the plated cells adhered to substratum. The adhesion was diminished to 10% with Sol-Lu. With MAb to Int β1 the adhesion diminished to 5%. With the combination of Sol-Lu and MAb to Int β1, as well as with Sol-Lu and MAb to Int αvβ3, the adhesion was less than 5%. The inhibitory effect of MAbs to Int α2, α3, and α6 on the adhesion of HPAE and HDME cells to Lm-511 was negligible as single agents, as well as in various combinations. The results suggest that adhesion of human endothelial cells to Lm-511 is mediated by Lu together with β1 and αvβ3 integrins.

Because of the limited availability of native human Lm-511, a mixture of Lms-511 and -521, produced by pepsin digestion from human placenta, has been used as an adhesion substratum in many cell adhesion studies. For comparison, we performed the quantitative cell adhesion experiments also with this laminin. In the adhesion of
HPAE cells, approximately 45% of the plated cells adhered to this substratum. The adhesion diminished to 35% with Sol-Lu. With MAb to Int β₁, the adhesion diminished to 30%. With the combination of Sol-Lu and MAb to Int β₁, the adhesion was less than 20%. In the adhesion of HDME cells, however, 35% of the plated cells adhered to Lm-511/521. The adhesion diminished to 25% with Sol-Lu. With MAb to Int β₁, the adhesion was 10%. When combined with Sol-Lu, the inhibitory effect of MAb to Int β₁ did not increase substantially. MAbs to Int α₂, α₃, α₆, and αβ₃ had smaller inhibitory effects on the adhesion as single agents, as well as in various combinations. The results suggest that the adhesion of human endothelial cells to commercial Lm-511/521 is mediated by Lu together with β₁ integrins.

In contrast to our results, earlier studies have presented evidence that endothelial cells adhere to Lm-511 predominantly via Int α₃β₁ (Doi et al. 2002, Fujiwara et al. 2004). We repeated the experiments outlined above with the function-blocking MAb PIB5 to Int α₃ with another function-blocking MAb 3G8 to the Int α₃ subunit, but it also failed to inhibit the cell adhesion to Lm-511 or Lm-511/521 alone or in combination with Sol-Lu. We therefore also studied by quantitative cell adhesion assay whether the endothelial cells have a functional Int α₃. As Lm-322 is known to be a high-affinity ligand for Int α₃β₁ (Tsuji 2004, Nishiuchi et al. 2006), native human Lm-332 was used as an adhesion substratum. Of the plated HDME cells, 20% adhered to this laminin. With the MAb PIB5 to Int α₃, the adhesion diminished to 10%. MAb to Int β₁ prevented the adhesion totally.

As the results showed that Lu, Int α₅, and Int β₁ are involved in adhesion of endothelial cells to Lm-511, we studied the distribution of these receptors in endothelial cells adhering to Lm-511 by indirect immunofluorescence microscopy. In a 2-h adhesion to Lm-511 in the absence of cycloheximide, immunoreactivity for Int α₅ was uniformly distributed on most of the cells, but in some cells it was located in tiny fibrillar structures. Immunoreactivity for Int β₁ was localized to small fibrillar structures. Immunoreactivity for Lu had a uniform, punctate distribution on the cells. In a 2-h adhesion to Lm-511 in the presence of cycloheximide, immunoreactivity for Int α₅ and Int β₁ was diffusely distributed on the cells, and immunoreactivity for Lu showed a uniform, punctate distribution on the cells.
7.6 BASEMENT MEMBRANE CONSTITUENTS IN LYMPHATIC VESSELS OF NORMAL HUMAN TISSUES (III)

An important role for BM proteins has been suggested with regard to development and function of lymphatic vessels (Pepper and Skobe 2003, Ji 2006). To gain insight into the potential roles of BM constituents in lymphatic endothelia, we evaluated the presence of laminins and some other BM constituents in lymphatic vessels of the skin, gastrointestinal tract, and ovary. As it has been generally problematic to distinguish lymphatic capillaries from blood capillaries (Sleeman et al. 2001), we first evaluated methods for identifying lymphatic vessels and for distinguishing them from blood capillaries.

LYVE-1-immunoreactive vessels were found in all skin, gastrointestinal tract, and ovary specimens, as well as in ovarian carcinomas (see section 7.7). All vessels presenting immunoreactivity for LYVE-1 had thin, irregular vessel walls, weak reactivity for FVIIIIR:Ag and TRITC-UEA-I, and net-like immunoreactivity for PECAM-1. Immunoreactivity for fibrillin-1 was found in fibres, running parallel to LYVE-1-immunoreactive lymphatic endothelia. All LYVE-1-immunoreactive vessels lacked immunoreactivity for smooth muscle myosin, which was used to identify smooth muscle-like cells (Díaz-Flores et al. 1991). LYVE-1 immunoreactivity was also observed in certain isolated cells, which were distinguishable from lymphatic vessels according to their morphology and their immunoreactivity for macrophage marker CD68 and occasionally also for CD163. Blood vessels, on the other hand, presented thicker vessel walls, stronger reactivity for FVIIIIR:Ag and UEA-I, more uniform reactivity for PECAM-1, and lacked immunoreactivity for LYVE-1.

Immunoreactivity for podoplanin was found in skin in lymphatic vessels, which showed similar characteristics to the LYVE-1-immunoreactive vessels. Podoplanin antiserum did not detect any lymphatic vessels in the gastrointestinal tract or ovary. MAb to VEGFR-3 did not detect lymphatic vessels in our specimens, but, in double-labeling with LYVE-1 antiserum, it showed immunoreactivity with some cell-like structures near LYVE-1-immunoreactive vessels, e.g. near the tips of lacteals of intestinal villi. Thus, LYVE-1 was chosen for further experiments for identification of lymphatic capillaries.

The distributions of BM proteins in lymphatic vessels were evaluated by indirect
immunofluorescence microscopy and also by laser scanning confocal microscopy. The results are summarized in Study III, Table 1. In all skin, ovary, and gastrointestinal tract specimens, patchy immunoreactivity for Lm α4, β1, β2, and γ1 chains was found in contact with LYVE-1-immunoreactive lymphatic endothelia. The immunoreactivity for the Lm β2 chain was prominent compared with that for the Lm β1 chain. In the skin and ovary, LYVE-1-immunoreactive lymphatic vessels lacked immunoreactivity for the Lm α5 chain. In the digestive tract, lymphatic vessels showed either lack of immunoreactivity (3 esophagus, 1 stomach, and 2 small intestine specimens) or traces of immunoreactivity (1 esophagus, 3 stomach, 1 small intestine, and 3 colon specimens) for the Lm α5 chain, but in two specimens (1 small intestine and 1 colon specimen) they showed immunoreactivity for the Lm α5 chain. In all skin, ovary and gastrointestinal specimens, LYVE-1-immunoreactive lymphatic capillaries lacked immunoreactivity for Lm α1, α2, α3, β3, and γ2 chains. Among other BM proteins, patchy immunoreactivity for nidogen-1, collagen type α1/2(IV), and collagen type XVIII was found in contact with all LYVE-1 immunoreactive lymphatic endothelia, whereas all lymphatic capillaries lacked immunoreactivity for collagen type VII. In general, immunoreactivity for BM constituents in lymph vessels was much weaker than that found in blood vessels.

