Compositional Differences between Infant and Adult Human Corneal Basement Membranes

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Purpose. Adult human corneal epithelial basement membrane (EBM) and Descemet's membrane (DM) components exhibit heterogeneous distribution. The purpose of the study was to identify changes of these components during postnatal corneal development.

Methods. Thirty healthy adult corneas and 10 corneas from 12-day- to 3-year-old children were studied by immunofluorescence with antibodies against BM components.

Results. Type IV collagen composition of infant corneal central EBM over Bowman's layer changed from α1-α2 to α3-α4 chains after 3 years of life; in the adult, α1-α2 chains were retained only in the limbal BM. Laminin α2 and β2 chains were present in the adult limbal BM where epithelial stem cells are located. By 3 years of age, β2 chain appeared in the limbal BM. In all corneas, limbal BM contained laminin γ3 chain. In the infant DM, type IV collagen α1-α6 chains, perlecan, nidogen-1, nidogen-2, and netrin-4 were found on both faces, but they remained only on the endothelial face of the adult DM. The stromal face of the infant but not the adult DM was positive for tenascin-C, fibrillin-1, SPARC, and laminin-332. Type VIII collagen shifted from the endothelial face of infant DM to its stromal face in the adult. Matrilin-4 largely disappeared after the age of 3 years.

Conclusions. The distribution of laminin γ3 chain, nidogen-2, netrin-4, matrilin-2, and matrilin-4 is described in the cornea for the first time. The observed differences between adult and infant corneal BMs may relate to changes in their mechanical strength, corneal cell adhesion and differentiation in the process of postnatal corneal maturation. (*Invest Ophthalmol Vis Sci.* 2007;48:4989–4999) DOI:10.1167/iovs.07-0654

Basement membranes (BMs) are a specialized form of extracellular matrix (ECM) that separate parenchymal from stromal cells and are important for cell adhesion, migration, differentiation, and signal transduction.[1–3] The cornea contains a complex ECM that undergoes extensive remodeling during embryonic and postnatal development. It comprises the epithelial basement membrane (EBM), an adjacent collagenous Bowman's layer, a stromal ECM composed of orthogonal collagen lamellae and several distinct proteoglycans, and an endothelial BM, or Descemet's membrane (DM). The corneal ECM and BMs contribute to the transparency and refractive properties of the cornea and play major roles in various corneal cell functions.

Many ECMs including BMs undergo considerable changes in development. Both corneal BMs have been shown to increase in thickness during the transition from fetal to adult stages,[4–6] especially, the DM that acquires a posterior nonbanded region after birth.[5–8] These changes have been revealed by light and electron microscopy, but the molecular alterations of BM components responsible for corneal BM maturation remained largely unknown.

In recent years, the complexity of corneal BMs has been appreciated, largely because of the availability of specific anti-

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bodies against most of the components and their isoforms. Studies from our group and others have shown that adult human continuous corneal EBM differs in composition in its various regions.[9–16] It appears to have regional horizontal heterogeneity among the central part, limbus, and conjunctiva with respect to the distribution of type IV collagen and laminin isoforms, as well as thrombospondin-1 and types XII and XV collagen.[10,14,16,17] The DM was also shown to be vertically heterogeneous with respect to type IV collagen and laminin, as well as fibronectin.[10] It remained unclear whether these regional differences in corneal BM structure appeared early in development or were acquired later in life. To answer this question, we compared infant and adult human corneas immunohistochemically, with attention to BM components. Both corneal BMs displayed distinct signs of postnatal maturation with shifts in the expression of major components in specific regions of the EBM and at both stromal and endothelial faces of the DM.

Materials and Methods

Tissue

Ten infant corneas obtained at autopsy from 5 individuals (aged 12 days to 3 years) and 30 healthy adult human corneas obtained at autopsy from 27 individuals (aged 13–75 years) were received from the National Disease Research Interchange (NDRI, Philadelphia, PA) within 30 hours of death. The NDRI has a human subject protection protocol that is approved and enforced by the managerial committee operating under National Institutes of Health oversight. All such tissues are thus qualified as exempt from Institutional Review Board review (exemption 4). Immediately on arrival, the corneas were bisected, placed in OCT compound (Miles, Elkhart, IN), frozen in liquid nitrogen, and stored at −80°C. Nonfixed 5-μm cryostat sections collected on glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA) were subjected to indirect or double-indirect immunofluorescence analysis, as described previously.[10] Photographs were taken by microscope (BX40; Olympus USA, Melville, NY). Routine specificity controls[10] were negative. Samples with only very low levels of background staining were analyzed. In some experiments, to unmask type IV collagen epitopes, sections were air dried for 5 minutes, after which they were denatured in 6 M urea at pH 3.5 for 1 hour at 4°C, washed, and ultimately fixed with 1% formalin for 5 minutes at room temperature before staining.[10] To unmask laminin epitopes, sections (with or without fixation in acetone at −20°C for 10 minutes) were pretreated with 0.05% SDS solution in PBS for 5 to 10 minutes at room temperature.[18] Monoclonal antibodies were used as undiluted hybridoma supernates or at 10 to 20 μg/mL as purified IgGs, and polyclonal antibodies were used at 20 to 30 μg/mL. At least two independent experiments were performed for each marker, with identical results. All infant corneas and nearly all adult corneas were stained for all markers. The staining patterns were mostly reproducible within each group of corneas. Some variations in staining intensity were observed only in the central EBM for some laminin chains.

Antibodies

Monoclonal and polyclonal antibodies against type IV collagen α1-α6 chains; laminin chains α1-α5, β1-β3, and γ1-γ2; nidogen-1 and -2; BM-40/SPARC/osteonectin; perlecan core protein domain IV; fibronectin 8th type III repeat and ED-A domain (cellular fibronectin); types VII, VIII, XII, XVII, and XVIII collagen; netrin-4 (β-netrin); various tenascin-C isoforms; fibrillin-1; thrombospondin-1; vitronectin; matrilin-2 and -4; α- and β-dystroglycan; integrin subunits α_6 and β_4; α-enolase, and cornea-specific keratin 3 have been described (see Table 1 for references). Antibodies against laminin γ3 chain (Steiner-Champliaud et al., unpublished data, September 2001) will be described in detail elsewhere. Laminin isoform nomenclature follows recent recommendations.[52] Cross-species adsorbed fluorescein- and rhodamine-conjugated secondary antibodies were from Chemicon International (Temecula, CA).

