Distribution of Laminins in the Developing Human Eye

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Purpose. To examine the distribution of laminin (Ln) chains in basement membranes (BMs) of the human cornea, lens, and retina in fetal development.

Methods. Ten fetal eyes (9–20 weeks of gestation [wg]) were serially sectioned and treated with specific antibodies against the Ln-α1, -α2, -α3, -α4, -α5, -β1, -β2, -β3, and -γ1 chains.

Results. The BM of the corneal epithelium was reactive for Ln-α3, -α5, -β1, and β3 chains through all ages, whereas the Ln-α1 chain was present at 9 to 12 wg and the Ln-α4 chain from 10 wg. The Descemet’s membrane (DM) was labeled with the Ln-α1 and -α4 chains at 10 to 17 wg, the Ln-α5 chain from 10 wg, the Ln-β1 chain at 11 to 17 wg, and the Ln-β3 chain from 17 wg. The Ln-α1, -α5, -β1, and -β2 chains were present in the lens capsule and the internal limiting membrane (ILM) through all ages. The Bruch’s membrane (BrM) was immuno-reactive for the Ln-α3, -α4, -α5, -β1, and -β2 chains through all ages, whereas the Ln-α1 chain was absent from 20 wg onward. The Ln-α2 chain was not detected in the eye, but it was present in the extraocular muscles.

Conclusions. BMs play an important role during morphogenesis, in that they influence cell proliferation, migration, and tissue differentiation. Lns are the major noncollagenous component of BMs. The presence of four different α chains, three β chains, and one γ chain of Ln in the eye reveals a high degree of complexity from the early stages of development and suggests an important role for the different Ln chains in human ocular differentiation. (Invest Ophthalmol Vis Sci. 2006;47:777–785) DOI:10.1167/iovs.05-0367

The development of the human eye comprises a series of complex events spanning the embryonic, fetal, and postnatal periods. 1 During embryogenesis, the optic pit forms as a lateral outpouching of the neural tube that is attached to the forebrain through the optic stalk. The optic pit enlarges and forms the optic vesicle. This invaginates during the fourth week and forms a two-layered optic cup that differentiates further into the different retinal layers. A thickened lens plate formed by the overlying surface ectoderm invaginates and forms the lens vesicle. Neural crest cells that migrate dorsolaterally later give rise to various structures in the eye and orbit. A period of differentiation and growth starts at the beginning of the third gestational month. During this period, the vitreous, the lens, and the structures of the angle and the pericellular mesenchyme develop, and the retina, optic nerve, and anterior rim of the optic cup mature. Some ocular structures, such as the macula, complete their differentiation after birth. 1–3

Basement membranes (BMs) are specialized forms of extracellular matrix that form early during tissue development and provide mechanical support for parenchymal cells. They are typically present at the interface between parenchymal cells and connective tissue, underlie both epithelial and endothelial cells, and surround individual muscle, nerve, and fat cells. The different components in the BMs interact with cells through cell surface receptors. These interactions play an important role during morphogenesis, as they influence cell proliferation, survival, migration, and differentiation. 4,5 In some organs, the BMs regulate diffusion of large solutes and migration of cells by forming a barrier (e.g., the blood–brain barrier). They are also regarded as depots of growth factors. 6

In the developing eye, the optic cup has a BM that lines its surface and is continuous with the invaginating layer at 4 to 5 weeks of gestation (wg). This BM forms the future internal limiting membrane (ILM) of the retina and the Bruch’s membrane (BrM), the BM of the retinal pigment epithelium (RPE). 7 Another BM lines the surface ectoderm that forms the lens capsule and the BM of the anterior epithelium of the future cornea. 7 The lens capsule is the BM that encloses the lens vesicle in which the epithelial cells all project inward. During the seventh week of gestation, the posterior epithelial cells elongate and gradually occlude the lumen of the vesicle. These are called primary lens fibers. 8,9 When these primary lens fibers attach to the anterior epithelium, tight and gap junctions appear between them. 1 The lens epithelium remains over the anterior surface and the equatorial zone. The anterior portion of the lens capsule continues to thicken throughout life. 8 At 5 wg, after separating from the lens vesicle, the surface ectoderm differentiates into a two-cell-thick epithelium, the primitive corneal epithelium, with a well-formed basal lamina. At 8 wg the endothelial cells of the cornea start to form Descemet’s membrane (DM), which at this stage is a patchy accumulation of BM material. 1

