Role of the Lewis\(^\text{x}\) Glycan Determinant in Corneal Epithelial Cell Adhesion and Differentiation

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Running Title:
Le\(^\text{x}\) in Corneal Epithelial Cell-Cell Adhesion

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ABSTRACT

In this study we demonstrate that in corneal epithelium there is cell-cell contact-regulated expression of a 145-kD glycoprotein bearing the glycan determinant Le\(^\alpha\) (Gal\(\beta\)(1,4)[Fuc\(\alpha\)(1,3)]GlcNAc). This glycoprotein (Le\(^\alpha\)-GP) was expressed in confluent/contact-inhibited cultures but not in sparse cultures of corneal epithelium. In contrast, a 135-kD glycoprotein bearing precursor, unfucosylated, lactosamine-containing glycans (Gal\(\beta\)1-4GlcNAc\(\beta\)1-R) was expressed in sparse cultures. Immunofluorescence staining and confocal microscopy of confluent cultures revealed that in corneal epithelium, Le\(^\alpha\)-antigen is located in high density at sites of cell-cell adhesion. In in vitro cell-cell adhesion assays, anti-Le\(^\alpha\), but not anti-sialyl-Le\(^\alpha\) mAbs, inhibited the formation of corneal epithelial cell-cell adhesion. Also, when added to confluent cultures, antibodies to Le\(^\alpha\) disrupted the monolayer and caused tightly packed polygonal cells to round up.

Analysis of the expression of Fut genes that encode \(\alpha\)(1,3) fucosyltransferases, the enzymes which generate the Le\(^\alpha\) determinant, revealed that confluent/contact-inhibited cultures of rabbit corneal epithelium contain markedly elevated levels of Fut4 and Fut3/5/6 gene transcripts compared to sparse cultures. These data suggest that the Fut4 and Fut3/5/6 genes are targets of cell-cell contact-regulated signals and that Fut gene products direct cell-cell contact-associated expression of Le\(^\alpha\) on the Le\(^\alpha\)-GP in corneal epithelium. Immunohistochemical analysis revealed that the expression of Le\(^\alpha\) antigen in the epithelium of adult and developing corneas is related to the stage of differentiation of the cells. While early differentiated cells robustly expressed Le\(^\alpha\), relatively undifferentiated cells did not, and the expression level was relatively low in terminally differentiated cells. Overall, these data provide evidence that a Le\(^\alpha\)-bearing glycoprotein plays a role through the Le\(^\alpha\) determinant in corneal epithelial cell-cell adhesion, and suggest that Le\(^\alpha\)-mediated cell-cell interactions contribute to mechanisms which mediate corneal epithelial cell differentiation.
INTRODUCTION

Corneal epithelium is a prototype stratified squamous epithelium. Its major function is to provide a barrier against fluid loss and pathogen entrance. This function requires the cells of the epithelium to remain tightly adherent to one another, as well as to the underlying basal lamina. The structural integrity of corneal epithelium is requisite for normal vision. Abnormality or loss of corneal epithelial cell-cell adhesion may lead to the development of a number of ocular surface disorders including corneal epithelial dysplasia and dysmaturation (1-3), corneal epithelial hyperplasia and corneal epithelial ingrowth, which is a rare but disastrous complication that can occur following intraocular surgery (4-6). In many of these conditions, enlarged intercellular spaces may be seen ultrastructurally in the epithelium (1-6).

Numerous studies on skin epithelium have shown that the loss of cell-cell adhesions in the epidermis produces life-threatening blistering skin diseases known as pemphigus foliaceus and pemphigus vulgaris (7-9). An understanding of the molecular mechanisms of epithelial cell-cell adhesion is also crucial to the understanding of complications relating to the failure of re-epithelialization of wounds. In response to injury, cells at the leading edge undergo a phenotypic conversion characterized by a dramatic reorganization of the cytoskeleton, disruption of stable intercellular adhesion, and redistribution of adhesion-related molecules. In fact, the breakage of the stable intercellular contacts is prerequisite for initiating the phase of re-epithelialization. Following re-epithelialization, reversion to the epithelial phenotype including the reformation of stable intercellular contacts must occur if the function of the epithelium is to be fully restored. The molecules which mediate cell-cell adhesion contacts also play crucial roles in important biological processes such as tissue morphogenesis and cell differentiation (reviewed in 9-12).

In normal, intact epithelium, intercellular adhesion is brought about by a variety of cell
adhesion molecules, including cadherins, desmogleins and desmocollins (7-12). Cell-cell adhesion might also be mediated by the carbohydrate determinant Lewis\(^x\) (Le\(^x\); Gal\(\beta\)(1,4)[Fuc\(\alpha\)(1,3)]GlcNAc). Several studies have suggested that Le\(^x\)-side chains of plasma membrane glycoconjugates play an essential role in cell-cell interactions during embryogenesis in the mouse (13-17). In the developing mouse embryo, the Le\(^x\) antigen appears after the third cleavage (8-cell stage) at the time of onset of compaction, and disappears around the 32-cell stage after completion of the compaction process. Multivalent lysyllysine conjugates of Le\(^x\) (Lacto-N-fucopentaose III, LNFP III) cause individual blastomeres of fully compacted 16-cell embryos to round up and lose their close apposition of membranes (14). Eggens et al. (15) and Kojima et al. (16) demonstrated that LNFP III also inhibits the intercellular adhesion of teratocarcinoma cells. In these cells and in developing mouse embryos, Le\(^x\)-Le\(^x\) interactions occurring between opposing homotypic cell surfaces have been postulated to mediate cell-cell adhesion (15,16). Several other studies have also implicated Le\(^x\) as a possible adhesion molecule during embryogenesis (18-20). In the present study we show that, in rabbit corneal epithelium: (i) there is cell-cell contact-regulated expression of a Le\(^x\) bearing 145-kD glycoprotein (Le\(^x\)-GP), and of \(\alpha(1,3)\)fucosyltransferase genes which mediate the synthesis of the Le\(^x\)-side chains, (ii) Le\(^x\)-antigen is located in high density at sites of cell-cell adhesion, (iii) antibodies to Le\(^x\) inhibit the formation of cell-cell adhesion contacts and (iv) there is cell differentiation stage-specific and developmentally regulated expression of the Le\(^x\) antigen.
EXPERIMENTAL PROCEDURES