Results

The compositions of normal human adult corneal epithelial BM and DM have been previously examined in immunohistochemistry studies.[10–12,14,17,19,53–60] Briefly, EBM was found to be positive throughout the central cornea and limbus (and also conjunctiva) for laminin-332 and -511 (possibly, also for laminin-111 and -311), nidogen-1, perlecan, fibronectin (total and cellular), and types IV (α5-α6 chains) and VII collagen. The central part of the corneal EBM with the underlying Bowman's layer in addition was positive for types IV (α3-α4 chains) and XII (long-form) collagen, thrombospondin-1, and vitronectin (not corroborated here possibly due to tissue fixation differences). In contrast, limbal and conjunctival EBM was also positive for types IV (α1-α2 chains) and XV collagen, laminin chains α2 and β2 (compatible with laminin-211, -121, -221, and -521), tenascin-C, fibrillin-1, and BM-40/SPARC, but lacked type IV collagen α3-α4 chains or the long form of type XII collagen.

In DM, the stromal face was found to be positive for fibronectin, vitronectin, and types IV (α1-α2 chains) and VIII collagen. The endothelial face stained for types IV (α3-α6 chains) and XII collagen, laminin-511, nidogen-1, thrombospondin-1, and perlecan.

In this report, the staining patterns of many of the studied components in both EBM and DM were found to be different between infant and adult corneas. It should be noted that the observed differences in the BM composition of infant compared with adult corneas could still be seen in 3-year-old corneas. In contrast, 13-year-old corneas already had the adult distribution of all studied markers.

Epithelial Basement Membrane

Results are summarized in Table 2. Both infant and adult human corneal central EBM were positive for chains of laminin-311 (α3β1γ1), -332 (α3β3γ2), -411 (α4β1γ1), and -511 (α5β1γ1); nidogen-1 and -2; perlecan; types IV (α5-α6 chains), VII, XII (both forms), XVII, and XVIII collagen; thrombospondin-1; matrilin-2 (Fig. 1, right column) and -4; and the hemidesmosomal component $\alpha_6\beta_4$ integrin (data not shown). Weak staining was also seen for SPARC/BM-40, laminin γ3 chain (Fig. 1, left column), and the laminin receptors, α- and β-dystroglycans (data not shown). Staining for laminin α1 chain (component of laminin-111) was weak and inconsistent and could be revealed by only one of three antibodies. Therefore, its presence in the corneal EBM cannot be documented with certainty; some data indicate that it might be expressed only in the embryonic EBM.[19,61] Distinct EBM staining for laminin α4 chain was revealed by two of five antibodies. These antibodies (377 and 1101+) did not stain muscle tissue from a laminin α4 chain null mouse confirming lack of cross-reactivity with other chains. Epithelial BM staining was only distinct after a mild pretreatment with SDS[18] suggesting that the respective epitopes were masked. This may explain the recently documented lack of central EBM staining (with limbal EBM positivity) using another α4 antibody, FC10.[62]

In the limbal EBM, the following components were found by immunostaining of both adult and infant corneas (Table 2): chains of laminin-311, -332, -333, -411, -423, -511, and -523; nidogen-1 and -2; perlecan, types IV (α5-α6 chains), VII, XII (short form only), XVII, and XVIII collagen; SPARC/BM-40 (weak); tenascin-C; fibrillin-1; matrilin-2 and -4; and $\alpha_6\beta_4$ integrin (data not shown). Strong staining was seen for laminin γ3 chain (Fig. 1, middle column) that also continued in the conjunctival BM. The data on adult corneas agree well with results

TABLE 1. Antibodies Used in This Study

Antigen	Antibody	Reference/Source
Collagen IV α1(IV)-α2(IV) chain triple helix	Mouse mAb M3F7	DSHB
α1(IV) chain NC1 domain	Rat mAb H11	Ref. 19
α2(IV) chain NC1 domain	Rat mAb H22	Ref. 19
α3(IV) chain NC1 domain	Rat mAb H31	Ref. 19
α4(IV) chain NC1 domain	Rat mAb H43	Ref. 19
α5(IV) chain NC1 domain	Rat mAb H52	Ref. 19
α6(IV) chain NC1 domain	Rat mAb H63	Ref. 19
Laminin α1 chain	Mouse mAb 161EB7	Ref. 20
	Rabbit pAb 317	Ref. 21
	Rabbit pAb 1057 (VI/V)	Ref. 22
Laminin α2 chain	Mouse mAb 1F9	Ref. 23
Laminin α3 chain	Mouse mAb BM165	Ref. 24
Laminin α4 chain	Rabbit pAb 377	Ref. 25
	Rabbit pAb 1100+ (LG1-3)	Ref. 26, 27
	Rabbit pAb 1101+ (LG4-5)	Ref. 27
	Rabbit pAb C0877	Ref. 28
	Mouse mAb FC10	Ref. 29
Laminin α5 chain	Mouse mAb 4C7	Chemicon International
Laminin β1 chain	Rat mAb LT3	Ref. 30
Laminin β2 chain	Mouse mAb C4	DSHB
Laminin β3 chain	Mouse mAb 6F12	Ref. 24
Laminin γ1 chain	Rat mAb A5	Ref. 31
Laminin γ2 chain	Mouse mAb D4B5	Chemicon International
	Rabbit pAb J20	Ref. 32
Laminin γ3 chain	Rabbit pAb 6296	Steiner-Champliaud, et al., unpublished
	Rabbit pAb 6297	Steiner-Champliaud, et al., unpublished
Laminin-332	Rabbit pAb 4101	Ref. 24
Netrin-4/β-netrin	Mouse mAb 61	Ref. 33
Collagen VII	Mouse mAb 4D2	Ref. 34
Collagen VIII α1 chain	Mouse mAb 9H3	Seikagaku America
Collagen XII long form	Mouse mAb I1C8	Ref. 14
Collagen XII total	Mouse mAb 3C7	Ref. 35
Collagen XVII	Rabbit pAb R67	Ref. 36
Collagen XVIII	Rabbit pAb	Ref. 37
Entactin-1/nidogen-1	Mouse mAb A9	Ref. 38, 39
Entactin-2/nidogen-2	Rabbit pAb 1080	Ref. 40
Perlecan core protein	Rat mAb A7L6	Ref. 41
Fibronectin 8th type III repeat	Mouse mAb 568	Ref. 20
Fibronectin ED-A domain	Mouse mAb IST-9	Sera-Lab
Vitronectin	Mouse mAb BV2	Chemicon International
Thrombospondin-1	Mouse mAb P10	Chemicon International
SPARC/BM-40/osteonectin	Goat pAb Gt78	Ref. 42
	Mouse mAb 236	Ref. 43
	Rabbit pAb 996	Ref. 44
Matrilin-2	Rabbit pAb	Ref. 45
Matrilin-4	Rabbit pAb	Ref. 46
Tenascin-C	Mouse mAbs*	Ref. 47, 48
Fibrillin-1	Mouse mAb 11C1	Chemicon International
α-Dystroglycan	Mouse mAb 8B4	Chemicon International
	Mouse mAb 6C1	Chemicon International
β-Dystroglycan	Rabbit pAb AP83	Ref. 49
	Mouse mAb 43DAG1/8D5	Novocastra Laboratories
Integrin α6	Mouse mAb NKI-GoH3	Chemicon International
Integrin β4	Mouse mAb 3E1	Chemicon International
Keratin 3	Mouse mAb AE5	Ref. 50
α-Enolase	Mouse mAb 4G10	Ref. 51

