α-2,3 Sialylation Differentiate the Limbal and Corneal Epithelial Cell Phenotypes

J. Mario Wolosin*† and Yu Wang*

Purpose. The initial differentiation event for the corneal epithelial cell lineage occurs as the limbaly localized stem cells yield, through mitosis, the highly proliferative, transiently amplifying corneal peripheral cells. This differentiation is characterized by the expression of tissue-specific cytokeratins, as well as the loss of α-enolase and pigmentation. All these are intracellular events. The aim of this study was to identify and characterize, through lectin analysis, changes in cell surface properties associated with differentiation.

Methods. Cryostat sections of the limbo-corneal area from freshly dissected pigmented rabbit corneas were stained with fluorescent lectins.

Results. Peanut lectin (PNA; binds to Ser/Thr-GalNAc-β-1,3-Gal, if the Gal residue is not sialylated) stained the plasma membrane of all layers of the conjunctiva and limbus but was excluded from corneal cell membranes. *Maackia amurensis* agglutinin (MAA; binds to sialic acid attached to galactose through α-2,3 bonds in either N-glycans or O-glycans) stained exclusively corneal cell plasma membrane. After complete tissue desialylation, all corneal plasma membranes became PNA positive with equal stain intensity across both sides of the limbo-corneal margin. The binding of the agglutinins from *Limax flavus* (binds unselectively to sialic acid) and *Sambucus nigra* (binds to sialic acid attached through α-2,6 bonds) to the basement membrane displayed a large increase at the corneal side of limbo-corneal demarcation.

Conclusions. Limbal (stem) cells express on the cell surface unsialylated galactose residues that are recognized by PNA and that lack any sialic acid bound through α-2,3 bonds. The initial differentiation involves sialylation of these residues and the concurrent appearance of α-2,3 sialic acid residues, suggesting expression or activation of α-2-3 sialytransferase. Changes in basement membrane composition, charge, or both may underpin this expression. Invest Ophthalmol Vis Sci. 1995;36:2277-2286.
vivo. More recently, the presence of high levels of α-enolase protein were observed in the basal limbal (C) bar = 32 \mu m.

Under stationary growth conditions, neither axis, complete disappearance of pigment occurs within one substantial amount of this protein.

However, evidence is accumulating for the involvement of stratifying cells nor corneal cells contain a sub-

On demand they undergo preferential proliferation, and that these cells remain pigmented but that, along the basal axis, complete disappearance of pigment occurs within one or two cell rows. (A,B) Bar = 40 cells. study shows that under stationary conditions these cells replicate slowly but on demand they undergo preferential proliferation, and that these cells are able to reestablish a normal corneal epithelium after its complete removal in vivo. More recently, the presence of high levels of α-enolase protein were observed in the basal limbal cells. Under stationary growth conditions, neither stratified limbal cells nor corneal cells contain a substantial amount of this protein.

The distribution of cytokeratins and enolase, as well as the proliferative differences between limbal and corneal cells described above, reflect modifications of the intracellular milieu with differentiation. Yet, developmental and differentiation processes are frequently accompanied by carefully controlled changes in cell membranes, particularly changes in the expression of cell surface glycoconjugates. Glycoconjugate modifications occur similarly during different stages of maturation in the epidermis. The biologic roles of these glycoconjugates have not yet been fully established. However, evidence is accumulating for the involvement of such sequences in cell-to-cell and cell-to-substrate interactions that may affect cell differentiation through transmembrane signaling. Therefore, to probe for the existence of glycoconjugate changes associated with the initial differentiation events in the cells of the corneal epithelial lineage, a battery of sequence-specific lectins was applied to the limbo-corneal zone of the pigmented rabbit.