Preparation of rabbit corneal epithelial cell cultures

Rabbit corneal epithelial cells were grown in tissue culture using eyes from Pel-Freez Biologicals (Rogers, AK) as previously described (21,22). Sparse/exponentially growing cultures, and confluent/contact-inhibited cell cultures were analyzed. Sparse cultures were collected 3 to 4 days after starting the culture at which time 30%-50% of the culture dish was populated with rapidly dividing cells that were migrating away from explants (Fig. 1). Confluent cultures were collected 10 to 12 days after starting the culture when 90%-95% of each dish was populated with tightly packed, polygonal cells (Fig. 1).

Western blot analysis of Le\(^x\)-glycoproteins of confluent and sparse cultures of corneal epithelium

MAb MMA, which is specific for Le\(^x\) (Galβ1-4[Fucα1-3]GlcNAcβ1-R) (23) and mAb 1B2 which binds to terminal N-acetyllactosamine disaccharide (Galβ1-4GlcNAcβ1-R), the unfucosylated precursor of Le\(^x\) (24) were used. Hybridomas secreting both mAbs were purchased from American Type Culture Collection (Rockville, MD). To isolate fractions enriched in Le\(^x\)-containing glycoproteins (Le\(^x\)-GPs), 1.0 ml of cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0] containing 150 mM NaCl, 0.1% Nonidet P-40, and 0.5% deoxycholic acid) was added to 10-cm dishes of washed confluent and sparse primary cell cultures. After 30 min. on ice, cell extracts were clarified by centrifugation (30 min, 100,000g, 4°C), and the protein concentration was estimated using the BCA protein assay reagent (Pierce, Rockford, IL). Clarified extracts (~7.0 mg protein in 1.0 ml) were incubated with 50 µl of agarose beads conjugated with *Aleuria aurantia* lectin.
(AAL, a fucose-specific lectin; Vector Labs, Burlingame, CA) for 1 h at 4°C. After the incubation period, the lectin-agarose beads were washed five times with RIPA buffer and 26 µl of electrophoresis sample buffer (50 mM Tris-HCl containing 2% SDS, 10% glycerol, and 100 mM dithiothreitol pH 6.8) was added. The samples were boiled for 4 min, centrifuged, and the supernatants were electrophoresed on 10% polyacrylamide gels in the presence of SDS. Proteins from the gels were blotted onto nitrocellulose paper. To identify Le^x^- GPs of corneal epithelium, gel blots were treated with 2% bovine serum albumin (BSA) in PBS (2h, room temperature) to block nonspecific binding and were then incubated with mAb MMA (undiluted hybridoma fluid, overnight, 4°C). Gel blots were developed with peroxidase-labeled antimouse IgM using a chemiluminiscent detection system (25) (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). To determine whether there is a correlation between the contact-inhibition of cell growth and the expression of the Le^x^-GP, cell cultures at various densities (day-4, sparse; day-10, early confluent; day-15, confluent and day-18, post confluent) were incubated in SHEM medium containing ^3^H-thymidine (2µCi/ml, 6.7 Ci/mmol) for 16 to 18 h. At the end of the incubation period, cells were extensively rinsed with PBS and harvested for estimating ^3^H-thymidine incorporation and Western blot analysis.

To determine whether glycoproteins containing precursor, non-fucosylated, lactosamine glycans are expressed in corneal epithelium, Western blot analysis was performed using mAb 1B2 which reacts with terminal (Galβ1-4GlcNAcβ1-R)-side chains. Briefly, detergent extracts of cell cultures of rabbit corneal epithelium were incubated with agarose beads conjugated with a β-galactose-specific lectin, *Ricinus communis* agglutinin-I (RCA-I). The RCA-I-bound glycoproteins were electrophoresed, and gel blots were analyzed for reactivity with mAb 1B2 as described above.
Immunohistochemical localization of Le$^x$ in confluent cultures of corneal epithelium

Explant cultures of corneal epithelium were grown to confluence on coverslips. For immunostaining, cells were fixed in 4% paraformaldehyde (15 min) and were then sequentially treated with 3% H$_2$O$_2$ (10 min) to block endogenous peroxidase, then 2.5% goat serum in PBS (20 min), primary anti-Le$^x$ antibody (MMA, undiluted hybridoma fluid; anti-SSEA-1, 20 µg/ml [2h]) and FITC-antimouse goat IgM (1:400 to 1:800 [1h]). Fluorescence was visualized in a Leica confocal laser scanning microscope (Exton, PA). The images were acquired using Tcsnt software from Leica.