* Seven mAbs were used that recognized the fibronectin type III repeats of tenascin-C: BC-8 (repeats 4-5), BC-10 (6-7), BC-2 (A1 and A4), αA2 (A2), αA3 (A3), αB (B), and αD (D).

in many previous reports. In contrast to the adult corneas, infant corneas displayed some staining for fibronectin (data not shown), perlecan, and type VII collagen in Bowman's layer (Fig. 2). In some cases, very short, regularly spaced, delicate streaks running perpendicular to the EBM plane could be clearly seen in Bowman's layer (Fig. 2, top). At the infant corneal periphery only, Bowman's layer was also positive for tenascin-C splice variants containing insertional FN-III repeats A1, D, and, to a lesser extent, B (data not shown). Adult corneas were negative in these areas.

Contrary to the adult corneas, infant corneas were positive for type IV collagen α1-α2 chains in the central portion (Fig. 3, left column). Conversely, the central EBM displayed very weak staining for α3-α4 chains of type IV collagen (abundant in the

TABLE 2. Distribution of BM Components in Adult and Infant Corneal Epithelial BM

Component	Adult EBM		Infant EBM	
	Central	Limbus	Central	Limbus
Type IV collagen chains	α3-α6	α1,α2,α5,α6	α1,α2,α5,α6*	α1,α2,α5, α6
Laminin chains	α1†‡,α3-α5,β1,β3, γ1,γ2,γ3†	α2-α5,β1-β3,γ1-γ3	α1†‡,α3-α5,β1,β3,γ1-γ3	α3-α5,β1,β2§,β3,γ1-γ3
Possible laminin isoforms	111‡,311,332,333†, 411,511	211,213,221,311,321,323,332, 333,411,421,423,511,521,522, 523	111‡,311,332,333,411,511	311,321§,323§,332,333,411, 421§,423,511,521§,522§, 523§
Nidogen-1/entactin-1	+	+	+	+
Nidogen-2/entactin-2	+	+	+	+
Fibronectin	+	+	+	+
Perlecan	+	+	+	+
Type VII collagen	+	+	+	+
Type XII collagen	+ (Both forms)	+ (Short form)	+ (Both forms)	+ (Short form)
Type XVII collagen	+	+	+	+
Type XVIII collagen	+	+	+	+
SPARC/BM-40/osteonectin	±	±	±	±
Thrombospondin-1	+	−	+	−
Tenascin-C	−	+	−	+
Fibrillin-1	−	+	−	+
Matrilin-2	+	+	+	+
Matrilin-4	+	+	+	+
β-Dystroglycan	±	+	±	+

Bold, components with different distribution in adults versus infants. Previous laminin classification relates to the new one as follows (with chain structure in parentheses): former laminin-1 (α1β1γ1) is now laminin-111, laminin-2 (α2β1γ1) is laminin-211, laminin-3 (α1β2γ1) is laminin-121, laminin-4 (α2β2γ1) is laminin-221, laminin-5 (α3β3γ2) is laminin-332, laminin-6 (α3β1γ1) is laminin-311, laminin-7 (α3β2γ1) is laminin-321, laminin-8 (α4β1γ1) is laminin-411, laminin-9 (α4β2γ1) is laminin-421, laminin-10 (α5β1γ1) is laminin-511, laminin-11 (α5β2γ1) is laminin-521, laminin-12 (α2β1γ3) is laminin-213, laminin-13 (α3β2γ3) is laminin-323, laminin α3β3γ3 (no number) is laminin-333, laminin-14 (α4β2γ3) is laminin-423, laminin α5β2γ2 (no number) is laminin-522, and laminin-15 (α5β2γ3) is laminin-523.

* Weak and irregular staining for α3 and α4 chains was seen in some corneas.

† Weak staining.

‡ Uncertain presence.

§ Appears between 6 months and 3 years of age. Grading is as follows: −, lack of staining; ±, weak staining in some cases; +, distinct staining in most or all cases.

adult corneas), which could be revealed only after pretreatment of the sections with urea (Fig. 3, right column). Cellular fibronectin staining was minimal or absent in the EBM, in contrast to its distinct presence in the adult corneas (data not shown). In contrast to adult corneas, infant corneas exhibited no laminin α2 chain in the limbal (and conjunctival) EBM (Fig. 4, left column). Laminin β2 chain appeared in corneal limbal BM between 6 months and 3 years of age (data not shown).