METHODOLOGY

Cryostat Sectioning and Staining

Corneas, including limbal rims, were dissected from 2-kg Dutch belted rabbits soon after euthanasia. Limbo-
corneal sections were embedded in Tissue-Tek (Miles, Naperville, IL) and frozen by immersion in liquid nitrogen-cooled 2-methyl butane. Twelve-micrometer sections were then cut at −20°C in an International (Needham Heights, MA) cryostat and deposited on Cell-line (Newfield, NJ) slides. The sections were either processed immediately or stored at −70°C. For staining with lectins, the sections were fixed for 2

![FIGURE 1. Comparison of cytokeratin K12 binding and pigment at the limbo-corneal margin of the Dutch belted rabbit. (A) Section immunostained with anti-K12 and photographed under epi-illumination. (B) Same section photographed under transmitted light. The arrow points to the depigmentation border along the limbo-corneal basal axis. Note that at the same point, basal cells convert from K12 negative to K12 positive. All cells become K12 positive on stratification within the limbus. Many of these cells retain full pigmentation (arrowheads). (C) Fine detail of the depigmentation border obtained using a high numerical aperture immersion oil objective in a different section. Note that stratifying cells remain pigmented but that, along the basal axis, complete disappearance of pigment occurs within one or two cell rows. (A,B) Bar = 40 \mu m; (C) bar = 32 \mu m.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933183/)

![FIGURE 2. A primer for relevant glycoconjugation patterns in proteins and the epitopes recognized by various lectins.](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933183/)
minutes with 10% buffered formalin, washed, and permeabilized by a 10-minute incubation in 0.1% Triton X-100 dissolved in phosphate-buffered saline (PBS) containing 1 mm Ca\(^{2+}\) (Ca-PBS). After a 3- × 5-minute slide wash in a large volume of Ca-PBS, non-specific binding sites were blocked by incubation in 5% bovine serum albumin (1 × crystallized; Sigma, St. Louis, MO), dissolved in Ca-PBS. Blocked sections were then overlaid with 100 µl of 0.05 mg/ml fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TMR)- conjugated lectins for 30 minutes at room temperature in a humid chamber. For immunostaining, the blocked sections were overlaid for 45 minutes with 100 µl of either a rabbit polyclonal K12-antibody (a generous gift of Dr. M. Kurpakus, Wayne State University, Detroit, MI), or a mouse monoclonal Thomsen–Friedenreich antibody (a generous gift of Dr. S. Hakomori, University of Washington, Seattle, WA). After a 3- × 15-minute slide wash, the sections were overlaid with 100 µl of 1:400 diluted TMR-conjugated goat anti-rabbit immunoglobulin G or FITC-conjugated goat anti-mouse immunoglobulin G (Sigma), respectively. All stained samples were washed in Ca-PBS (3 × 10 minutes), mounted in Fluoromount (Fisher, Fair Lawn, NJ), and examined and photographed in an Olympus (Tokyo, Japan) IMT-2 microscope equipped with fluorescent epi-illumination. Unless indicated otherwise, all micrographs within each figure shown represent samples processed in parallel from embedding to photographic processing.

The agglutinins from Arachis hypogaea (peanut; PNA), Maakia amurensis (MAA), Artocarpus integrifolia (jakalin; AIA), Limax flavus (LFA), Sambucus nigra (SNA), in native or derivatized form, were purchased from EY Labs (San Mateo, CA.). Canavalia ensiformis (concavalin A [ConA]) was obtained from Sigma.

**Enzymatic Treatments**

Bovine serum albumin preblocked sections were desialylated by overnight incubation at room temperature with 100 µl of 6 U/ml of protease-free *Vibrio cholera* n-acetyl neuraminidase (Boehringer–Mannheim, India).