Laminins (Lns) are the major noncollagenous component of BMs. They are heterotrimERIC extracellular matrix glycoproteins composed of one α chain, one β chain, and one γ chain, joined together through a long α-helical coiled–coil region. 9 To date, the cDNA sequences of five α-, three β-, and three γ-chains have been reported in humans, forming 15 different Ln isoforms. 4 Lns are vital for the assembly of BMs and interact with the type IV collagen network via nidogen and other extracellular matrix molecules. The expression of Ln chains is regulated both spatially and temporally, 10 suggesting that the different Ln isoforms have distinct roles to fulfill. They have
been shown to affect tissue development and integrity in diverse organs.\textsuperscript{4} Natural mutations in any of the genes coding for subunits of Ln type-5 (\(\alpha_3\beta_3\gamma_2\)) can result in junctional epidermolysis bullosa.\textsuperscript{11,12} A reduced expression of Ln-\(\alpha_2\) chain has been reported in Walker-Warburg syndrome, a muscular dystrophy that also involves the eye.\textsuperscript{13} Antibodies to Ln-5 are present in 10\% of cicatricial pemphigoid, a blistering and scarring disorder with predilection for the mucous membranes of the eyes and mouth.\textsuperscript{14}

The spatial and temporal patterns of distribution of Ln chains in the BMs of the human eye during fetal development are, to the best of our knowledge, currently unknown. Previous studies in other developing vertebrates and in adult humans suggest an important role for Ln chains in ocular BMs.\textsuperscript{15–28} We investigated the distribution of nine Ln chains in the BMs of fetal human eye. We found distinct spatial and temporal patterns of distribution for each of the different Ln chains, except Ln-\(\alpha_2\), indicating that they are likely to have an

\textbf{FIGURE 1.} Cross sections of human fetal corneas at different gestational ages showing immunoreactivity for the (A–C) Ln-\(\alpha_1\), (D–F) Ln-\(\alpha_3\), (G–I) Ln-\(\alpha_4\), (J–L) Ln-\(\alpha_5\), (M–O) Ln-\(\beta_1\), (P–R) Ln-\(\beta_3\), and (S–U) Ln-\(\gamma_1\) chains in the BM of the corneal epithelium (arrow). In all micrographs, the corneal stroma is located at the bottom of the image. Note that the Ln-\(\alpha_1\) chain was no longer present at 14 wg (C) and that the BM had a patchy appearance in the early stages, with Ln-\(\alpha_1\) (A), Ln-\(\alpha_4\) (G), and Ln-\(\beta_1\) chains (M).
important role in the maturation and maintenance of the different eye structures.

**MATERIALS AND METHODS**

Ten eyes were obtained from human fetuses at 9, 10, 11, 12, 14, 16, 17, and 20 wg, after legal interruptions of pregnancy. The samples were collected with the approval of the Ethics Committee of the Medical Faculty, Umeå University, after informed consent was obtained and in accordance with the tenets of the Declaration of Helsinki. Gestational age was dated from the first day of the last menstrual period and was further confirmed by ultrasound before abortion in most cases. The eyes were mounted in embedding medium (Tissue-Tek OCT; Miles, Elkhart, IN), rapidly frozen in propane chilled with liquid nitrogen (−110°C) and stored at −80°C until use. Serial cross sections, 5-μm thick, were processed for immunohistochemistry, with the following previously characterized antibodies: polyclonal antibodies hLN-α1G4/G5 against the Ln-α1 chain; monoclonal antibodies (mAb) 163DE4 against the Ln-α1 chain; mAb 5H2 against the Ln-α2 chain (provided by Eva Engvall, The Burnham Institute, La Jolla, CA); mAb BM-2 against the Ln-α3 chain; mAb 168 FC10 against the Ln-α4 chain; mAb 4C7 against the Ln-α5 chain; mAb 1928 against the Ln-β1 chain (Chemicon, Temecula, CA); mAb C4 against the Ln-β2 chain; mAb 6F12 against the Ln-β3 chain; and mAb 113BC7 against the Ln-γ1 chain.