Cornea-specific keratin 3 was similarly distributed in infant and adult corneas. All epithelial cells in the central part and suprabasal cells in the limbus were stained. However, the staining of peripheral corneal basal cells in infant corneas was weak or negative over larger distances toward the center, in comparison to that in adult corneas (Fig. 4, right column). Apparently this difference was not due to the regional heterogeneity in its expression[63] because all infant corneas showed similar patterns. A marker of limbal basal epithelial cells, α-enolase, was confined to the limbus in most infant corneas, similar to that of adults (data not shown).

Descemet's Membrane

Results are summarized in Table 3. In infant corneas, fibronectin, both total and cellular, was found at the stromal face, as in the adult corneas (data not shown). Thrombospondin-1 and type XVIII collagen also exhibited the adult staining pattern in infant corneas, at the endothelial DM face (data not shown). Other major BM components showed different patterns between infant and adult DM. Type IV collagen (α1-α6 chains), nidogen-1 and -2, laminin-411 and -511, perlecan, and netrin-4 were found on both DM faces of infant corneas ("railroad" pattern), contrary to the endothelial face location in the adult (Fig. 5). The major DM component, type VIII collagen, was observed mostly on the endothelial face, contrary to the adult corneas where it was mostly seen on the stromal face (Fig. 6, left column). Staining for the long form of type XII collagen was positive on the endothelial DM face in the infant but not in the adult corneas. In contrast, adult corneal stroma was stained more prominently for the long form of type XII collagen (characteristic beads-on-a-string pattern) than was infant corneal stroma (Fig. 6, right column). Similar developmental accumulation of this protein was previously reported in the rabbit.[35]

Some components were observed only in infant but not in adult corneas. The stromal DM face stained for laminin-332, tenascin-C, and fibrillin-1 (Fig. 7, LM-332, TN-C, and FIB-1, respectively) in infant corneas only; adult corneas displayed no staining. Staining was seen only with antibodies to total tenascin-C and to FN-III repeat A1 (Fig. 7, column TN-C A1). These data indicate that in infant corneal DM, only tenascin-C variants without insertional repeats or with insertional repeat A1 (see Ref. 64) were present. Matrilin-4 was present on both DM faces in the infant corneas but had largely disappeared in the adult DM (Fig. 7, right column).

DISCUSSION

The results showed that in the infant human corneas studied the composition of both corneal BMs, the EBM and DM, is reproducibly different from that of adult corneas. In the infant corneal EBM, the central part above the Bowman's layer contained little type IV collagen α3-α4 chains (revealed only after

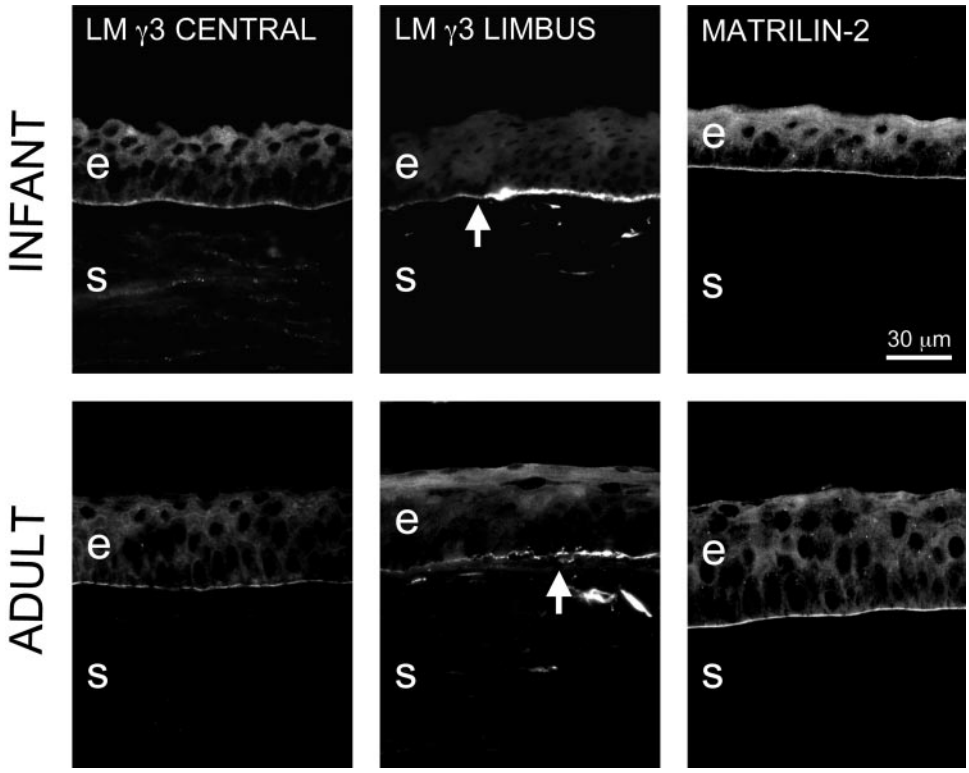

FIGURE 1. New BM components in infant and adult human corneas. Antibodies against laminin γ3 chain (LM γ3; pAb 6296 is shown) showed weak staining of infant and adult central corneal EBM (*left*). Limbal BM staining was more prominent (*middle*); some capillary BMs were also positive. Bowman's layer is located to the *left*; its end is marked by an *arrow*. Matrilin-2 (*right*) was present in both infant and adult central EBM. e, epithelium; s, stroma.

urea treatment) but stained well for α1-α2 chains, opposite to the adult central EBM pattern.[10] The distribution of α1-α2 type IV collagen in the infant corneas is in keeping with continuing expression of α1(IV) mRNA during postnatal life.[65] Subsequently, α1-α2 type IV collagen synthesis may be developmentally inhibited at the posttranscriptional level, or the epitopes may become masked, because various monoclonal antibodies no longer detected it in the adult central corneas.[9,10,53,65,66] α3-α4(IV) chains accumulate in the EBM later in postnatal life, in agreement with previous data.[54] Because α5-α6(IV) chains are expressed in both infant and adult central corneal EBM, they appear to be regulated differently from α3-α4(IV) chains, and probably form trimers mostly without α3-α4(IV) chains.