Cell-cell adhesion assay

In this study, two different anti-Le$^x$ antibodies, mAbs MMA (23) and anti-SSEA-1 (26), were used. MAb CSLEX-1, which binds to sialyl-Le$^x$ (27) served as control. One milligram each of mAb anti-SSEA-1 and CSLEX-1 were provided by Dr. Barry Potvin (Albert Einstein College of Medicine, N.Y). MAb MMA (15 mg) was produced using the CELLMAX System (Cellco, Spectrum Labs, Rancho Dominguez, CA) in media consisting of DMEM and 5% fetal bovine serum. The antibody concentrations were measured by radial immunodiffusion (RID) using the mouse IgM-NL-RID kit (The Binding Site Inc., San Diego, CA). Prior to use, all antibodies were dialyzed against EMEM:F12 (1:1) and were diluted as needed using dialyzed EMEM:F12 containing 5% fetal bovine serum (MFS).

To determine the effect of anti-Le$^x$ mAbs on cell-cell adhesion, primary cultures of rabbit corneal epithelium were trypsinized and were plated in 24-well plates (3 x 10$^5$ cells/well in 0.6 ml) in SHEM media (22). After allowing the cells to adhere for 3 h, they were rinsed with EMEM:F12(1:1) and incubated overnight in the MFS media containing mAb MMA or anti-SSEA-1 (0.8 mg/ml). At the end of the incubation period, the cells were washed with PBS, fixed with 1% glutaraldehyde and evaluated under a phase-contrast microscope for the presence of round cells with intercellular spaces.
instead of tightly packed contact-inhibited polygonal cells. Control experiments involved incubation of the cells in MFS medium alone or in the medium containing mouse IgM (CalBiochem, San Diego, CA) or mAb CSLEX-1 (0.8 mg/ml). To determine whether the effect of the antibody was dose-dependent, cultures were incubated with varying concentrations of mAb MMA (0.05 mg/ml to 0.8 mg/ml).

In one experiment, to determine whether anti-LeX mAb was able to disrupt a contact-inhibited monolayer, mAb MMA was added to tightly packed monolayer cultures of corneal epithelium. This assay was carried out as described above except that cell cultures in 24-well plates were incubated overnight instead of for 3 h in serum containing SHEM media to obtain monolayers consisting of tightly packed polygonal cells. These monolayers were rinsed with EMEM:F12 (1:1) and were then incubated with MFS medium in the presence and absence of mAb MMA (0.05 to 0.8mg/ml) and IgM (0.8mg/ml) for 20-24 h. The cultures were then evaluated under a phase-contrast microscope for cell rounding and lifting of cell clumps from the culture dish.

RNA isolation

Poly(A)⁺ RNA from confluent and sparse cultures of corneal epithelium and from CHO cell mutants LEC11 (28) and LEC30 (29) was isolated using FastTrack 2.0 Kit mRNA Isolation System (Invitrogen, Carlsbad, CA). The RNA preparation was treated with RQ, RNase-free DNase (2U/µg RNA, Promega, Madison, WI) for 15 min at 37°C and RNA was repurified by phenol:chloroform extraction and ethanol precipitation. The yield of poly(A)⁺ RNA was approximately 25 µg/10⁸ cells.

Genomic DNA preparation

One gram of fresh liver (mouse or rabbit) was cut into small pieces and was incubated with 12 ml of digestion buffer consisting of 100 mM NaCl, 10 mM Tris HCl pH 8.0, 25 mM EDTA, 0.5%
SDS, and 0.1 mg/ml proteinase K for 16h at 55°C. The digest was extracted twice with saturated phenol and twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated by the addition of 2 volumes of 7.5M sodium acetate and 2 volumes of 100% ethanol. After rinsing the precipitate with 70% ethanol, genomic DNA was dissolved in TE buffer and stored at 4°C.

**RT-PCR amplification of Fut genes expressed by rabbit corneal epithelium**

α(1,3)fucosyltransferase genes known to direct the synthesis of the Le^x^ epitope include Fut3-Fut6 and Fut9. The human Fut3, Fut5 and Fut6 genes share 90% sequence identity and cannot be differentiated from one another by RT-PCR or Northern blot analysis with coding region probes or primers. On the other hand, Fut4 and Fut9 are considerably different from one another as well as from Fut3/5/6 (30-32). To determine which Fut genes were expressed in corneal epithelium, oligonucleotide primers corresponding to the conserved regions of Fut4, Fut3/5/6 and Fut9 were designed using Oligo 6.0 software (Molecular Biology Insights, Cascade, CO) unless stated otherwise. Nucleotide sequences of primers used are shown below.

\[
\begin{align*}
\text{Fut4:} & \quad \text{Forward: 5'}-\text{TAYCTRGCNTTTGAGAACTC} \\
& \quad \text{Reverse: 5'}-\text{GGAAGTAGCGACGATAGAC} \\
\text{Fut3/5/6:} & \quad \text{Forward: 5'}-\text{GCCCTACGGCTGGCTSGAGC} \\
& \quad \text{Reverse: 5'}-\text{GTGATGTAGTCGGGGTGCARGGAGTTCTC} \\
\text{Fut9:*} & \quad \text{Forward: 5'}-\text{CAGCTGGGATCTGACTAACTTACC} \\
& \quad \text{Reverse: 5'}-\text{CCACATGAATGAATGAATCAGCTGG}
\end{align*}
\]