The bound antibodies were visualized with standard indirect fluorescence technique, with the secondary antibodies conjugated with a fluorochrome (Alexa 488; Molecular Probes, Leiden, The Netherlands; and Cy3; Jackson ImmunoResearch Laboratories, West Grove, PA). The primary antibody was omitted in control sections. These sections remained unstained. The sections were studied under a microscope (Nikon, Tokyo, Japan), and the staining intensities for each separate antibody were recorded as weak, moderate, or strong. Exposure times were determined, and background-level adjustments were made to the micrographs to reflect the true staining observed.

**FIGURE 2.** Staining patterns in DM shown in cross sections at different fetal ages and immunoreactive for the (A–C) Ln-α1, (D–F) Ln-α4, (G–I) Ln-α5, (J–L) Ln-β1, (M–O) Ln-β3, and (P–R) Ln-γ1 chains (arrows). Note that vessels on the lens capsule (D, E, arrowheads) showed strong immunoreactivity for the Ln-α4 chain, but that the Ln-α4 chain was absent from the DM at 20 wg (F). Ln-α5 chain reactivity showed a patchy pattern at 10 wg + 4d (G), and the Ln-β1 chain was found in the DM from 11 wg (J–L). CoStr, corneal stroma; AC, anterior chamber; L, lens.
RESULTS

BM of the Corneal Epithelium

The BM of the corneal epithelium reacted strongly with the Ln-α1 chain between 9 and 12 wg (Figs. 1A, 1B). Immunoreactivity for the Ln-α3 chain was distinctly present in the BM of the corneal epithelium through all ages (Figs. 1D–F). Ln-α3 chain immunoreactivity was detected from 10 wg, although it was weak at 20 wg (Figs. 1G–I). The Ln-α5 and -β1 chains were present in the BM at all ages (Figs. 1J–O). This BM showed strong immunoreactivity for the Ln-β3 (Figs. 1P–R) and Ln-γ1 (Figs. 1S–U) chains at 10 to 20 wg. The Ln-α2 and -β2 chains were not present in the BM of the corneal epithelium at the examined stages (data not shown).

Descemet’s Membrane

The DM (i.e., the BM of the corneal endothelium) was reactive for the Ln-α1 chain from 10 wg (Fig. 2A). The staining intensity was weak at 10 wg, distinct at 11 to 17 wg (Figs. 2A–C), and absent at 20 wg (results not shown). The Ln-α4 chain was found in DM at 10 to 17 wg, but at 17 wg the reactivity was weak (Figs. 2D–F). The Ln-α5 chain was found in DM from 10 wg, initially in a patchy pattern (Fig. 2G). The immunoreactivity pattern became more even and increased in intensity during the following weeks (Figs. 2H, 2I). The DM was also labeled with the Ln-β1 chain at 11 to 17 wg (Figs. 2K, 2L), although the staining was weak at 20 wg. At 17 and 20 wg, the DM was weakly immunoreactive for the Ln-β3 chain (Figs. 2N, 2O). Ln-γ1 chain immunoreactivity was present at 10 to 20 wg (Figs. 2P–R). Ln-α2, -α3, and -β2 chains were not found in the DM at these stages (not shown).

Corneal Blood Vessels

The BM of vessels in the corneal stroma were reactive for the Ln-α4 chain from 10 wg (Figs. 3A–D). At 20 wg, blood vessels were no longer detected in the central cornea, but the Ln-α4 chain was present in vessels in the peripheral stroma of the cornea (not shown).

Lens Capsule

The lens capsule was immunoreactive for Ln-α1, -α5, -β1, -β2, and -γ1 chains at all ages (Figs. 4A–L, 4P–R). At 9 to 10 wg, the posterior part of the capsule appeared thicker and more intensely positive for the Ln-β2 chain than the anterior part, whereas at 12 wg, the anterior part of the capsule was more intensively reactive. Thereafter, the whole capsule was strongly immunoreactive for the Ln-β2 chain.

The Ln-α2, -α3, -α4, and -β3 chains were not detected in the lens capsule at the examined stages. However, the contours of the epithelial lens cells were weakly labeled with the antibody against the Ln-α3 chain, forming a honeycomb pattern at all ages (Figs. 4M–O). The blood vessels around the lens capsule were positive for the Ln-α4 chain at all ages (not shown).