In adult corneas, basal epithelial precursor (stem) cells are localized in the limbus, although in the embryonic and newborn corneas they may be also present in the central part.[50,67,68] Limbal epithelial stem cells strongly adhere to placental type IV collagen,[69] composed mostly of the α1-α2 chains[70] typical of adult limbal BM. Limbal explants on amniotic membranes also make α1-α2 type IV collagen.[71] In addition, embryonic stem cells undergo epithelial differentiation on the same α1-α2 type IV collagen and then can be used for replacement of injured limbus.[72] These data suggest that limbal stem cells and their more abundant early progeny (transient amplifying cells) may contribute to a unique BM composition with respect to type IV collagen isoforms and also to some other molecules (e.g., specific laminin isoforms containing α2, β2, and γ3 chains, tenascin-C, fibrillin-1, types XII [long form] and XV collagen, and thrombospondin-1).[10,14,16,35,57,64,68,73] The horizontal EBM heterogeneity between the limbus and the central part that develops during embryonic and postnatal life could reflect the need for limbal stem cells and transient amplifying cells to maintain a specific BM composition to preserve their undifferentiated state. Interactions of stem cells with the BM appear to be regulated through specific integrin receptors.[74,75] Patches of some limbal BM components (agrin, SPARC/BM-40, tenascin-C, and versican) were reported to co-

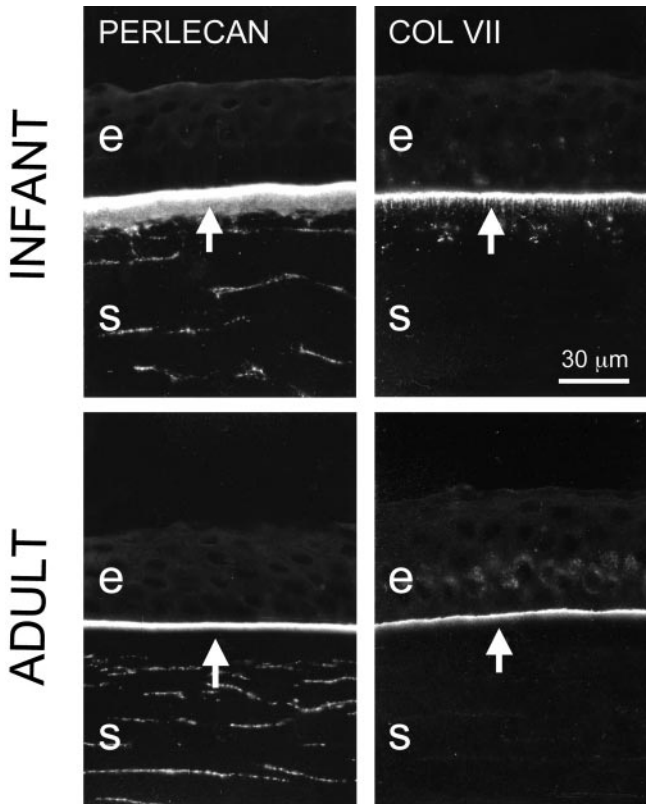

FIGURE 2. BM components and Bowman's layer. Infant corneas displayed distinct staining for perlecan, total fibronectin, and collagen VII (COL VII) in Bowman's layer (*arrows*). Such staining was absent in adult corneas. e, epithelium; s, stroma.

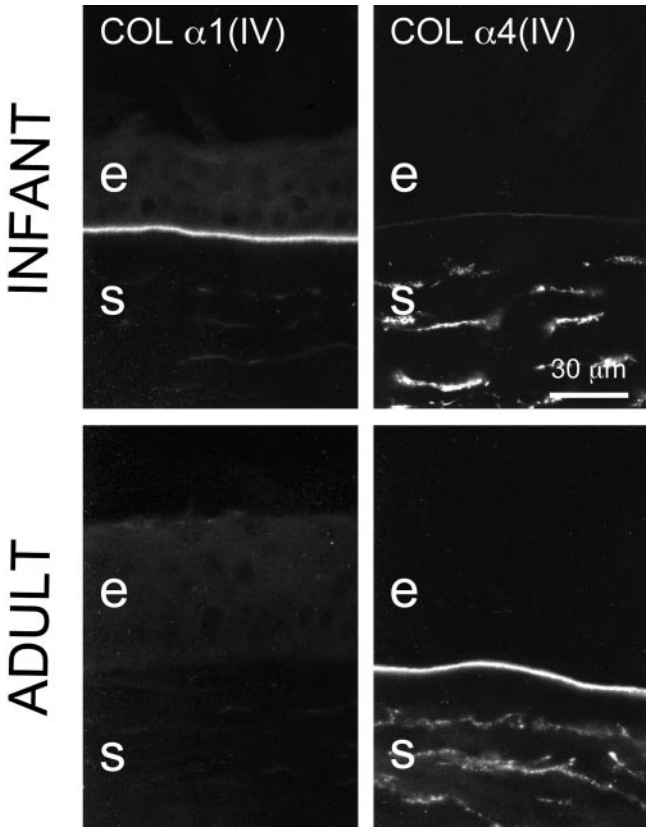

FIGURE 3. Collagen type IV chains in infant and adult human corneas. Infant corneas contained the α1(IV) and α2(IV) (not shown) chains in the central part, contrary to their absence in adult corneas. However, the infant central EBM displayed very weak staining for the α3(IV) (not shown) and α4(IV) chains that were abundant in the adult central corneas. e, epithelium; s, stroma.

localize with p63/ABCG2-positive and Cx43-negative cell clusters in the limbal basal epithelium (Kruse FE et al. *IOVS* 2005; 46:ARVO E-Abstract 2081). These BM proteins may be products and markers of putative stem cells that are a minor population of limbal basal epithelial cells. The other BM components differentially expressed in the limbus (described in the Results section) may be related to a bigger population of transient amplifying cells.