*from Kudo et al. 1998 (31)

For RT-PCR amplification, the Access RT-PCR System of Promega (Madison, WI) was used.
This is a single-tube two-enzyme system in which reverse transcription and polymerase chain reaction are coupled in the same tube. Reaction mixtures were prepared by combining 0.2 mM dNTP mix, 1 µM each of gene specific downstream and upstream primers, 1mM MgSO₄, AMV reverse transcriptase (0.1 unit/µl), Tfl DNA polymerase (5 unit/µl), AMV/Tfl reaction buffer, poly(A)+ RNA prepared from corneal epithelium (0.7 to 1.0 µg) and nuclease-free water to 50 µl. Reactions were incubated at 48°C for 45 min to synthesize first strand cDNA, denatured at 94°C for 2 min, and subjected to 30 cycles of PCR amplification. Annealing temperatures were 50°C for Fut4, 63°C for Fut3/5/6, and 60°C for Fut9. PCR products were fractionated on 1% agarose gels, DNA fragments were isolated from the gel using the Concert Rapid Gel Extraction System (Gibco-BRL, Grand Island, NY), cloned into the pCR 2.1-TOPO vector using the TOPO TA Cloning Kit (Invitrogen), sequenced and used as probes for Northern blot analysis.

**Northern blot analysis**

Poly(A)+ RNA isolated from confluent and sparse cultures (3 µg each) was fractionated on 1% agarose gels containing formaldehyde and transferred onto a Hybond nylon membrane (Amershem Pharmacia Biotech, Piscataway, NJ). The blot was prehybridized overnight in a solution consisting of 0.05M PIPES, 0.1M NaCl, 0.05M NaPO₄, 1mM EDTA, 5% SDS, 60 µg/ml herring sperm DNA. The PCR probes prepared as described in the previous section, were labeled with [³²P]α-dCTP using the Prime-It RmT Random Primer Labeling Kit (Stratagene, La Jolla, CA). Unincorporated nucleotides were removed from the labeled probes using the Push Column Beta Shield Device and NucTrap probe purification columns (Stratagene). The specific activity of the labeled probes was approximately 10⁹ cpm/µg DNA. The probe was denatured at 100°C for 5 min and chilled on ice briefly and was then
added to prehybridization buffer to the final concentration of $10^6$ cpm/ml. Hybridization was performed overnight at 60°C. Blots were washed with 2X SSC buffer containing 0.1% SDS for 30 min at 60°C and were then subjected to autoradiography. Approximate densities of various components in the autoradiographs were estimated using the Bio-Image Whole Band Analysis System (Millipore, Ann Arbor, MI).

**Immunohistochemical localization of Le^x-glycoconjugates in developing rabbit corneas**

New Zealand white rabbits (Milbrook Farms, Amherst, MA) were used throughout this study. All animal treatments in this study conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Vision Research and the recommendations of the NIH Guide for the Care and Use of Laboratory Animals. Rabbit eyes were removed at different stages of development including 21 and 27 days of gestation, and from 1-, 7-, 10-, 12-, 14-day and 1-, 2-, and 3-month old offspring (N=3 or more at each stage). Depending on the age of the animal, either the eyes or the corneas with 2- to 3-mm scleral rims were embedded in optimal cutting temperature (OCT) medium immediately upon collection. Prior to embedding, convex shaped buttons made of OCT medium were prepared using a mold. These buttons were used as supports during embedding to protect the shape of the corneas. The embedded tissues were stored at -80°C until used for sectioning.

Unfixed longitudinal cryostat sections (5µ thick) of whole eyes or of corneas were incubated sequentially with 3% H$_2$O$_2$ (37°C, 10 min) and a solution of mouse liver powder (100 µg/ml, 10 min) to block endogenous peroxidase activity and nonspecific binding, respectively. The sections were subsequently incubated with mAb MMA (undiluted hybridoma fluid, 1h), biotinylated anti-mouse IgM (1:200, Vector Labs, Burlingame, CA), a freshly prepared complex of avidin D, and biotinperoxidase and diaminobenzidine (DAB) - H$_2$O$_2$ reagent (21). For negative controls, sections
were treated with an irrelevant IgM mAb, mAb 2D4 (ATCC), which is specific for asialo GM₂, a glycolipid not expressed in rabbit corneal epithelium (Panjwani et al, unpublished observations). To determine whether the Leˣ antigen is widely expressed in epithelium of adult tissues, a variety of rabbit epithelial tissues including skin, conjunctiva, tongue, esophagus and bladder were also stained with the mAb MMA.