### 7.7 BASEMENT MEMBRANE CONSTITUENTS IN LYMPHATIC VESSELS OF OVARIAN CARCINOMAS (III)

We also aimed to evaluate whether a difference exists between normal and tumour-associated lymphatic vessels with respect to BM protein distribution. As many tumour types lack intratumoural lymphatic vessels (Mouta Carreira et al. 2001, Williams et al. 2003, Koukourakis et al. 2005), ovarian carcinomas were used as an example of carcinomas with abundant intratumoural lymphatic vessels (Birner et al. 2000, Yokoyama et al. 2003, Ueda et al. 2005). According to the most recent classification, ovarian tumours comprise over 100 histologically distinct tumour types. In Western countries, over 90% of malignant ovarian tumours are surface epithelial-stromal tumours, which comprise serous, mucinous, endometrioid, clear cell, transitional cell, squamous cell, mixed epithelial, undifferentiated, and unclassified tumours (Tavassoli and Devilee 2003).
In ovarian carcinomas, LYVE-1-immunoreactive lymphatic vessels were found especially in the peripheral stromal areas. In endometrioid ovarian carcinomas, many LYVE-1-immunoreactive lymphatic capillaries were also found in thin cords of stroma between the islets of carcinoma cells. In the endometrioid ovarian carcinomas, most lymphatic vessels lacked immunoreactivity for Lm α1 and α2 chains. In some specimens, in which the stroma showed immunoreactivity for Lm α1 (5/10 of specimens) or Lm α2 (3/10 of specimens) chains, patchy immunoreactivity for these laminins chains was also detected in co-localization with the LYVE-1-immunoreactive endothelium of some lymphatic capillaries. The LYVE-1-immunoreactive lymphatic endothelia showed immunoreactivity for Lm α4, α5, β1, β2, and γ1 chains, nidogen-1, collagen type α1/2(IV), and collagen type XVIII. Immunoreactivity for the Lm β1 chain was prominent compared with that for the Lm β2 chain. All LYVE-1-immunoreactive lymphatic capillaries of ovarian carcinomas lacked immunoreactivity for Lm α3, β3, and γ2 chains, as well as for collagen type VII. The BM protein distribution in lymphatic vessels of serous and mucinous carcinomas was comparable with that found in endometrioid carcinomas.

Because the immunoreactivity pattern for the Lm α5 chain suggested variability in laminin content in lymphatic vessels of different tissues, we wanted to analyse this issue in more detail. We therefore studied the distribution of Lu, the most specific receptor of the Lm α5 chain (Kikkawa and Miner 2005), in lymphatic vessels. In the skin, ovary, and gastrointestinal tract, LYVE-1-immunoreactive lymphatic capillaries lacked immunoreactivity for Lu, whereas in ovarian carcinomas immunoreactivity for Lu was detected in co-localization with LYVE-1-immunoreactive lymphatic endothelia.

7.8 LOCALIZATION OF α4 CHAIN LAMININS IN RENAL CELL CARCINOMAS (IV)

As the role of α4 chain laminins in carcinomas remains unknown (Fujita et al. 2005, Määttä et al. 2005), we set out to study the distribution of α4 chain laminins in RCCs.

The distribution of α4 chain laminins in RCCs was first evaluated by an alkaline phosphatase-anti-alkaline phosphatase technique. In oncocytomas, immunoreactivity for the Lm α4 chain was localized to the region of endothelial BMs of blood capillaries and larger blood vessels. Immunoreactivity was also found in the BM region around tumour cell islets. Thin cords of stroma between tumour cell islets lacked immunoreactivity, but wider stromal areas around larger blood vessels showed immunoreactivity for the Lm α4 chain.

In conventional (clear cell) RCCs, metastases of conventional RCCs, and papillary RCCs, the immunoreactivity of the stroma, BMs of tumour cell islets, and endothelial BMs of capillaries could not with certainty be distinguished due to the continuous nature of the immunostaining. To distinguish between immunoreactivity in these locations, the distribution of the Lm α4 chain in RCC was also studied by double-labelling immunofluorescence microscopy and laser scanning confocal microscopy.

In double-labelling, TRITC-UEA-I was used to identify blood vessel endothelia. The Lm α1 chain is typical for epithelial BMs (Virtanen et al. 2000) and is also found in BMs of tumour cell islets in RCCs (Rissanen et al. 2003). Thus, affinity-purified rabbit antiserum to the Lm α1 chain was used to identify BMs of tumour cell islets. The results are summarized in Study IV, Table 1. In oncocytomas, immunoreactivity for the Lm α4 chain was found in colocalization with TRITC-UEA-I-reactive endothelia. Cords of stroma between tumour cells islets lacked immunoreactivity for the Lm α4 chain, but stroma around larger blood vessels did show immunoreactivity. The BMs of tumour cell islets showed immunoreactivity for the Lm α4 chain. In all grade 1-3 conventional RCCs and metastases of grade 1 and 3 conventional RCCs, immunoreactivity for the Lm α4 chain was found in co-localization with TRITC-UEA-I-reactive endothelia of blood vessels and as patches in stroma. Lm α1 chain-immunoreactive BMs were observed around tumour cell islets in 10/10 grade 1, 14/15 grade 2, and 4/6 grade 3 conventional RCCs, and in 2/2 metastases of conventional RCCs. Immunoreactivity for the Lm α4 chain was found in co-localization with these Lm α1 chain-immunoreactive BMs in 7/10 grade 1, 12/14 grade 2, and 4/4 grade 3 conventional RCCs, and
in 2/2 metastases of conventional RCCs. In papillary RCC, immunoreactivity for the Lm α4 chain was found in co-localization with TRITC-UEA-I-reactive endothelia of blood vessels and as patches in stroma. Lm α1 chain-immunoreactive BMs of tumour cell islets were found in 2/2 papillary RCCs. Immunoreactivity for the Lm α4 chain was seen in co-localization with Lm α1 chain-immunoreactive BMs in 1/2 papillary RCCs.