---

**FIGURE 3.** Staining patterns for five different lectins in the limbo-corneal zone of the Dutch belted rabbit. Each corneal section was photographed under fluorescent epi-illumination (top section) and transmitted light (bottom section) to allow correlation of the location of the lectin stain with cellular pigmentation. (A) PNA; (B) MAA; (C) AIA; (D) SNA; (E) LFA. The arrows in D and E point to increases in staining at the basement membrane plane. Bar = 100 µm. PNA = peanut lectin; MAA = Maakia amurensis; AIA = Artocarpus integrifolia; SNA = Sambucus nigra; LFA = Limax flavus.
FIGURE 4. Dual PNA-MAA staining of the limbo-corneal zone. FITC-PNA (green) and TMR-MAA (red) conjugates were used. (A) Low-magnification view inclusive of the limbo-conjunctival margin. (photographed with a 4 × 0.15 numerical aperture objective). Note the absence of MAA binding to the conjunctival cells, with the exception of discretely distributed large cells (arrows). Photographed at higher magnification (lower left corner, oil immersion, 25 × 0.8 numerical aperture), these cells exhibited exocytotic profiles suggesting goblet cells. The apparent stain-free zone at the limbo-corneal margin is generated by the screening effect of the heavy pigmentation). (B) High magnification of A showing in detail the relationship between PNA and MAA staining and pigmentation. Note that at the epithelial surface, the MAA staining and depigmentation extend deep into the limbal zone. (C) A wide view of the limbo-corneal zone. The limbal zone folded on itself during sectioning. This folding facilitated the inclusion of a wider area within a single frame. PNA-positive suprabasal cell (large arrow) can be seen in the cornea. The difference in gross structure between the corneal and limbal stromae and the profusion of PNA-positive cells within the latter are noticeable. (A) Bar = 1200 μm; (A inset, B) bar = 266 μm; (C) bar = 200 μm. PNA = peanut lectin; MAA = Maakia amurensis; FITC = fluorescein isothiocyanate; TMR = tetramethylrhodamine isothiocyanate.
Sialylation in Corneal Epithelium

napolis, IN), dissolved in 0.1% bovine serum albumin–100 mM cacodylate buffer, pH = 6.0, before lectin staining. For the selective modification of terminal galactose residues, sections were incubated overnight at room temperature with 30 U/ml galactose oxidase (Sigma) in the bovine serum albumin–cacodylate buffer, followed by a 30-minute incubation with 1 mM sodium borohydride dissolved in PBS, pH 7.4. All experiments conformed to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

One of the prominent phenotypic characteristics of the limbal epithelial cells is their pigmentation. This feature is particularly pronounced in the Dutch belted rabbit. To establish the relationship, if any, between this feature and cellular differentiation, its spatial correlation to the expression of one of the corneal-specific cytokeratins, K12, was determined (Fig. 1). The nasal and temporal limbal zones, where the pigmentation band reaches maximal width and intensity, were used. The following observations were made: (i) Along the basal cell axis, the change in cytokeratin expression is fully coincident with that in pigmentation. (ii) These two changes occur in a sharp manner, within one or two rows of cells. (iii) Within the limbal zone, stratification induces the abundant expression of K12, but along this spatial axis, cellular pigmentation changes (i.e., decreases) occur in a gradual manner. Given these topologic features, pigmentation provides a convenient way to visualize the limbal basal zone, identify accurately the limbo-corneal margin, and establish whether any given suprabasal cell is of limbal or corneal origin. For this reason, bearing in mind the spatial relationship between this attribute and the differentiation-defining distribution of K12, all lectin studies in this article were made with reference to cellular pigmentation.

Of 16 different lectins (6 of which are shown in this article) tested, only a few displayed noticeable transitions at the limbo-corneal margin. Figure 2 presents in a graphic format the binding characteristics of these lectins. PNA, a lectin that recognizes the GalNac-β-1,3-Gal sequence, providing the galactose group is not sialylated, stained all pigmented cells (Figs. 3a, 4). The stain localized predominantly to the plasma membrane and displayed a marked increase with stratification. On the conjunctival side, the stain extended beyond the pigmentation zone (Fig. 4a). In contrast, on the epithelial side of the plasma membrane, staining ceased abruptly at the limbo-corneal margin. In the cornea, PNA sites were found only within the wing cells as a weak, highly variable, Golgi-like stain (Fig. 4c, arrowhead). On the extreme, in some specimens, a continuous row of wing-like cells in a monolayer-like arrangement was stained. In other specimens, only a few individual suprabasal cells bound PNA. This binding is caused by a phenomenon unrelated to the limbo-corneal issues studied in this article; thus, it will be illustrated and dealt with elsewhere.