Internal Limiting Membrane

Because of its relationship to the vitreous body, the ILM was fragile and sometimes damaged or lost in the tissue sections. The ILM was labeled with antibodies against the Ln-α1, -α5, -β1, -β2, and -γ1 chains (Figs. 5A–O) and unlabeled with mAbs against the Ln-α2, -α3, -α4, and -β3 chains through all ages (not shown).

Bruch’s Membrane

BrM is traditionally described with the choroid, but its innermost portion is actually the BM of the RPE. 8 It was immunoreactive for the Ln-α3, -α4, -α5, -β1, -β2, and -γ1 chains through all ages (Figs. 6D–U). Initially, the Ln-α1 chain was found in the whole BrM (Fig. 6A). After 10 to 12 wg, immunoreactivity in BrM was predominantly found in the anterior part of the eye, and it was absent at 20 wg (Fig. 6C). Immunoreactivity for the Ln-α3 chain was found in BrM through all ages, although in a patchy and uneven pattern (Figs. 6D–F). There was also a weaker immunoreactivity in the retina itself, forming a network around the cells (Figs. 6D–F). Immunoreactivity for the Ln-α4 chain in BrM was uneven and mostly in the anterior part of the eye at 9 to 10 wg (Fig. 6G). From 14 to 16 wg, immunoreactivity for Ln-α4 chain in BrM was stronger and it became more difficult to separate from that of the underlying blood vessels (Fig. 6I). The Ln-α5 chain was found in BrM throughout all examined ages (Figs. 6I–L). Immunoreactivity for the Ln-β1 chain was uneven (Figs. 6M–O). The Ln-β2 chain was found in BrM in the anterior part of the eye at 9 to 11 wg and in the posterior part from 16 wg (Figs. 6P–R). Ln-γ1 chain reactivity was strong through all ages (Figs. 6S–U).

The interphotoreceptor matrix (IPM), just proximal to the RPE was positive for the Ln-α3 and -β2 chains (Figs. 6D–F, 6P–R). Ln-α2 and -β3 chains were not present in the BrM at these stages (not shown).

DISCUSSION

The discovery of naturally occurring Ln mutations and gene targeting of Ln over the past years has profoundly changed our understanding of BM function. Defects of different Ln chains can cause ocular disease, and therefore a detailed study of the Ln isoforms involved in human ocular development is important to further understand the role of Lns in normal and diseased eyes.
Ln-1 (α1β1γ1) is the major Ln during very early embryogenesis, and the Ln-α1 chain is present in many locations during development but is largely absent in adult tissues. In our study, the Ln-α1 chain was detected both with polyclonal and monoclonal antibodies in the BMs of all examined tissues in the eye at the earliest stages, but there were important temporal differences as it disappeared from the BM of the corneal epithelium after 12 wg, from DM after 17 wg, and from BrM at ~20 wg. This difference of approximately 5 weeks between the corneal epithelial BM and the DM indicates independent regulation of the expression of the Ln-α1 chain in these two parts of the cornea. Virtanen et al. have shown a restricted distribution of Ln-α1 chain in epithelial BMs of fetal and adult human tissues. During development, the Ln-α1 chain is transiently present in some epithelial BMs and epithelial–mesenchymal interfaces in many tissues, but it is absent in adulthood. This has been proposed to reflect the role of this chain in the polarization of epithelial cells. The Ln-α1 chain, detected with a polyclonal antibody against Ln-1, has been reported in BrM, ILM, and the retinal vasculature of adult rat and human eyes. Previous data collected with mAb 4C7 showing a wider distribution of the Ln-α1 chain should be reinterpreted in the light that this mAb in fact detects the Ln-α5 but not the Ln-α1 chain.