Recently, a shift from the expression of Lm-421 to expression of Lm-411 has been suggested during progression of malignancy in gliomas and breast carcinomas (Ljubimova et al. 2001, 2004, Fujita et al. 2005). We therefore evaluated the distribution of Lm β1, β2, and γ1 chains in a subset of our specimens, including two oncocytomas, three grade 1, three grade 2, and three grade 3 conventional RCCs, two metastases of conventional RCCs, and two papillary RCCs. In oncocytomas, conventional RCCs, and metastases of conventional RCCs, immunoreactivity for Lm β1, β2, and γ1 chains was found in all locations where the Lm α4 chain was present. In papillary RCCs, immunoreactivity for Lm β1 and γ1 chains was found in all locations where the Lm α4 chain was present, whereas immunoreactivity for the Lm β2 chain was observed in BMs of blood vessels, but not in stroma or in Lm α1 chain-immunoreactive BMs of tumour cell islets.

**7.9 PRODUCTION OF α4 CHAIN LAMININS BY RENAL CARCINOMA CELLS (IV)**

Presence of the Lm α4 chain in BMs of tumour cell islets suggested that α4 chain laminins could be produced by RCC cells. We therefore evaluated whether RCC cells produce α4 chain laminins.

Northern blot analysis of RNA extracted from RCC cell lines Caki-1, Caki-2, and ACHN with an RNA probe to the Lm α4 chain showed a band of approximately 6.5 kb. The band was weakest in Caki-2 cells and strongest in Caki-1 cells.

In Caki-1, Caki-2, and ACHN cells grown under normal cell culture conditions, weak immunoreactivity for the Lm α4 chain was found diffusely throughout the cells. When protein secretion was inhibited by pre-exposing the RCC cells to monensin, prominent immunoreactivity for the Lm α4 chain was seen in cytoplasmic vesicles. After monensin treatment, immunoreactivity for Lm β1 and γ1 chains was also observed in cytoplasmic vesicles, whereas negligible immunoreactivity for the Lm
β2 chain was found throughout the cells.

Immunoprecipitation of culture medium of Caki-1, Caki-2, and ACHN cells with MAb 113BC7 to the Lm γ1 chain and Western blot analysis of the precipitates with affinity-purified rabbit antiserum to the Lm α4 chain showed broad bands of M_r 200 000 and 220 000, and a minor band of M_r 180 000. The expression in Caki-1 and ACHN cells was prominent compared with the expression in Caki-2 cells. In previous studies, Western blotting of the Lm α4 chain has shown variable broad bands of M_r 180 000–220 000. The variability in M_r has been suggested to result from variability in protein cleavage sites and modification of the protein by glycosylation and glycosaminoglycans (Talts et al. 2000, Geberhiwot et al. 2001, Hayashi et al. 2002, Kortesmaa et al. 2002).

7.10 RENAL CARCINOMA CELLS’ ADHESION TO AND MIGRATION ON LAMININ-411 (IV)

To study the potential functional consequences of Lm α4 chain expression in RCC, we evaluated the effect of recombinant human Lm-411 on adhesion and migration of RCC cells.

In quantitative cell adhesion assay, the adhesion of Caki-1, Caki-2, and ACHN cells to 1-20 µg/ml of recombinant human Lm-411 was negligible. By contrast, all three RCC cell lines adhered significantly to 1 µg/ml of Fn (p<0.01; compared with negligible adhesion to BSA-coated wells). Maximal adhesion to Fn was achieved with 5 µg/ml coat concentration.

The experiments were then performed with a combination of 5 µg/ml of Fn and varying concentrations of recombinant human Lm-411. The adhesion of Caki-1 cells to Fn was significantly (p=0.001) inhibited with 5 µg/ml of Lm-411, and over 95% inhibition was achieved with 15 µg/ml. The adhesion of Caki-2 cells to Fn was also significantly inhibited (p=0.003) with 5 µg/ml of Lm-411, and over 90% inhibition was achieved with 20 µg/ml. The adhesion of ACHN cells to Fn was significantly (p=0.004) inhibited with 10 µg/ml of Lm-411, and over 60% inhibition was achieved with 20 µg/ml.

The migration of RCC cells was investigated with a fluorescence live cell imaging technique. Within 2 h, Caki-1 cells migrated significantly (p<0.005) more on Lm-411 (mean 231 µm, ±SD 123 µm, median 216 µm) than on Fn (mean 143 µm, ±SD 98 µm,
median 120 µm). Caki-2 cells migrated significantly (p<0.005) more on Lm-411 (mean 200 µm, ±SD 55 µm, median 207 µm) than on Fn (mean 54 µm, ±SD 33 µm, median 43 µm). Also ACHN cells migrated significantly (p<0.005) more on Lm-411 (mean 484 µm, ±SD 62 µm, median 480 µm) than on Fn (mean 73 µm, ±SD 30 µm, median 66 µm). On Lm-411, the zigzag movement was pronounced compared with that on Fn. Prominent movement continued for approximately 1.5 h (180/240 images) on both proteins and ceased thereafter.
8. DISCUSSION

8.1 DISTRIBUTION OF α4 CHAIN LAMININS IN DEVELOPING AND ADULT HUMAN TISSUES

The first aim of this study was to create a MAb against the laminin α4 chain to evaluate the distribution of this protein in human tissues. The results indicated that MAb 168FC10 reacts specifically with recombinant hybrid Lm-411 in EIA and Lm-411 from human platelets in immunoprecipitation. The results excluded reactivity with other laminin isoforms (Lm-511/521) and isolated Lm β1 and γ1 chains. Because other α4 chains containing laminin isoforms, such as Lm-421, were unavailable for this kind of experiment, it could not be confirmed whether this MAb reacts with the α4 chain or with the Lm-411 trimer. The fact that this MAb reacted with both native human Lm-411 and recombinant hybrid Lm-411 (containing mouse Lm β1 chain) suggested that the reactivity was not dependent on the exact characteristics of the β chain. Thus, we consider that the immunoreactivity pattern of this MAb reflects the tissue distribution of the Lm α4 chain.

When we evaluated the distribution of the Lm α4 chain in developing and adult human tissues, we found immunoreactivity for this Lm chain in co-localization with all blood vessel endothelia, with the exception of glomerular capillaries. Previous data on the presence of the Lm α4 chain in blood vessel endothelia have been somewhat controversial. By in situ hybridization, Iivanainen et al. (1995) did not detect Lm α4 chain mRNA in blood vessel endothelia in human tissues. By immunohistochemistry, however, Lm α4 chain has been found in vasculature of human skeletal muscle, brain, and bone marrow (Gu et al. 1999, Patton et al. 1999, Ljubimova et al. 2001). In mouse tissues, Sorokin and co-workers (Frieser et al. 1997) detected by in situ hybridization Lm α4 chain mRNA in blood vessel endothelia in, for instance, the aorta, brain, and skin. They concluded that Lm α4 chain is expressed only in a subset of endothelia, but based on the same evidence they later stated that the Lm α4 chain is expressed by all endothelial cells (Hallmann et al. 2005). By immunohistochemistry, the Lm α4 chain has been found in mouse blood vessel endothelia of skin, lung, heart, skeletal muscle, kidney, intestine, and brain (Iivanainen et al. 1997, Miner et al. 1997, Lefebvre et al. 1999, Sasaki et al. 2001, Sixt et al. 2001a). These discrepancies could be due to differences in
the methods used, in situ hybridization showing the localization of high mRNA expression and immunohistochemistry the localization of the protein. Our results on the presence of the Lm α4 chain in most, if not all, non-glomerular endothelial BMs are supported by abnormal structure of endothelial BMs of Lm α4 chain-deficient mice (Thybol et al. 2002).