FIGURE 5. Effect of desialylation on the PNA binding at the limbo-corneal zone. (A) Control sample. (B) Treated sample; note that the corneal cells have become PNA-positive. (C) Treated sample incubated with 1 mg/ml AIA before incubation with fluorescein isothiocyanate-PNA; note that the desialylation-induced PNA binding has been blocked fully by the preincubation with the O-glycoconjugate-specific lectin. Bar = 200 μm. PNA = peanut lectin; AIA = Artocarpus integrifolia.
Maakia amurensis, lectin that recognizes α-2,3-linked sialic acid\(^2\) in both N- and O-linked glycoconjugates, produced a stain that was mutually exclusionary to that of PNA (Figs. 3b, 4), that is, there was no limbal staining, and, in the limbo-corneal zone, the MAA binding started where PNA binding ended. The base-to-apex increase in staining intensity within the cornea was similar to that shown by PNA in the limbus. We also observed this mutually exclusionary pattern of PNA and MAA staining in albino rabbits (not shown), which indicates that the PNA–MAA staining changes are not directly determined or affected by the changes in pigmentation. The PNA and MAA staining was not modified to a visible extent by the incubation of the freshly cut unfixed sections in a 2:1 methanol:chloroform mix for 30 minutes at room temperature, conditions that could be expected to cause substantial extraction of lipid components but were markedly affected by treating the sections with trypsin. Thus, it is reasonable to conclude that the staining changes reflect changes in polypeptide-linked glycoconjugates. On the conjunctiva, MAA failed to stain the predominant, PNA-positive, epithelial cells (Fig. 4a). However, MAA bound intensely to goblet cells easily recognized by their large round shape. Finally, this lectin produced a differential staining of the limbal and corneal stroma, which was fully coincident with the depigmentation border at the epithelial level.

Because α-2,3 sialylation of the Gal residue in the GalNAc-β-1,3-Gal sequence (see Fig. 2) will abolish PNA recognition and simultaneously will generate MAA binding sites, such a sialylation could underpin the observed exclusionary binding of PNA and MAA. This possibility was tested by treating corneal sections with the wide spectrum sialidase from V. cholera (Fig. 5). The treatment resulted in the near complete elimination of LFA and MAA binding sites (not shown). After it, there was a marked increase in the PNA staining of the corneal plasma membrane. Furthermore, the intensity of PNA staining was essentially the same on both sides of the limbo-corneal margin, suggesting that the sum of exposed and cryptic PNA-recognition sites remains largely unchanged with cellular displacement along the basal axis.

The direct role of terminal galactose on the binding of PNA was confirmed by the complete disappearance of PNA staining (Fig. 6) after selective chemical modification of terminal galactose,\(^1\) as described in Methods. The specificity of the method toward galactose was confirmed by the apparent absence of effect on the binding of two other lectins, MAA and ConA (not shown).

The types of glycoconjugates involved in the PNA and MAA recognition sites had to be resolved as well. Although the primary recognition structures for PNA reside in O-linked glycoconjugates, competition studies in vitro have shown that this lectin can bind with lower affinity to the GluNac-β-1,4-Gal sequence present in the multiple antennary ends of N-linked glycoconjugates (Fig. 2). To resolve this issue, AIA, a lectin highly specific for O-glycoconjugates, was used.\(^3\) This lectin recognized all ocular surface epithelial cells (Fig. 3c) and showed a base-to-apex distribution similar to that shown by PNA in the limbus and MAA in the cornea, respectively. Additionally, at the stromal level, AIA produced a differential limbal staining similar to that seen with MAA. Preblocking of the cryostat sections with AIA prevented the binding of PNA, MAA, and AIA itself (Fig. 7). The binding of PNA to the sialidase-treated cornea also was abolished (Fig. 5c). In contrast, the binding of lectins that recognized common N-linked glycoconjugate epitopes, ConA (recognizes α-glucoside and α-mannoside residues; Figs. 7d, 7h), and succinyl wheat germ agglutinin (rec-
Sialylation in Corneal Epithelium

recognizes β-1,4 GlcNAc oligomers, not shown), was not affected by the AIA treatment.