The distribution of type IV collagen in the infant central corneal EBM resembles that of the adult limbal EBM that is probably produced by the epithelial cells.[55] Such EBM composition may favor the existence of epithelial stem cells and early transient amplifying cells in the maturing cornea.[68] Accordingly, the corneal epithelial differentiation marker, keratin 3, is absent from the basal cells of the embryonic central cornea until birth.[67] It was also less abundant in the peripheral basal cells of the infant versus adult corneas (Fig. 4). Basal cells of the infant central corneal epithelium might therefore exist at a similar level of differentiation as most of the basal cells of the adult limbus. However, they seem to be somewhat more differentiated by α-enolase expression. During postnatal maturation, corneal epithelial basal cells begin to accumulate type IV collagen α3-α4 chains in the EBM with concomitant decrease in the production of limbal α1-α2(IV) chains. Our data suggest that the differentiation level of the corneal epithelial basal cells influences the expression pattern of type IV collagen isoforms in the EBM (see Ref. 68). Supporting this hypothesis are our previous results on the limbal pattern of type IV collagen and keratin 3 expression of the central epithelial cells in epithelial plugs over radial keratotomy scars.[76] Cell proliferation, as determined by proliferating cell nuclear antigen (PCNA) staining, may not be an important factor in the regulation of postnatal BM protein expression because after 6 months of life, PCNA-positive cells are already confined to the limbal area.[77]

Laminin α2 and β2 chains were not seen in the infant EBM, whereas they were both found in the limbal and also conjunctival EBM in the adult corneas. These chains may be produced by stromal cells (hence, their absence from central corneas), and their presence could reflect stromal rather than epithelial maturation in the postnatal cornea, a situation previously described in the intestine for α2 chain.[78,79] Together with type IV collagen chains (described earlier), laminin α2 and β2 chains seem to be developmentally regulated in the cornea, although the exact timing and mechanisms of their appearance in the adult life are unknown. These findings may be one of the first indications of significant maturation (further differentiation) of epithelial and stromal limbal and conjunctival cells and their mutual BM during postnatal development. This system could be useful for the study of developmental regulation of specific integrin receptors for laminin. One integrin, $\alpha_6\beta_4$, was expressed in an adult pattern in infant corneas (data not shown), but others (such as $\alpha_3\beta_1$or $\alpha_2\beta_1$) might change their expression levels and/or patterns during corneal maturation.

In DM, infant corneas exhibited laminin-511, nidogen-1 and -2, type IV collagen α1-α6 chains, perlecan, and netrin-4 on both DM faces. However, in adult corneas, these components were located only on one DM face. α1-α2(IV) chains were found on the stromal face, and all others, on the endothelial face. Type VIII collagen was primarily located on the endothelial DM face in infant corneas, but was found mostly on the stromal face in the adult corneas. The mechanisms of these changes are not known.

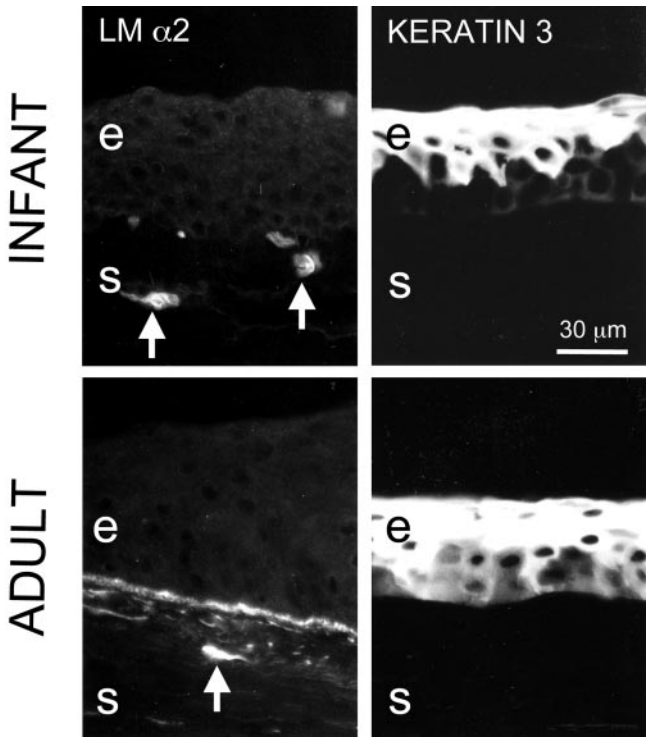

FIGURE 4. Specific laminin chains and keratin 3 in infant and adult peripheral cornea. Infant corneas, unlike adult corneas, did not stain for laminin chains α2 (LM α2) and β2 (not shown) in the limbal EBM. The peripheral basal epithelium did not stain for keratin 3 in infant corneas, in contrast to adult corneas. *Arrows*: LM α2-positive limbal vessels. e, epithelium; s, stroma.

TABLE 3. Distribution of BM Components in Adult and Infant Descemet's Membrane

Component	Adult DM		Infant DM	
	Stromal Face	Endo. Face	Stromal Face	Endo. Face
Type IV collagen chains	α1,α2	α3–α6	α1–α6	α-1–α6
Laminin chains	None	α4,α5,β1,γ1	α3–α5,β1,β3,γ1,γ2	α4,α5,β1,γ1
Possible laminin isoforms	None	411,511	311,332,411,511	411,511
Nidogen-1/entactin-1	−	+	+	+
Nidogen-2/entactin-2	−	+	+	+
Perlecan	−	+	+	+
Netrin-4/β-netrin	−	+	+	+
Matrilin-4	−	±†	+	+
Tenascin-C	−	−	+	−
Fibrillin-1	−	−	+	−
Type VIII collagen	+	−	−	+
Type XII collagen	−	+ (Short form)	−	+ (Both forms)
SPARC/BM-40/osteonectin*	±	−	+	−
Type XVIII collagen	−	±	−	±
Thrombospondin-1	−	+	−	+
Fibronectin	+	−	+	−
Vitronectin	+	−	+	−

Bold, components with different distribution in adults versus infants. Endo., endothelial.