The results showed that the Lm α4 chain is found in human BMs of skeletal muscle cells during development, but not in adults. In developing skeletal muscle, the Lm α4 chain revealed a striated immunoreactivity pattern in exact co-localization with the Lm α2 chain. The Lm α2 chain is a constituent of costameres. Costameres are focal adhesion-like cell-ECM adhesions that form a force-transmitting link from sarcomeres to sarcolemma and further to Lm-211-containing BMs. Costameres appear in striated distribution on striated muscle cells (Ervasti 2003). Thus, the results suggest that the Lm α4 chain is localized to costameres in developing skeletal muscle. In contrast to normal skeletal muscle, in Lm α2 chain-deficient muscular dystrophy in humans and in mice, the Lm α4 chain is found in BMs of skeletal muscle cells also in adults (Patton et al. 1997, 1999). This has raised the question of whether the Lm α4 chain could to some extent functionally compensate for the Lm α2 chain in muscle (Patton et al. 1997). In Lm α2 chain-deficient muscular dystrophy and certain other muscular dystrophies, extraocular muscles are not affected (Kaminski et al. 1992, Porter and Karathananas 1998). BMs of adult extraocular muscle fibres have later been shown to comprise Lm α4 and α5 chains, as well as the Lm α5 chain receptor Lu (Kjellgren et al. 2004). In Lm α2 chain-deficient mice, the expression of the Lm α4 chain in extraocular muscles is pronounced compared with that of the Lm α5 chain, suggesting that the Lm α4 chain may protect extraocular muscles in Lm α2 chain-deficient muscular dystrophy (Nyström et al. 2006).

Previous results on the presence of the Lm α4 chain in BMs of heart muscle cells in mice have been contradictory (Iivanainen et al. 1997, Miner et al. 1997, Talts et al. 2000). Our findings showed that in man while the expression of Lm α4 chain in BMs of heart muscle cells declines during development, it is still detectable in adults. This emphasizes a difference between the two striated muscle types, skeletal and heart muscle, with respect to Lm distribution. Later, lack of the Lm α4 chain in mice has been shown to lead to a cardiomyopathy-like condition comprising a higher heart weight,
increased end-diastolic and end-systolic diameters, decreased fractional shortening of the left ventricle, intermittent bradycardia, and sinus arrhythmia in echocardiography. Histologically, the hearts of Lm α4 chain-deficient mice presented with increased size, degeneration, uneven cell borders and uneven distribution of dystroglycans and sarcoglycan in cardiomyocytes, fibrosis, and widening of intercellular spaces (Wang J et al. 2006), suggesting a role for α4 chain laminins in the function of heart muscle. Recently, mutations in the Lm α4 chain causing deletion or conformational change in the LG region have been described in human patients with dilated cardiomyopathy, but not in the control population, implying a role for α4 chain laminins in pathogenesis of this disease (Knöll et al. 2007).

The results showed that BMs of visceral smooth muscle cells are immunoreactive for the Lm α4 chain in both developing and adult tissues. BMs of vascular smooth muscle cells revealed immunoreactivity for the Lm α4 chain during development, but the immunoreactivity declined before adulthood. This also emphasizes a difference between the two smooth muscle types, visceral and vascular, with respect to laminin distribution. A similar distribution pattern, including the consistent presence in BMs of visceral smooth muscle, but a developmental decrease in BMs of vascular smooth muscle, has previously been described for the Lm β1 chain (Glukhova et al. 1993), suggesting that the developmentally-regulated laminin in vascular smooth muscle is Lm-411. In addition to smooth muscle, immunoreactivity for the Lm α4 chain seemed to be a shared feature of other contractile tissues such as the myoid cell layer of the testis and the mesangium of kidney glomeruli.

Considering the neuromuscular system, immunoreactivity for the Lm α4 chain was also found in the epineurium and perineurium of peripheral nerves, as well as in co-localization with the Lm α2 chain in the endoneurium (Leivo and Engvall 1988, Sanes et al. 1990). In addition to the previously described effects on localization of synaptic specializations at neuromuscular junctions (Patton et al. 2001), later studies have shown that lack of the Lm α4 chain in mice leads to ataxia, tremor, and impaired myelination of axons (Wallquist et al. 2005, Yang et al. 2005). In vitro, Lm-411 promoted proliferation and protrusion formation of Schwann cells (Wallquist et al. 2005, Yang et al. 2005). Lm-411 did not promote axon growth of motor neurons, but promoted neurite outgrowth from sensory neurons (Fried et al. 2005, Wallquist et al. 2005). Thus,
in the endoneurium, α4 chain laminins seem to affect myelination and possibly also axon growth of peripheral nerves.

Studies on the kidney were among the first to imply a role for laminins in epithelial development (Ekblom et al. 1980, 1998; Klein et al. 1988, Miner and Yurchenco 2004). Based on in situ hybridization, the Lm α4 chain was suggested to be expressed in mice in developing but not in adult kidneys (Iivanainen et al. 1997, Sorokin et al. 1997a). By immunohistochemistry Miner et al. (1997) detected the Lm α4 chain in mice in the area of developing but not mature nephrons. We found immunoreactivity for the Lm α4 chain also in adult human nephrons, particularly in the mesangium. Mesangial cells function in regulation of glomerular capillary flow, maintenance of glomerular structure, and production of, for instance, vasoactive substances and ECM components, and are involved in the pathogenesis of many renal diseases (Herrera et al. 2006). A subsequent in vitro study suggested that α4 chain laminins promote adhesion and differentiation of mesangial cells (Hansen and Abrass 2003). In a linkage disequilibrium study, the Lm α4 chain was found to be among the candidate genes for diabetic nephropathy (Ewens et al. 2005).

Further, previous studies on mouse tissues have suggested the presence of the Lm α4 chain in connective tissues, but not in BMs of epithelia (Iivanainen et al. 1997, Miner et al. 1997, Sorokin et al. 1997a, Lefebvre et al. 1999). We found immunoreactivity for the Lm α4 chain in the region of epithelial BMs in the epidermis, medullary tubules in the kidney, esophageal glands, gastric glands, intestinal glands, salivary glands, and pancreatic acini. In agreement with our findings, Sasaki et al. (2001) reported immunoreactivity for the Lm α4 chain in epidermal BM of mice. Miner et al. (2004b) have later detected immunoreactivity for the Lm α4 chain in acinar BMs of the mouse pancreas. The phenotype of mice lacking both Lm α4 and α2 chains suggests that the Lm α4 chain may have a role in polarization of pancreatic acinar cells (Miner et al. 2004b).