In our study, the Ln-α2 chain was present in extraocular muscles from 10 wg. The Ln-α2 chain has been reported in developing and adult human skeletal muscle and peripheral nerve, and it has also been identified in the developing and adult brain and in the retinal vasculature. The Ln-α3 chain was detected in the BM of the corneal epithelium at all examined ages, and we speculate that it may be part of Ln-5 which is known to associate with hemidesmosomes and to play an important role in their formation. Basal cells in the corneal epithelium adhere to the underlying BM via hemidesmosomes. This Ln isoform is relevant in eye disease, as the staining patterns of the chains of Ln-5 (α3β3γ2)
have been reported to be affected in corneas with keratoconus. A mutation in the Ln-α3/H9251 gene causes laryngo-onychocutaneous (LOC) syndrome, which affects the eye with aggressive pterygium, symblepharon, and corneal scarring. Our data showed that the Ln-α3/H9251 chain is present in BrM during development and supports earlier discussions about hemidesmosomes’ keeping BrM and the RPE together. The site of cleavage in RPE detachment is between the BM of the RPE and the inner collagenous membrane of BrM rather than between RPE and its BM. Immunoreactivity for Ln-α3/H9251 chain has been reported as prominent in the ILM, the tips of the photoreceptors, and around cell bodies of outer and inner nuclear layers, and as diffuse in the inner plexiform layer (IPL) of the human adult retina. The presence of the Ln-α3 chain around lens epithelial cells, retinal cells, and more distinctly in the IPM indicates that Lns are not only found in true BMs but are also likely to play an important role in attaching cells to each other in the developing human eye. Previous studies have reported a similar distribution of the Ln-α3 and Ln-β2 chains in the floor plate of the neural tube at nearly the same stage, suggesting that both chains contribute to neural development. Of note, we saw similar staining patterns for these chains in the developing human retina. The Ln-α4 chain is widely distributed in most BMs of both epithelia and endothelia, and we found the Ln-α5 chain to be widely expressed in the human eye. Libby et al. reported the presence of the Ln-α5 and -α3 chains in BrM, ILM, retinal vasculature, and neural retina and proposed that Lns are essential in retinal adhesion as components of IPM. The Ln-β1 chain is another chain associated with early development; in fact, embryogenesis cannot proceed in its absence. Ln-1 (α1β1γ1) binds to many cell surface receptors, including the α6 integrin subunit. Targeted disruption of the α6 integrin gene results in perinatal lethality due to defects in epithelial adhesion, and ectopic neuroblastic outgrowths in the ocular vitreous body have also been noted. We found the Ln-β1 chain in the BMs of all examined ocular tissues. In a previous study, the Ln-β1 chain was detected in the BM of retinal vasculature but not in the IPM or neural retina, whereas
Lnβ2 chain was present in both the IPM and external limiting membrane (ELM), as well as in the retinal vasculature in the adult human eye.

The Lnβ2 chain has been detected around cells in the inner nuclear layer (INL) of the adult human retina and in the ILM, and the Lnβ3 chain has been detected in the IPM.15

We found the Lnβ2 chain in both the BM of the retina and in the lens capsule through all examined ages. The Lnβ2 chain has been shown to play a role in directing photoreceptor development and is an important component of the IPM in the developing rat retina.21 In our study, the Lnβ2 chains were also found in the IPM, together with the Lnα3 chain, supporting previous results that suggest the presence of a novel laminin, Ln-13 (α3β2γ3), in rat and adult human retina.15 The importance of the Lnβ2 chain in the formation of the eye is illustrated by Pierson syndrome, a human ocular syndrome caused by Lnβ2 deficiency and characterized by hypoplasia of the ciliary and pupillary muscles, hypoplasia of the iris and ciliary body, lens malformation, corneal and retinal anomalies, and renal failure.28
The detection of the Ln-β3 chain in the corneal epithelial BM in addition to the Ln-α3 chain strengthens previous findings, suggesting the presence of Ln-5 and hemidesmosomes in the cornea. In the DM, we saw a shift between Ln chains. The immunoreactivity for the Ln-β1 chain was weak at higher ages, and instead the Ln-β3 chain appeared. We did not detect the Ln-β3 chain in the retina at these ages, and therefore the presence of Ln-5 (α3β3γ2) is unlikely. Instead, our results support earlier studies suggesting the presence of Ln-13 (α3β2γ5) in the retina.

The widespread distribution of the Ln-γ1 chain both temporally and spatially in the fetal ocular BMs is consistent with the earlier suggestion that it is the most widely expressed Ln chain.

In summary, our data showed that the Ln-α1, -α3, -α4, -α5, -β1, -β2, -β3, and -γ1 chains are present in the BMs of the developing human eye. They showed distinct spatial and temporal patterns of distribution, indicating a high degree of complexity and suggesting an important role for different Ln chains in human ocular differentiation. The simultaneous presence of different Ln chains from the same class in a given BM of the developing eye suggests the possibility that one Ln isoform may be able to substitute for another, thereby decreasing the impact of Ln-deficient diseases in the human eye.

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References