* Only one antibody showed reactivity in this location.

† Some cases were negative. Grading is as follows: −, lack of staining; ±, weak staining in some cases; +, distinct staining in most or all cases.

Alterations in the distribution of DM components during postnatal development may reflect cellular differentiation and/or proliferation changes, especially in the case of type VIII collagen, a major DM component. It is made by proliferating corneal endothelial cells in culture but its production is inhibited on confluence.[80] Human corneal endothelial cells largely cease to proliferate in the last trimester of fetal life and after birth.[81] Therefore, this collagen's network may not be made by endothelial cells after birth and may be gradually distanced from them as DM thickens. It can still remain there because of increased stability of its hexagonal network to degradation compared with the trimer.[82] In adult life, it could also be produced by posterior stromal keratocytes, which is supported by data on knockout mice for α1(VIII) and α2(VIII) chains that display severe stromal alterations.[83]

DM components found on both sides in the infant corneas may be initially laid down by stromal and endothelial cells. During postnatal corneal maturation, the stromally located components (e.g., laminin-332, tenascin-C, fibrillin-1, netrin-4, and matrilin-4) may be degraded and replaced by those (e.g., fibronectin) made by the differentiated adult stromal cells. It would be interesting to verify this hypothesis by in situ hybridization on corneas at various stages of postnatal development. It is noteworthy that developmental vertical heterogeneity with respect to type IV collagen chains was observed in glomerular BM,[84,85] which is also a product of more than one cell type.[86]

Tenascin-C and fibrillin-1 could be detected on the stromal face of the DM only in infant corneas. Both of these proteins can reappear in DM of adult corneas affected by bullous kera-

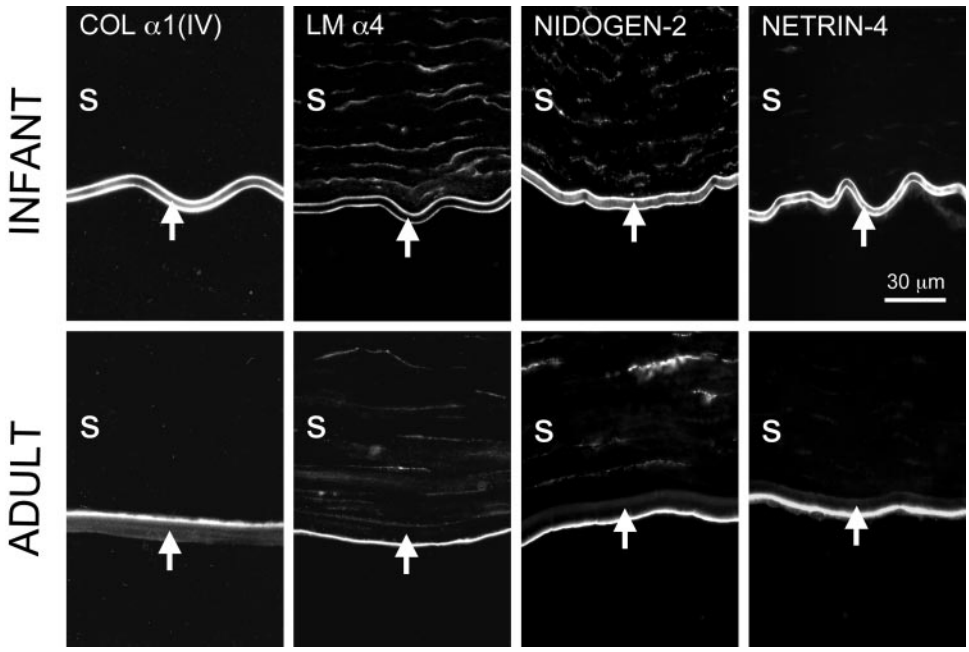

FIGURE 5. Different distribution of BM components in infant and adult DM. Type IV collagen α1 chain (COL α1(IV)), nidogen-2, the α4 chain of laminin 411 (LM α4; pAb 1101+), and netrin-4 were found on both faces of the DM (*arrows*) of infant corneas ("railroad" pattern). In adult corneas, these and other (see Table 3) BM components were found only on one DM face.

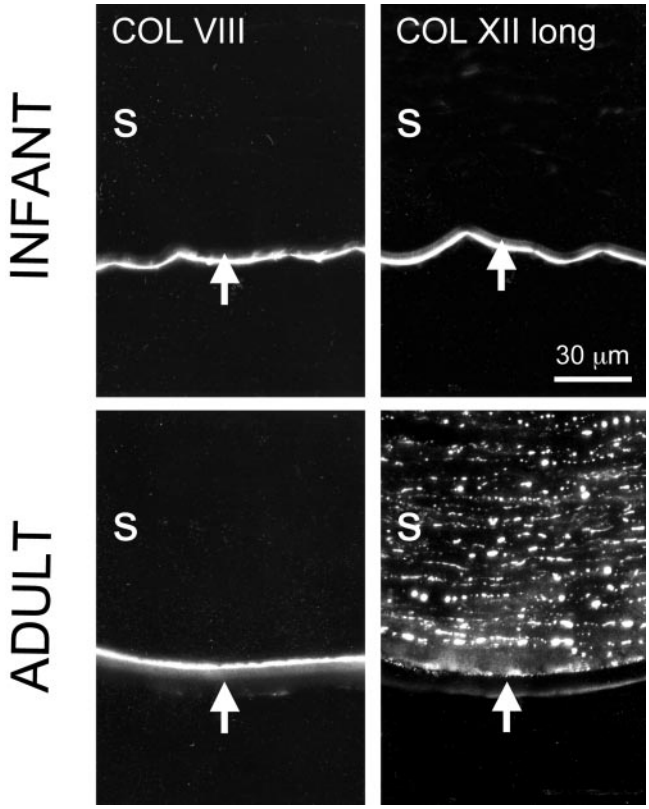

FIGURE 6. Collagen types VIII and XII in infant and adult DM. Infant and adult DM (*arrows*) displayed inverse patterns of type VIII collagen (COL VIII). Only infant DM contained the long form of type XII collagen (COL XII long; mAb I1C8) on its endothelial face. s, stroma.