8.2 ADHESION OF HUMAN ENDOTHELIAL CELLS TO LAMININS OF ENDOTHELIAL BASEMENT MEMBRANES

The results of Study I showing that α4 chain laminins are prominent in endothelial BMs of most, if not all, non-glomerular blood vessels elicited our interest in the role of lam-
inins in endothelial BMs. Immunofluorescence, immunoprecipitation, and Western blotting in Study I showed that human endothelial cells produce and deposit α4 chain laminins. Further, evaluation of protein synthesis by immunoprecipitation in Study II revealed that human endothelial cells also produce α5 chain laminins. Considering the suggestion by Sorokin and co-workers that the expression of α4 and α5 chain laminins is upregulated by inflammatory cytokines, growth factors, or angiostatic agents (Frieser et al. 1997, Sixt et al. 2001a, Hallmann et al. 2005), we observed production of these laminins by human endothelial cells under normal cell culture conditions.

To gain insights into the functions of α4 and α5 chain laminins in endothelia, we evaluated the ability of these laminins to promote adhesion of endothelial cells. Human endothelial cells barely adhered and neither spread nor formed cell-ECM adhesion complexes on recombinant human Lm-411. Native human Lm-511 promoted the adhesion and spreading of endothelial cells better than recombinant human Lm-411, but not as well as human fibronectin. The endothelial cells adhering to Lm-511 formed tiny fibril-like adhesion structures immunoreactive for both talin and fibronectin. Mature focal adhesions are usually devoid of fibronectin, whereas fibronectin immunoreactivity is typical for fibrillar adhesions, cell-ECM adhesion complexes associated with fibronectin fibrillogenesis (Avnur and Geiger 1981, Chen and Singer 1982, Zamir et al. 1999, 2000, Katz et al. 2000, Pankov et al. 2000). Thus, the results suggested that endothelial cells form fibrillar adhesions on Lm-511. Detection of fibronectin-immunoreactive adhesion structures under fibronectin-free experimental conditions suggested, however, that during the adhesion assays the endothelial cells produce proteins, which could have effects on the results. Endothelial cells indeed produced endogenous proteins, such as fibronectin and α5 chain laminins, within 2-3 h, and the production of these proteins could be prevented with cycloheximide. In the presence of cycloheximide, the proportion of cells adhering and spreading on either Lm-511 or fibronectin was slightly decreased. Immunoreactivity for talin was found diffusely in the cells. This could, however, raise the question of whether cycloheximide prevents the formation of cell-ECM adhesion structures. Although findings of studies about the effect of cycloheximide on the formation of cell-ECM adhesion complexes have been somewhat controversial (Tarone et al. 1985, Kabir et al. 2002), the prevailing view is that cycloheximide does not prevent their formation (Pankov et al. 2000, Cukierman et al. 2001,
Kabir et al. 2002). In agreement, our results showed that addition of fibronectin to the Lm-511 coat induced the formation of typical focal adhesions in the presence of cycloheximide. Thus, our findings indicate that neither recombinant human Lm-411 nor native human Lm-511 promote the formation of any specific cell-ECM adhesion complexes, suggesting that these proteins do not promote firm adhesion (Zamir et al. 1999, Geiger et al. 2001, Zaidel-Bar et al. 2004).

In contrast to our results, Fujiwara et al. (2004) reported that human dermal microvascular endothelial cells show elongated morphology and protrusions on Lm-411 isolated from T98G human gliomablastoma cells, and focal adhesions on Lm-511/521 isolated from A549 human lung adenocarcinoma cells. A possible reason for this discrepancy could be the production of endogenous proteins during the adhesion assay, which Fujiwara et al. (2004) did not take into consideration. Gonzales et al. (2001) suggested that immortalized human bone marrow endothelial cells or growth factor-stimulated human microvascular endothelial cells form focal adhesion-like adhesion complexes, in which immunoreactivity for the Lm α4 chain and integrin αv are in partial co-localization. These adhesion complexes were not, however, formed in cells adhering to α4 chain laminins, but in normal subconfluent cell cultures in cells adhering to glass, to proteins of their culture medium, and to their own ECM products. Thus, the formation of these adhesion complexes was not shown to be induced by α4 chain laminins.

The adhesion of human endothelial cells to recombinant human Lm-411 was significantly decreased by function-blocking MAbs to α6 and β1 integrin subunits, suggesting that the adhesion of endothelial cells to Lm-411 is mediated by integrin α6β1. This is in agreement with other studies showing that the adhesion of immortalized mouse brain capillary endothelial cells and bovine adrenal microvascular endothelial cells to recombinant hybrid Lm-411 as well as the spreading of human dermal microvascular endothelial cells on Lm-411 isolated from T98G human glioblastoma cells are inhibited by function-blocking MAbs to α6 and β1 integrin subunits (Kortesmaa et al. 2000, Fujiwara et al. 2004). In contrast, attachment of immortalized human bone marrow endothelial cells and human umbilical vein endothelial cells to recombinant fragments of Lm α4 chain LG domains, produced in bacteria, was shown to be inhibited by function-blocking MAbs to αvβ3 and β1 integrins, and the combination of function-block-
ing MAbs to α₃ and α₆ integrins (Gonzales et al. 2001, Gonzalez et al. 2002, Lian et al. 2006). This discrepancy could arise, for instance, from differences in glycosylation between the recombinant fragments produced in bacteria and the recombinant or native Lm-411 produced in human cells. Alternatively, it could result from conformational changes or exposure of cryptic binding sites in the LG domain fragments compared with whole Lm-411 molecules.

As to the potential receptors of α5 chain laminins, previous immunohistochemical studies have localized Lu to blood vessels (Parsons et al. 1997, Schön et al. 2000). Moulson et al. (2001) found Lu around vascular smooth muscle cells. Garin-Chesa et al. (1994) suggested that human umbilical vein endothelial cells produce a M₉₀ 000 isoform of Lu. Our Western and Northern blot results showed that human endothelial cells express both Lu isoforms of M₇₈ 000 and 85 000, which correspond to the Lu isoforms detected in human red blood cells, tumour cells, and many human tissues (Parsons et al. 1987, Daniels and Khalid 1989, Rahuel et al. 1996). The long-tail isoform of Lu, encoded by the smaller 2.5 kb transcript (Rahuel et al. 1996, El Nemer et al. 1997), was predominantly expressed by human endothelial cells. On endothelial cells, Lu presented a punctate immunoreactivity pattern, which did not resemble the distribution of any known cell-ECM adhesion complexes.