RESULTS

Cell-cell contact-regulated expression of Leˣ-GP in corneal epithelium

Confluent and sparse cultures of corneal epithelium were analyzed to determine whether there is cell-cell contact-regulated expression of glycoproteins containing Leˣ determinants. Under the phase-contrast microscope, confluent cultures contained polygonal cells with marked contact inhibition, whereas sparse cultures contained mostly nonpolarized cells (Fig. 1). Protein yield of sparse and confluent cultures was ~1.7 and ~7 mg per 100-mm dish, respectively. To assess whether there is a correlation between the expression level of Leˣ-containing glycoproteins and cell-cell adhesion in vitro, detergent extracts of three different preparations of sparse (exponentially growing) and confluent (contact-inhibited) cultures of corneal epithelium were analyzed for reactivity with mAb MMA by Western blot analysis. When total extracts were analyzed, no mAb MMA-reactive components were detected. Therefore, fractions enriched in fucose-containing glycoproteins were prepared by incubating total extracts with agarose beads conjugated with AAL, and the proteins bound to the lectin were analyzed for reactivity with mAb MMA. A neoglycoprotein, Leˣ-bovine serum albumin (V-Labs Inc., Covington, LA) served as a positive control (Fig. 2, panel MMA, lane
BSA-Le\textsuperscript{x}). One major mAb MMA-reactive glycoprotein (145-kD) was detected in the AAL-bound fraction of three independently prepared confluent cultures (Fig. 2, panel MMA, lane C). In contrast, in three independently prepared sparse cell culture extracts, no mAb MMA-reactive glycoproteins were detected (Fig. 2, panel MMA, lane S). There was a direct correlation between the expression of the Le\textsuperscript{x}-GP and the contact-inhibition of cell growth (Fig. 2, bottom panel). Rapidly dividing 4-day sparse cultures did not express Le\textsuperscript{x}-GP. The expression of Le\textsuperscript{x}-GP was first seen in 10-day old early confluent cells which exhibited significantly less \textsuperscript{3}H-thymidine incorporation compared to sparse cultures. Even at this stage the expression of the Le\textsuperscript{x}-GP was relatively low. The expression level of the Le\textsuperscript{x}-GP was most robust in 15-day tightly packed, fully confluent cultures which exhibited only background levels of \textsuperscript{3}H-thymidine incorporation (Fig. 2, bottom panel). The expression of Le\textsuperscript{x}-GP was somewhat reduced in 18-day post-confluent cultures which had begun to lift from the culture dish in some areas.

To determine whether glycoproteins containing Le\textsuperscript{x}-precursor lactosamine units are present in the sparse cell cultures of corneal epithelium, samples enriched in galactose-containing glycoproteins were prepared by incubating total extracts with agarose beads conjugated with RCA-I, and the proteins bound to the lectin were analyzed for reactivity with mAb 1B2 by Western blot analysis. A major mAb 1B2-reactive component (~135-kD) was detected in extracts of both sparse and confluent cultures (Fig. 2, panel 1B2). These data suggest that in corneal epithelium there may be cell-cell contact-regulated α(1,3) fucosylation of the Le\textsuperscript{x}-precursor on the ~135-kD glycoprotein.

**Le\textsuperscript{x}-antigen is expressed in corneal epithelial cells at sites of cell-cell adhesion**

To localize the Le\textsuperscript{x}-antigen in corneal epithelium, confluent cultures grown on coverslips were immunostained with two different Le\textsuperscript{x}-mAbs and were examined by confocal laser scanning
microscope. While in some cases there was random staining of the entire cell membrane, in most cases the expression level of the antigen was significantly higher at sites of cell-cell adhesion (Fig. 3A). Both anti-Le\(^x\) mAbs, anti-SSEA-1 (Figs. 3Ai and 3Aii) and MMA (Figs. 3Aiii and 3Biv) gave similar results.

**Anti-Le\(^x\) antibodies but not anti-sialyl-Le\(^x\) perturb the formation of corneal epithelial cell-cell adhesion contacts**

To determine whether the anti-Le\(^x\) antibodies inhibit the formation of cell-cell adhesion contacts, corneal epithelial cells were seeded in 24-well culture plates, allowed to adhere to the culture dish and were then incubated in culture medium in the presence and absence of various antibodies and purified IgM. Cultures incubated in medium alone (Fig. 3Bi) and medium containing anti-sialyl-Le\(^x\) mAb CSLEX-1 (Fig. 3Bii) or IgM (not shown) exhibited tightly packed polygonal cells with marked contact inhibition. In contrast, cultures incubated in the presence of anti-Le\(^x\) mAbs, MMA (Fig. 3Biii) and anti-SSEA-1 (Fig. 3Biv) contained a large number of round cells. The cell rounding was more pronounced in cultures incubated in the presence of MMA compared to those incubated with anti-SSEA-1. Overall, cultures incubated with MMA and anti-SSEA-1 contained ~80% to 90% and ~50% to 60% cells with round morphology respectively. The inhibitory effect of mAb MMA on the formation of cell-cell adhesion was dose-dependent (Fig. 3Ci). For each antibody, duplicate wells were used. Experiments with mAb MMA and IgM were performed at least three times with reproducible results. Due to limited availability of the antibodies, mAbs anti-SSEA-1 and CSLEX-1 were used only in one experiment.

To determine whether the anti-Le\(^x\) is able to disrupt the cell-cell adhesion contacts, monolayer cultures of corneal epithelium consisting of tightly packed polygonal cells in 24-well plates were
treated overnight with mAb MMA. In this experiment, mAb MMA was found to induce rounding and lifting of cells from the culture dish in a dose-dependent manner (Fig. 3Cii).