topathy and Fuchs' endothelial dystrophy.[11,87] These conditions are characterized by the inability of corneal endothelial cells to pump fluid efficiently out of the cornea resulting in corneal swelling. It is possible that the infant endothelium also cannot pump out fluid as efficiently as adult endothelium, leading to the accumulation of tenascin-C and fibrillin-1 in the infant DM. Previously, tenascin-C isoforms were also found in the central corneas of fetal and infant eyes, and their expression diminished with postnatal aging.[88] However, in the Maseruka et al. paper,[88] more isoforms were seen in infant corneas than we have observed and epithelial (rather than ECM) staining was notable. Moreover, they did not observe the DM staining described in the present study. These discrepancies are probably due to the use of cryosectioned tissues in our study versus paraffin-embedded sections in the Maseruka et al. paper.

To the best of our knowledge, we provide the first account of the distribution of laminin γ3 chain, nidogen-2, matrilin-2, matrilin-4, and netrin-4 in human corneas (Figs. 1, 5, 7). Staining for the laminin γ3 chain was strong in limbal and conjunctival BM, similar to that of α1-α2(IV) chains and laminin α2 and β2 chains (Table 2). The staining in the EBM was weak and irregular, especially in adult corneas. In several tissues, this chain was found at non-BM locations.[89–91] However, in skin, testis, retina-choroid, and kidney, γ3 chain was observed in BMs including Bruch's membrane and epidermal BM[92,93] and was markedly reduced in mouse testis in the absence of laminin α2 chain.[94] It is not known which laminin isoforms containing the γ3 chain are present in limbal BM, but this region has all α and β chains that were previously shown to complex with γ3 to form laminin-213, -333, -423, and -523.[52,90,91]

Nidogen-1 and -2 are close homologs and both bind to laminin.[95] In the human cornea, nidogen-2 was codistributed with nidogen-1/entactin and was a prominent component of corneal epithelial and limbal vascular BMs. Nidogen-1 and -2 were also both observed around keratocytes. Staining of a human meningioma (data not shown) revealed that nidogen-2 was present in both tumor stroma and vascular BMs, but nidogen-1 was seen only in the vessel walls. These data exclude antibody cross-reactivity supporting the presence of both nidogens in the infant and adult corneal EBM and DM.

Matrilin-2 and -4 have been found in noncorneal BMs, such as skin epithelial BM.[45,46] These von Willebrand factor A-like domain-containing ECM adapter proteins interact with various BM components[96] and may reinforce corneal BMs, especially the infant DM, where matrilin-4 is found in a "railroad" pattern. Netrin-4 (also known as β-netrin), a BM protein with homology to laminin, may have a similar function in the DM.

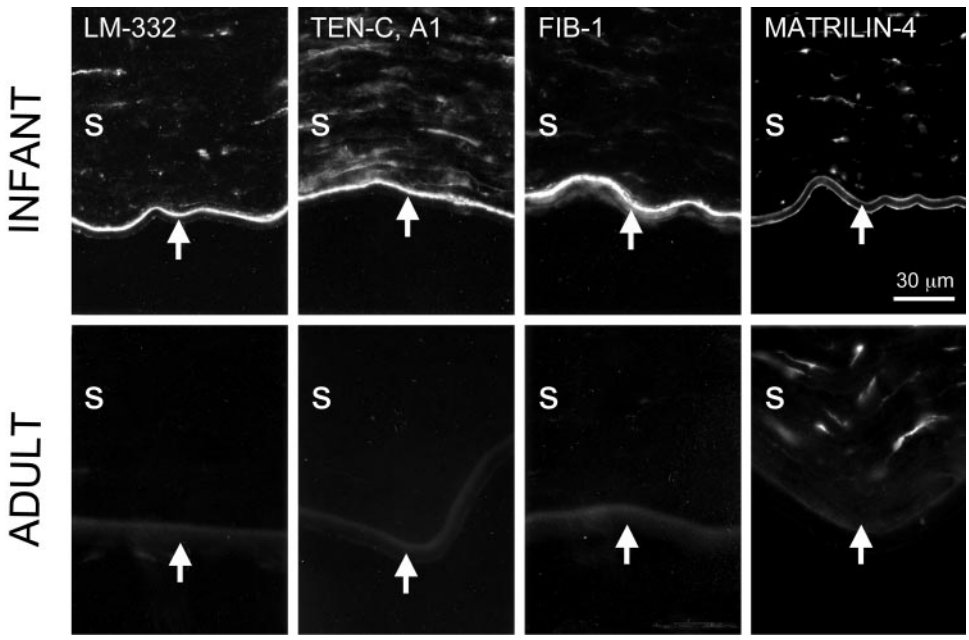

FIGURE 7. Differential expression of specific BM components in infant and adult DM. Infant DM (*arrows*) contained laminin-332 (LM-332), tenascin-C (TEN-C, alternatively spliced repeat A1 is shown), fibrillin-1 (FIB-1), and matrilin-4, in contrast to adult DM. s, stroma.

Our results indicate that human corneal BMs undergo significant compositional changes from the infant to the adult, possibly related to the differentiative and/or proliferative processes of contributing cells. It is important to identify mechanisms responsible for these changes, for a better understanding of the pathogenesis of certain corneal diseases. BM structure alterations have been described in many common corneal disorders, such as keratoconus, Fuchs' endothelial dystrophy, and bullous and diabetic keratopathies.[7,11,56,58,62,64,97–101] Elucidation of the underlying abnormalities in BM gene and protein expression may provide a means to alleviate symptoms or to prevent the development of these common vision-threatening diseases.

Note Added in Proof

While this article was in press, the paper appeared (Schneiders FI, Maertens B, Böse K, et al. Binding of netrin-4 to laminin short arms regulates basement membrane assembly. *J Biol Chem.* 2007;282: 23750–23758) showing the importance of laminin-binding netrin-4 for proper BM assembly.

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