We also investigated whether the PNA recognition site consisted of the minimal elongation sequence Ser/Thr-GalNAc-β-1,3-Gal or of a more complex structure. This sequence, the Thomsen–Friedenreich (T) antigen, is not normally present in mature tissues except in immunoprivileged sites. It is found frequently in early embryonic stages and in transformed cells, suggesting that it is an onco-developmental antigen. A well-characterized anti-T antigen failed to react with the PNA-positive cells (Fig. 8). Weak intracellular staining was observed sporadically at both the limbus and cornea in the PNA-positive suprabasal cells. However, the staining was clearly more localized and less frequent than that produced by PNA.

Relevant staining patterns for the limbo-corneal transition were generated also by the sialic acid-binding lectins, SNA (specific for sialic acid bound through α-2,6 bonds) and LFA (a bond type-independent sialic acid lectin). Both lectins reacted with limbal cell membranes, demonstrating the existence of substantial sialylation in these cells, and neither exhibited a qualitative staining difference across the limbo-corneal demarcation. On the other hand, the staining at the basement membrane level was much stronger in the cornea than in the limbus, in particular with SNA (Figs. 2d, 2e, arrows). The intense SNA stain was preserved in corneas deepithelialized by the heptanol exposure method (not shown), suggesting that it originates in the basement membrane itself and not in the basally facing membrane of the basal cell.

DISCUSSION

Limbal basal cells have been shown to be distinct from corneal basal cells in their pigmentation, their exclusion of tissue-specific cytokeratins, and the presence of high levels of α-enolase. This study extends these observations to include the absence of a specific form of protein sialylation as an identifier of the rabbit limbal cells. The even binding of AIA across both sides of the margin and the ability of this lectin to prevent PNA and MAA staining completely indicates that the α-2,3 sialylation occurs exclusively at Ser/Thr-linked conjugates. In addition, the identical levels of PNA binding across both sides of the limbo-corneal

FIGURE 7. Effect of preincubation of limbo-corneal sections with excess unlabeled AIA specifically to block O-linked glycoconjugates on the fluorescein isothiocyanate-lectin staining. (A to D) Controls. (E to H) Preblocked samples. (A,E) PNA. (B,F) MAA. (C,G) AIA. (D,H) ConA. (The latter sample was processed separately from the other specimens shown.) Preincubation with AIA has blocked the binding of the fluorescent derivatives of PNA, MAA, and AIA but has not affected ConA. Bar = 200 μm. (A to D) Controls. AIA = Artocarpus integrifolia; PNA = peanut lectin; MAA = Maakia amurensis; ConA = concanavalin A.

FIGURE 8. Limbal staining with an antibody specific for the underivatized Ser/Thr-Gal-β-1,3-GalNAc (Thomsen–Friedenreich antigen; see Fig. 2) sequence. The posterior limbus is shown, and sporadic weak staining in wing cells can be observed. Bar = 200 μm.
margin obtained after desialylation suggests that the reciprocally exclusionary binding of PNA and MAA at this location results from the specific addition of α-2,3-linked sialic acid residues to exposed galactose sites in glycoproteins rather than changes in the density or in the nature of the residues susceptible to glycosylation. De novo expression of an α-2,3 sialyltransferase when cells of the corneal lineage undergo displacement from the limbus to the cornea along the basal axis is a plausible source of such a phenomenon; several such enzymes differing somewhat in substrate specificity have been identified and cloned recently, and Gillespie et al have shown that an equivalent conversion of human thymocytes from PNA positive (PNA⁺) to PNA⁻ (and MAA⁺) during hemopoietic maturation is determined by the expression of one of these Gal-β1,3GalNAcα-2,3-sialyltransferases at the transcriptional level.

Cell differentiation events in the corneal epithelial cell lineage occur both when cells move from the limbus to the cornea and when they leave the basal cell monolayer, whether within the limbus or the cornea. The results presented in this article constitute, to our knowledge, the first evidence for the distinctiveness of these two processes. Within the basal cell layer, all four expression changes mentioned above appear to occur concurrently at the limbo-corneal demarcation. In contrast, a divergence in the responses associated with stratification is evident. As graphically illustrated in Figure 9, for the cytokeratin and α-enolase markers, intralimbal stratification elicited the same changes observed during cellular displacement toward the cornea. In contrast, the PNA staining was preserved (and no MAA binding appeared), suggesting that activation of the sialylation requires stimulatory inputs beyond those needed for the control of cytokeratin and α-enolase expression. (An equivalent conclusion may be derived in regard to pigmentation. However, in this case, straightforward interpretation of the observations is complicated by the intrinsic stability of the pigment granule; differences in the rate of their disappearance may be secondary to differences in anabolic cellular activity between stratifying cells and cells moving from the limbus to the cornea.)