**Corneal epithelial cells express transcripts for the Fut4 and Fut3/5/6 genes**

To assess which Fut genes are expressed in rabbit corneal epithelium, RT-PCR experiments were performed on four different preparations of poly(A)^+ RNA from confluent cultures. PCR products of the predicted size were cloned into a pCR2.1-TOPO vector and sequenced in both strands. All four RNA preparations of confluent cultures of corneal epithelium produced the expected size Fut4 (213 bp) and Fut3/5/6 (258 bp) products (Fig. 4A, lanes RCE). In both cases, when reaction mixtures lacked reverse transcriptase, no components were amplified (not shown). Poly(A)^+ RNA isolated from CHO cell mutants LEC30 and LEC11 served as positive controls for Fut4 (29) and Fut3/5/6 (28) respectively (Fig. 4A). Clustal W multiple sequence alignment analysis (Fig. 4B) showed that the deduced amino acid sequence of the rabbit Fut4 PCR fragment was closely related to that of human, mouse and hamster Fuc-TIV (human: 90% similarity, 88% identity; mouse and hamster: 87% similarity, 84% identity). The deduced amino acid sequence of rabbit Fut3/5/6 PCR fragment was most similar to that of human Fuc-TIII, Fuc-TV and Fuc-TVII (74% similarity and 71% identity to Fuc-TIII, V and VI each); the degree of similarity was somewhat less with the equivalent region of bovine Fuc-T and hamster Fuc-TVII (bovine: 68% similarity, 66% identity; hamster: 69% similarity, 65% identity). Numerous efforts were made utilizing a variety of annealing temperatures and cycling parameters to detect Fut9 gene transcripts in confluent cultures of rabbit corneal epithelium by RT-PCR. In no case was a Fut9-related PCR product detected. In contrast, when PCR was performed using rabbit genomic DNA, the expected size (500 bp) Fut9 PCR product (31) was produced (not shown). These data lead us to conclude that rabbit corneal epithelium expresses the
Fut4 and Fut3/5/6 genes but not the Fut9 gene. In the present study, no attempts were made to determine whether Fuc-TVII is expressed in corneal epithelium, because this enzyme fucosylates only sialated precursors. We have previously shown that sialyl-LeX is not present in the basal and middle cell layers of rabbit corneal epithelium (21).

**Corneal epithelial cell-cell adhesion contact-regulated expression of α(1,3) fucosyltransferase genes**

Northern blot analysis using polyA⁺ RNA from two different preparations each of confluent and sparse cultures was performed to determine whether in corneal epithelium there is cell-cell contact regulated expression of the Fut4 and/or Fut3/5/6 genes. In confluent as well as sparse cultures of corneal epithelium, a 3.3-kb Fut4 gene transcript and a 2.1-kb Fut3/5/6 gene transcript were detected (Fig. 5). In both preparations, confluent/contact-inhibited cultures were found to contain markedly elevated levels of Fut4 and Fut3/5/6 mRNA transcripts compared to sparse cultures which lack stable cell-cell contacts. Overall, compared to sparse cultures, confluent cultures contained ~6 times more Fut4 and ~3 times more Fut3/5/6 gene transcripts, respectively.

**Cell differentiation stage-specific expression of LeX determinant in adult rabbit corneas**

In the avascular corneal epithelium, stem cells are confined to a vascularized outer rim of the cornea known as the limbus, the transition zone between cornea and conjunctival epithelium (Fig. 6, regions marked L). These stem cells undergo differentiation as their daughter cells migrate centripetally from the limbus towards the center of the cornea (33-35). As a result there is differentiation-related hierarchy of cells with primitive stem cells in the limbus at the edge of the cornea and an increasing degree of maturation of cells towards the center of the cornea. This sequential arrangement of stem, early differentiated and terminally differentiated cells makes corneal
epithelium an excellent model in which to study the molecular mechanisms of epithelial cell differentiation.

The characteristic anti-Le\(^x\) staining pattern of adult corneal, limbal, and flanking conjunctival epithelium is shown in Figure 6. Because limbal epithelium overlies a "loose" connective tissue, it was easily differentiated from the corneal epithelium that overlies compact, plywood-like stroma (36). Conjunctival epithelium was identified based on the presence of the mucin-secreting goblet cells. The anti-Le\(^x\) mAb MMA did not react with limbal epithelium (Figs. 6A and 6Bi, regions marked “L”), but reacted intensely with peripheral corneal epithelium (Figs. 6A, 6Bi [arrowhead] and 5Biii). The Le\(^x\) immunoreactivity of corneal epithelium decreased towards the center of the cornea with an increasing degree of maturation of cells (Figs. 6A and 6Bvii). The immunoreactive distribution of Le\(^x\) determinant appeared to be largely membranous at the site of cell-cell and cell-matrix interactions (Figs. 6Biii and 6Bv). In the present study, a total of 17 rabbit corneas (from adult, 2-3 months old animals) were analyzed. Le\(^x\) immunoreactivity was not detected in limbal epithelium in any of the 17 corneas analyzed. The characteristic Le\(^x\) distribution pattern shown in Figure 6 indicating progressive decrease in the Le\(^x\) immunoreactivity towards the center of the cornea was seen in 14 of the 17 corneas analyzed. In the remaining three corneas, the entire epithelium stained weakly with no significant difference in the staining intensity between the central and peripheral regions. Le\(^x\) immunoreactivity was also detected in conjunctival epithelium of all corneas analyzed, but there was a characteristic difference between the Le\(^x\) distribution pattern of conjunctival and corneal epithelium. While, in corneal epithelium, Le\(^x\) immunoreactivity was predominantly detected in basal and middle cell layers (Figs. 6Bi[arrowhead], 6Biii and 6Bv), in conjunctival epithelium the Le\(^x\) expression was seen largely in superficial cell layers (Fig. 6Bi, black arrow). No Le\(^x\)-immunoreactivity was detected in
basal or suprabasal cell layers of conjunctival epithelium in any of the 17 specimens analyzed. For comparison purposes, three rabbit corneas were also stained with another anti-Le\textsuperscript{x} mAb, mAb 7A (37). Similar results were obtained with both mAbs 7A and MMA except that the staining intensity with mAb 7A was often less than that observed with mAb MMA. No staining was detected when tissue sections were treated with a control mAb (mAb 2D4, not shown).