Because function-blocking antibodies preventing the interaction of Lu with Lm α5 chain were unavailable, the role of Lu in the adhesion of endothelial cells to α5 chain laminins was studied by saturation of the Lu binding sites on the Lm α5 chain with Sol-Lu, as described by Kikkawa et al. (2003). The inhibitory effect of Sol-Lu was specific for α5 chain laminins. When used alone, Sol-Lu and function-blocking MAb to integrin β₁ subunit inhibited the adhesion markedly. With their combination, the adhesion was nearly abolished, suggesting that the endothelial cell adhesion to Lm-511 is mediated by both Lu and β₁ integrins. Interestingly, function-blocking MAb to integrin α₅β₃ did not hinder the adhesion to Lm-511 when used alone, but in combination with Sol-Lu it prevented the adhesion almost completely. This implies that integrin α₅β₃ is not essential for adhesion of endothelial cells to Lm-511. It could, for instance, partially replace the function of primary adhesion receptors, Lu or β₁ integrins, if the function of either one of them is prevented. Another explanation for this phenomenon could be the trans-dominant inhibition of other adhesion receptors, such as β₁ integrins.
via integrin $\alpha_v\beta_3$, as previously proposed by others (Diaz-Gonzales et al. 1996, Hynes 2002b). Thus, the results suggest that adhesion of human endothelial cells to Lm-511 is mediated by Lu together with $\beta_1$ and $\alpha_v\beta_3$ integrins. El Nemer et al. (2007) have later reported that on endothelial cells Lu could also mediate the interaction of these cells with sickle red blood cells by interacting with integrin $\alpha_4\beta_1$.

Fujiwara et al. (2004) have shown that spreading of human dermal microvascular endothelial cells on Lm-511/521 is completely inhibited by function-blocking MAb to integrin $\beta_1$, and 40% inhibited by function-blocking MAb to integrin $\alpha_3$ subunit. Doi et al. (2002) have shown that the adhesion of human saphenous vein endothelial cells and immortalized mouse brain capillary endothelial cells to recombinant human Lm-511 is partially inhibited by function blocking MAbs to integrin $\beta_1$ and $\alpha_3$ subunits. Our results on the role of integrin $\beta_1$ are in agreement with the aforementioned studies. Although we used the same two MAbs to integrin $\alpha_3$ subunit (PIB5 and 3G8) as in the previous studies, our results did not support the suggested primary role for integrin $\alpha_3$ subunit in the adhesion of human endothelial cell to Lm-511. The binding of an integrin depends not only on its expression and affinity, but also on its activation state and interaction with other proteins, such as CD151 (van der Flier and Sonnenberg 2001, Hynes 2002a, Nishiuchi et al. 2005). One of the high-affinity ligands for integrin $\alpha_3\beta_1$ is Lm-332 (Tsuji 2004, Nishiuchi et al. 2006). Lm-332 has been suggested to be present in endothelial BMs of certain capillaries (Määttä et al. 2004, Wang et al. 2004), in which case microvascular endothelial cells could interact with Lm-332 via integrin $\alpha_3\beta_1$. In quantitative cell adhesion assay, the adhesion of HDME cells to Lm-332 was indeed inhibited by MAb PIB5 to integrin $\alpha_3$ subunit. Thus, endothelial cells have a functional $\alpha_3$ integrin, although it appeared not to mediate adhesion to Lm-511.

The results showed that MAb to integrin $\beta_1$ inhibited the adhesion of HDME cells clearly better than HPAE cells, whereas the effect of Sol-Lu was more pronounced with HPAE cells than with HDME cells. Thus, microvascular and pulmonary artery endothelial cells seem to differ to some extent in their adhesion characteristics. Further, MAbs to integrin $\beta_1$ subunit and Sol-Lu had more pronounced effects on cell adhesion to native human Lm-511 than to the commercial Lm-511/521 preparation. Combination of Sol-Lu and function-blocking MAb to $\alpha_v\beta_3$ integrin did not inhibit adhesion to Lm-511/521, unlike to native Lm-511, indicating that experiments performed with the
commercial Lm-511/521 are not fully comparable with experiments performed with native Lm-511. The commercially available Lm-511/521 preparations are produced by pepsin digestion from placenta and contain partially degraded mixture of laminins. Proteolysis and variability in preparation contents have effects on experimental results (Wondimu et al. 2006).

Our results on lack of focal adhesion formation in endothelial cell adhesion to Lm-411 and Lm-511 indicate that these laminins do not promote firm adhesion. Adhesion in the absence of focal adhesion formation implies that these proteins may promote a migratory phenotype in the cells (Murphy-Ullrich et al. 2001). In agreement, α4 and α5 chain laminins have been suggested to promote activation of Rho family guanine triphosphatase Rac and migration of endothelial cells (Doi et al. 2002, Fujiwara et al. 2004). Lm-411 has been suggested to enhance angiogenesis, but the results are somewhat controversial (Thyboll et al. 2002, Zhou et al. 2004, Fujita et al. 2006, Li et al. 2006). As to the other potential functions of these laminins in endothelial BMs, α4 chain laminins appear to be permissive for leukocyte transmigration through endothelium, whereas α5 chain laminins seem to prevent it (Sixt et al. 2001a, Wang S et al. 2006). Similarly, Trypanosoma brucei, a parasite that causes human Sleeping Sickness, has recently been suggested to penetrate endothelial BMs in areas where α4 chain laminins are present, but α5 chain laminins are absent (Masocha et al. 2007).

8.3 DISTRIBUTION OF BASEMENT MEMBRANE CONSTITUENTS IN LYMPHATIC CAPILLARIES

The suggested important roles for BM proteins in blood vessel endothelia (Kalluri 2003, Davis and Senger 2005, Hallmann et al. 2005) elicited our interest in the role of these proteins in lymph vessels. We thus evaluated the presence of BM proteins in lymphatic vessels of the skin, gastrointestinal tract, ovary, and ovarian carcinomas. The results in the majority of normal tissue specimens showed that the BM region of lymphatic vessels is immunoreactive for Lm α4, β1, β2, and γ1 chains, type α1/2(IV) and XVIII collagens, and nidogen-1, but not for Lm α1, α2, α3, α5, β3, and γ2 chains, or collagen type VII.

According to the current concept, laminin polymerization is a prerequisite for BM assembly (Li et al. 2002, Yurchenco et al. 2004). Laminin α4 chain is truncated
in lacking N-terminal short-arm domains, with the exception of a few LE domains (Livanainen et al. 1995, Richards et al. 1996). Truncated laminins have been suggested to be incapable of polymerization (Cheng et al. 1997, Li et al. 2002, Yurchenco et al. 2004). In agreement, the presence of Lm α4 chain cannot compensate for the loss of Lm α2 chain in muscle cell BMs in Lm α2 chain-deficient muscular dystrophy in humans and in mice (Patton et al. 1997, 1999). Consistently, Lm-411 was unable to re-establish BM assembly in Lm γ1 chain-null mouse embryoid bodies, whereas Lms-111, -221, and -211/221 restored BM assembly (Murray and Edgar 2000, Li et al. 2002). Thus, the presence of only α4 chain laminins in the BM region of most lymphatic vessels may be the reason for discontinuity of endothelial BMs, previously defined by electron microscopy (Leak 1970), in these vessels. The inability of α4 chain laminins to polymerize requires further evaluation.