An obvious difference between the intralimbal stratification and the lateral displacement is that, in the latter case, the cells remained in contact with the extracellular matrix, a well-recognized effector of differentiation. In this context, Kolega et al and Ljubimov et al have reported differences between the composition of the limbal and corneal basement membranes; the latter study identified specific differences in the collagen type IV and laminin isoforms present in each case. Our current results with SNA and LFA point, in turn, to differences in sialic acid composition between both zones. The protein and carbohydrate observations may be interrelated; there is growing evidence for the involvement of glycosylation in the signaling function of laminin. Additionally, because LFA is a broad-spectrum sialic acid lectin and sialic acid is a substantial contributor to total surface charge, the corneal basement membrane may be more negatively charged than its limbal counterpart. These putative differences in basement membrane composition could be instrumental in inducing either activation or de novo expression of a sialyl transferase. On the other hand, it should be pointed out that glycosylation changes in integrins also have been linked to cell–matrix signals. Given the closeness of

FIGURE 9. Comparative description of the expression changes associated with limbal-to-corneal basal cell transition and with stratification within the limbus and the cornea. Cytokeratin and enolase are represented as particulate and soluble intracellular elements in A and B, respectively. Pigmentation is shown in C. PNA and MAA staining are represented as lighter and darker tracings of the plasma membrane, respectively, in D. Sharp changes in cytokeratin expression are shown to occur equally with cell displacement toward the cornea or stratification, whereas sharp changes in lectin binding or pigmentation are shown to occur only during lateral cell displacement. PNA = peanut lectin; MAA = Maackia amurensis.
these cell membrane proteins to the basement membrane, an investigation at the ultrastructural level will be needed to identify unambiguously the location of the SNA epitopes.

The functional purpose of the sialylation changes, or its consequences, also are worthy of analysis. With some exceptions, most notably the initial attachment of circulating lymphocytes to the vascular wall, the role of cell surface glycoconjugates in cell recognition, and the molecular mechanisms involved, remain to be elucidated. Exposed galactose residues may be involved in cell-to-cell cross-talk.\(^{40,41}\)

Alternatively, O-sialylation changes may be intended to regulate cell adhesiveness. The stem cells can be expected to incorporate enhanced mechanisms for biologic survival. A high level of mechanical strength and cohesiveness will be beneficial to resist both external abrasion and any lateral population pressure (because of a high level of proliferation at the peripheral corneal zone). In this context, we have observed that although, as reported, exposure to heptanol elicits a rapid exfoliation of the corneal epithelial sheet, the limbal epithelial cells remain firmly adherent to each other and to their basement membrane. On the other hand, for ocular surface function, it is important to reduce to a minimum the time span between the overt devitalization of a superficial cell and its exfoliation. Indeed, the rapidity of exfoliation of suprabasal corneal epithelial cells after loss of viability increases markedly as cells mature and approach the surface position. Sialylated O-glycans are suited particularly to perform or contribute to changes in cellular adhesiveness. The Ser/Threo-GalNAc bond confers polypeptide backbones a linear conformation, which can extend outward from the cell surface so that O-sialylated conjugates confer an expanded negative charge cloud to cell surfaces. Thus, the observed changes in cellular and basement membrane sialylation may underpin important functional differences between the morphologically similar corneal and limbal cells.

**Key Words**

basement membrane, corneal epithelium, differentiation, sialylation, stem cell

**References**

22. Wang WC, Cummings RD. The immobilized leukoag-
glutinin from the seeds of *Maakia amurensis* binds with high affinity to complex type Asn-linked oligosaccharides containing terminal acid-linked α-2,3 to penultimate galactose residues. *J Biol Chem.* 1988;263:4576–4585.