To determine whether the Le\textsuperscript{x} antigen is widely expressed in the stratified epithelia of adult tissues at the site of cell-cell adhesion, we surveyed a variety of epithelial tissues of three adult rabbits for Le\textsuperscript{x} expression by immunohistochemical analysis. In epithelia of six different tissues analyzed, Le\textsuperscript{x} immunoreactivity in the basal and the middle cell layers was seen only in the cornea. In other stratified epithelia, Le\textsuperscript{x} immunoreactivity was either not detected (epidermis) or was detected only in the superficial cell layers (conjunctiva, tongue, esophagus and bladder).

**Expression of Le\textsuperscript{x}-glycoconjugates in the cornea is developmentally regulated**

To further determine whether there is a correlation between Le\textsuperscript{x} expression and the differentiation stage of corneal epithelial cells, we analyzed the Le\textsuperscript{x} expression pattern of developing corneas. Frozen sections of 21-day and 27-day-old rabbit embryos and of offspring of various age groups (1 day - 12 weeks) were stained with mAb MMA. Table 1 shows a summary of the Le\textsuperscript{x} expression pattern of developing corneas. No Le\textsuperscript{x} immunoreactivity was detected in the corneas of rabbit embryos or of 1- to 10-day-old animals. At postnatal day 12, around the time of eyelid opening, Le\textsuperscript{x} immunoreactivity was transiently detected in central corneal epithelium in three of the seven corneas analyzed (Fig. 7A, Table 1). In these three corneas, no immunoreactivity was detected in peripheral (Fig. 7C) corneal epithelium. In fact, the Le\textsuperscript{x} distribution pattern detected in these corneas, i.e., robust expression in central corneal epithelium with the antibody staining diminishing
progressively towards the periphery of the cornea (Fig. 7), was exactly the opposite of that seen in the adult corneas (Fig. 6). No \( \text{Le}^x \) immunoreactivity was detected in epithelia of 14- or 15-day-old corneas (Table 1). Weak \( \text{Le}^x \) immunoreactivity of peripheral corneal epithelium was first detected in 1-month-old corneas. With increasing age of the animal, the \( \text{Le}^x \) immunoreactivity in peripheral corneal epithelium progressively increased. Between the second and third month of age, the \( \text{Le}^x \) distribution pattern approached that of adult corneas with peripheral epithelium staining intensely with the anti-\( \text{Le}^x \) mAb and epithelium in the central region either not staining or staining only weakly (Fig. 6, Table 1).

**DISCUSSION**

In the present study, we demonstrate cell-cell contact-regulated expression of a 145-kD glycoprotein bearing the \( \text{Le}^x \) determinant (\( \text{Le}^x\)-GP) in corneal epithelium. While confluent cultures of corneal epithelium were found to robustly express \( \text{Le}^x\)-GP, sparse cultures did not express detectable levels. \( ^3\text{H} \)-thymidine incorporation and analysis of \( \text{Le}^x\)-GP at various cell densities revealed a direct correlation between the contact-inhibition of cell growth and expression of \( \text{Le}^x\)-GP. Rapidly dividing cells exhibiting high levels of \( ^3\text{H} \)-thymidine incorporation did not express \( \text{Le}^x\)-GP, whereas contact-inhibited cultures which incorporated only background levels of \( ^3\text{H} \)-thymidine robustly expressed \( \text{Le}^x\)-GP. Using immunofluorescence staining in conjunction with confocal microscopy, we further demonstrated that in confluent cultures of corneal epithelium, \( \text{Le}^x \) antigen is located in high density at sites of cell-cell adhesion. In in vitro cell-cell adhesion assays, antibodies to \( \text{Le}^x \) but not to sialyl-\( \text{Le}^x \) inhibited the formation of cell-cell adhesion contacts. While cultures incubated with the anti-sialyl-\( \text{Le}^x \) and IgM successfully formed monolayer cultures consisting of tightly packed polygonal cells, those
incubated with anti-Le\(^x\) antibodies failed to form the monolayer and contained largely round cells. Moreover, when added to confluent cultures of corneal epithelium, anti-Le\(^x\) disrupted the monolayer, causing polygonal cells to round up and lose their close apposition with the neighboring cells.