Lymphatic capillaries of ovarian carcinomas showed consistent immunoreactivity also for the Lm α5 chain. The α5 chain laminins, which have an elongated N-terminal short arm (Doi et al. 2002), might enable laminin polymerization and BM formation in these vessels. The appearance of endothelial BMs in tumour lymphatic vessels, however, warrants further evaluation by electron microscopy. Among receptors of the Lm α5 chain, Lu seems to be the most specific for this ligand (Kikkawa and Miner 2005). In tissues, Lu has been suggested to be present only at sites where the Lm α5 chain is found in adjacent BMs. Thus, the distribution of Lu may reflect specific functions of α5 chain laminins (Moulson et al. 2001, Kikkawa and Miner 2005). Immunoreactivity for Lu was observed in lymphatic capillaries of ovarian carcinomas, but not in those of normal tissues; thus, a difference exists between normal and tumour lymphatic vessels.

In many previous studies, lymphatic vessels have been distinguished from blood vessels on the basis of their weak immunoreactivity for BM proteins (Barsky et al. 1983, Hultberg and Svanholm 1989, Wigle et al. 2002). In most studies describing weak or lack of immunoreactivity for laminins in lymphatic vessels, antibodies against EHS-Lm have been used. These antibodies have been suggested to detect Lm γ1 chain (Erickson and Couchman 2000). Our results revealed immunoreactivity for certain BM constituents in the BM region of lymphatic endothelia. In support of our findings, Sauter et al. (1998) have demonstrated the presence of collagen type IV in the discontinu-
ous endothelial BM of lymphatic capillaries by immunoelectron microscopy. Previous microarray studies have shown lower expression of Lm α3, α5, β1, β2, and γ2 chain, collagen type IV and XVIII, and nidogen mRNA in lymphatic endothelial cells than in blood vessel endothelial cells (Petrova et al. 2002, Podgrabinśka et al. 2002, Hirakawa et al. 2003). This is in line with our results indicating that immunoreactivity for BM constituents was generally weaker in lymphatic vessels than in blood vessels.

Considering that the endothelial BMs of most lymphatic vessels are discontinuous (Leak 1970, Sacchi et al. 1997, 1999), and thus, may not provide mechanical stability, what is the significance of BM proteins near the endothelia in lymphatic vessels? The α4 and α5 chain laminins have been suggested to promote migration of blood vessel endothelial cells and to regulate transmigration through blood vessel endothelium (Sixt et al. 2001a, Doi et al. 2002, Fujiwara et al. 2004, Wang S et al. 2006, Masocha et al. 2007). Despite their likely inability to polymerize (Yurchenco et al. 2004), α4 chain laminins have been proposed to regulate angiogenesis (Thyboll et al. 2002, Zhou et al. 2004, Fujita et al. 2006, Li et al. 2006). Nidogens, type α1/2(IV) and type XVIII collagens seem to be essential for development and function of at least certain blood vessels (Fukai et al. 2002, Pöschl et al. 2004, Böse et al. 2006). Also proteolytic fragments of type IV and XVIII collagens have been suggested to regulate angiogenesis (Ortega and Werb 2002). The presence of these proteins in the region of endothelial BMs in lymphatic vessels implies that they could at least in part serve similar roles in development and function of lymphatic vessels as those suggested for blood vessels.

8.4 DISTRIBUTION AND FUNCTION OF α4 CHAIN LAMININS IN RENAL CELL CARCINOMAS

In the course of this study, α4 chain laminins have been proposed to promote malignancy, invasion, and angiogenesis of human gliomas (Ljubimova et al. 2001, 2004, Khazenzon et al. 2003, Nagato et al. 2005, Fujita et al. 2006). Tissue-specific inhibition of the laminin α4 chain with antisense oligonucleotides has been tendered as a potential new treatment for gliomas and tumour angiogenesis (Khazenzon et al. 2003, Nagato et al. 2005, Fujita et al. 2006, Lee et al. 2006). A shift from the expression of Lm-421 to the expression of Lm-411 was associated with progression of human breast carcinoma (Fujita et al. 2005). To gain further insights into the role of α4 chain lam-
inins in carcinomas, we evaluated their distribution in RCCs.

The results showed that α4 chain laminins are consistently found in endothelial BMs and in stroma of oncocytomas, conventional RCCs, metastases of conventional RCCs, and papillary RCCs. In addition, α4 chain laminins were observed in the BMs of tumour cell islets in oncocytomas and most RCCs. In several previous microarray studies, Lm α4 chain mRNA has been suggested to be overexpressed in renal tumours, primarily conventional RCCs, compared with its expression in normal human kidneys (Boer et al. 2001, Young et al. 2001, Gieseg et al. 2002, Lenburg et al. 2003). The mRNA expression results have not been previously confirmed at the protein level. Despite the wide distribution of the Lm α4 chain in RCCs, shown in Study IV, and the relatively restricted distribution in the normal human kidney, shown in Study I, it is difficult to conclude whether there is overexpression in RCCs at the protein level. This is because the cell types of the normal human kidney are not directly comparable with those of RCCs. Based on cytological, histological, and immunohistochemical features, conventional RCCs have been proposed to originate from proximal tubules of nephrons (Thoenes et al. 1990, Lohi et al. 1998, Young et al. 2001). In this respect, our results that α4 chain laminins are not present in proximal tubules of adult kidney but are found in conventional RCCs suggest a possibility that neo-expression of α4 chain laminins occurs in RCCs. On the other hand, α4 chain laminins have been thought to be primarily produced by mesenchyme-derived cells such as endothelial cells, blood cells, smooth muscle cells, and fibroblasts (Pierce et al. 1998, Lefebvre et al. 1999, Geberhiwot et al. 2000, 2001, Pedraza et al. 2000, Talts et al. 2000). In this regard, we suspect that the prominent expression of the Lm α4 chain detected in particular in the vasculature and stroma of RCCs accounts for the overexpression of the α4 chain in RCCs suggested by microarray studies (Boer et al. 2001, Young et al. 2001, Gieseg et al. 2002, Lenburg et al. 2003). It is noteworthy, however, that despite their assumed epithelial origin RCC cells were capable of producing α4 chain laminins, indicating that they can participate in the production in α4 chain laminins in RCCs.

Immunoreactivity for the Lm α4 chain has been suggested to correlate with increasing malignancy, tumour recurrence, and poor patient survival in gliomas (Ljubimova et al. 2004). By contrast, we found no correlation between immunoreactivity for the Lm α4 chain and tumour grade in RCCs. In concordance, no clear correlation between
Lm α4 chain mRNA expression and tumour grade has been detected in the previous microarray studies on renal tumours (Boer et al. 2001, Young et al. 2001, Gieseg et al. 2002, Lenburg et al. 2003). Further, correlation between a shift from Lm-421 expression to Lm-411 expression and malignancy in gliomas and breast carcinomas has been proposed (Ljubimova et al. 2001, 2004, Fujita et al. 2005). We found immunoreactivity for both Lm β1 and β2 chains in the BMs of tumour cells islets, blood vessels, and stroma in conventional RCCs. In papillary RCCs, immunoreactivity for the Lm β1 chain was observed in BMs of tumour cell islets, blood vessels, and stroma, whereas immunoreactivity for the Lm β2 chain was restricted to BMs of blood vessels. Thus, in agreement with a previous study by Lohi et al. (1996), no correlation existed between Lm β chain expression and the tumour grade in RCCs. Based on localization, Lm α4 chain can be in the form of both Lm-411 and Lm-421 in RCCs.

To evaluate the potential functional consequences of Lm α4 chain expression in RCCs, we also studied the effects of Lm-411 on the adhesion and migration of RCC cells. The results of quantitative cell adhesion assays showed that recombinant human Lm-411 did not promote the adhesion of RCC cells. Instead, it inhibited the adhesion to fibronectin in a concentration-dependent manner. As observed by fluorescence live cell imaging, Lm-411 promoted undirected migration of RCC cells better than fibronectin. In addition to the migration-promoting activity of Lm-411, this could also reflect the inability of RCC cells to firmly adhere to this substratum. Such counteradhesive functions, which have been suggested to reflect an intermediate state of adhesion, have been previously described for matricellular proteins such as tenascin-C and SPARC (Murphy-Ullrich 2001, Bornstein and Sage 2002). In fact, α4 chain laminins seem to share some properties with matricellular proteins (Bornstein and Sage 2002); they are widely expressed during development, they appear to be primarily expressed in mesenchyme-derived tissues and tumour stroma, and deletion of their genes in mice produces a grossly normal phenotype, which is nevertheless susceptible to injury (Iivanainen et al. 1995, Miner et al. 1997, Lefebvre et al. 1999, Thyboll et al. 2002, Zhou et al. 2004, Määttä et al. 2005, Wallquist et al. 2005, Wang J et al. 2006). Concerning the suggestions that Lm α4 chain is overexpressed in invasive gliomas and that inhibition of Lm α4 chain prevents glioma invasion and angiogenesis, similar characteristics have been described also for SPARC (Khazenzon et al. 2003, Ljubimova et al. 2004,
Nagato et al. 2005, Fujita et al. 2006). The results on inhibition of cell adhesion and stimulation of cell migration suggest that, as previously described for matricellular proteins, Lm-411 has a counteradhesive function, and thus, could have a role in detachment and invasion of RCC cells.
9. SUMMARY AND CONCLUSIONS

To gain insights into the potential functions of α4 chain laminins in human tissues, we raised a MAb specific for the laminin α4 chain and studied the distribution of this laminin chain in developing and adult human tissues. Laminin α4 chain was found in all studied tissues. It was primarily localized to BMs of endothelia, adipocytes, nerves, muscle cells, and other contractile cells. It was also found in the BM region of certain epithelia in the skin, esophagus, stomach, intestine, pancreas, salivary glands, and kidney. The expression of α4 chain laminins appeared to be developmentally regulated, especially in skeletal, heart, and vascular smooth muscles. Later studies have provided evidence for essential roles of α4 chain laminins in many of the aforementioned tissues, as exemplified by a cardiomyopathy-like condition in laminin α4 chain-deficient mice (Wang J et al. 2006) and novel laminin α4 chain mutations in human patients with dilated cardiomyopathy (Knöll et al. 2007).

The prominent expression of α4 chain laminins in endothelial BMs of blood vessels elicited our interest in the role of laminins in endothelial BMs. The results showed that human endothelial cells produce α4 and α5 chain laminins. Human endothelial cells barely adhered to laminin-411, but adhered and spread on laminin-511. Neither α4 nor α5 chain laminins promoted formation of cell-ECM adhesion structures. Thus, α4 and α5 chain laminins do not promote firm adhesion, but may induce a migratory phenotype in endothelial cells. Other studies suggest that α4 chain laminins have an important role in the formation of blood capillaries (Thyboll et al. 2002, Zhou et al. 2004, Fujita et al. 2006, Li et al. 2006). Further, α4 and α5 chain laminins may regulate transmigration of inflammatory cells and possibly also of microbial pathogens through endothelium (Sixt et al. 2001a, Wang S et al. 2006, Masocha et al. 2007).

To gain insights into the potential roles of laminins in development and function of lymphatic vessels, we evaluated the distribution of laminins and certain other BM constituents in lymphatic vessels. The results showed that the endothelial BM region of all LYVE-1-immunoreactive lymphatic capillaries possesses laminin α4, β1, β2, and γ1 chains, type IV and XVIII collagens, and nidogen-1. Taking into account the suggested inability of α4 chain laminins to polymerize, and thus, to promote BM assembly (Yurchenco et al. 2004), the presence of only α4 chain laminins may at least in part explain the incomplete BM formation in the vessels. The consistent presence of the
laminin α5 chain and its receptor Lu in lymphatic vessels of ovarian carcinomas emphasizes a difference between lymphatic vessels of normal tissues and ovarian carcinomas. The roles of laminins and other BM constituents in the formation and function of lymphatic vessels require further evaluation.

Other studies have suggested that α4 chain laminins have an important role in progression of human gliomas and that tissue-specific inhibition of α4 chain laminins may offer a potential new treatment for gliomas and tumour angiogenesis (Ljubimova et al. 2001, 2004, Khazenzon et al. 2003, Nagato et al. 2005, Fujita et al. 2006, Lee et al. 2006). To evaluate the role of α4 chain laminins in human carcinomas, we studied their distribution in RCCs. Laminin α4 chain was prominent in the stroma and vasculature of all RCCs, and it was also found in the BMs of tumour cell islets in most RCCs. Laminin-411 did not promote the adhesion of RCC cells, but it did inhibit their adhesion to fibronectin. Laminin-411 also promoted RCC migration. The results suggest that α4 chain laminins have a counteradhesive function, and may thus promote detachment and invasion of RCC cells.

The major findings of this study indicate that α4 chain laminins are widely expressed in normal human tissues and RCCs and appear to have a counteradhesive function. The roles of endothelial laminins in vivo warrant further evaluation.
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