Cell-cell contact-regulated expression of Le\(^x\)-GP in corneal epithelium could be the result of either the \textit{de novo} synthesis of carrier protein or of the oligosaccharide chains carrying the Le\(^x\)-epitope. Le\(^x\)-epitope is synthesized by \(\alpha(1,3)\) fucosylation of oligosaccharide chains bearing terminal (Gal\(\beta\)1-4GlcNAc\(\beta\)1-R) disaccharides. Western blot experiments revealed that a 135-kD glycoprotein bearing the Le\(^x\) precursor disaccharide (Gal\(\beta\)1-4GlcNAc\(\beta\)1-R), is robustly expressed in sparse cultures of corneal epithelium. Thus cell-cell contact-regulated expression of the Le\(^x\)-GP in corneal epithelium appears to be due to the \textit{de novo} synthesis of the Le\(^x\)-epitope due to cell-cell contact-regulated \(\alpha(1,3)\) fucosylation of Le\(^x\)-precursor terminated side chains on the 135-kD glycoprotein. The \(\alpha(1,3)\)fucosyltransferases known to generate Le\(^x\) include Fut4, Fut9 and a family of highly homologous Futs referred to herein as Fut3/5/6 (30-32). Sequencing of RT-PCR products showed that transcripts for both Fut4 and Fut3/5/6 but not Fut9 are expressed in cultures of corneal epithelium. Northern blot analysis showed that in corneal epithelium there is cell-cell adhesion contact-regulated expression of both Fut4 and Fut3/5/6. Confluent/contact-inhibited cultures of corneal epithelium were found to contain markedly elevated levels of both Fut4 and Fut3/5/6 gene transcripts compared to sparse cultures which lack stable cell-cell contacts. These data lead us to conclude that the Fut4 and Fut3/5/6 genes are targets of cell-cell contact-regulated signals and that these Fut gene products direct cell-cell contact-associated expression of Le\(^x\) on the Le\(^x\)-GP in corneal epithelium.
Together, our findings suggest a role for the \( \text{Le}^x \)-GP in events which mediate corneal epithelial cell-cell adhesion. Previous studies have suggested that \( \text{Le}^x \)-side chains of plasma membrane glycoconjugates play an essential role in cell-cell interactions during embryogenesis in the mouse (13-17). Moreover, it has been suggested that the \( \text{Le}^x \) recognizing molecule in adjacent cells is \( \text{Le}^x \) itself, such that homotypic \( \text{Le}^x \)-\( \text{Le}^x \) interactions occurring between opposing cell surfaces mediate cell recognition during early development (15,16). It has been further shown that homotypic \( \text{Le}^x \)-\( \text{Le}^x \) interactions are catalyzed by \( \text{Ca}^{++} \) and other divalent cations (15,16,38,39). Alternatively, a hitherto unidentified \( \text{Le}^x \)-binding lectin may be present in corneal epithelium. Thus, it remains to be determined whether the \( \text{Le}^x \)-GP identified in this study represents a novel selectin ligand or whether a \( \text{Le}^x \)-\( \text{Le}^x \)-mediated cell-cell recognition system operates in the cornea.

In the present study, we also investigated the putative role of \( \text{Le}^x \) in corneal epithelial cell differentiation. There are numerous examples of studies showing that homotypic interactions between identical cells as well as heterotypic interactions between two different cell types induce the process of differentiation (12,40,41). We show here that the expression of \( \text{Le}^x \)-glycoconjugates in corneal epithelium correlates with the stage of differentiation of the cells. In the immunohistochemical study of adult rabbit corneas using anti-\( \text{Le}^x \) mAbs, no immunoreactivity was detected in limbal epithelium, which contains relatively undifferentiated cells. Intense immunoreactivity was detected in peripheral corneal epithelium, which contains early differentiated cells, and little or no immunoreactivity was seen in the terminally differentiated, central corneal epithelium. Such a region-specific expression leads us to speculate that the absence of \( \text{Le}^x \) in the limbal region may provide a mechanism by which stem cells of the cornea are maintained in the undifferentiated state and that the induction of \( \text{Le}^x \) may signal cells to enter the pathway of terminal...
If Le\(^x\) indeed plays a role in corneal epithelial cell-cell adhesion, it is reasonable to ask whether cell-cell interactions are perturbed in limbal epithelium which lacks Le\(^x\). Matic et al. (42) have reported that two gap junction connexin proteins, CX43 and CX50, which are abundantly expressed in corneal epithelium, are absent in the limbal epithelium. Also, functional gap junctions are absent in limbal epithelium. It is thus tempting to speculate that the lack or paucity of connexins and functional gap junctions in the limbal epithelium may be related to the absence of Le\(^x\) in this region. It is known that selectin-carbohydrate interactions during the rolling of leukocytes along the endothelium activate the leucocytes and upregulate its integrin receptors; this causes leucocytes to adhere to ICAM-1 and transmigrate through the capillary wall (32). It is thus conceivable that the Le\(^x\)-mediated cell-cell adhesion may facilitate the signals required for the induction of connexins in the corneal epithelium.

In the present study, we also examined Le\(^x\) expression pattern of developing corneas. It is known that in prenatal and early postnatal developing corneas, undifferentiated stem or stem-like cells are present across the entire corneal epithelium (43-47). We reasoned that if the lack of Le\(^x\) expression in limbal epithelium is related to the undifferentiated state of the cells, the corneal epithelium of fetal and young rabbits, like that of limbal epithelium of adult animals, would not react with anti-Le\(^x\) mAbs. Indeed, in our study, no Le\(^x\) immunoreactivity was detected in the epithelia of fetal and young rabbit corneas. The appearance of the Le\(^x\) antigen in peripheral corneal epithelium was first seen in the corneas of 1-month-old animals, and even at this stage, the Le\(^x\) expression level was relatively low. Only when rabbits reached about 10 weeks of age was the Le\(^x\) staining intensity of peripheral corneal epithelium, similar to that seen in adult corneas, detected. Consistent with the report of Zieske and Wason (47), our findings also suggest that the process of maturation of epithelial...
cells begins in the central cornea around the time of eyelid opening. In three of seven 12-day-old corneas, around the time of eyelid opening, Le\textsuperscript{x} immunoreactivity was detected in central corneal epithelium but not in peripheral corneal epithelium. This heterogeneity amongst rabbits of the same age group suggests transient Le\textsuperscript{x} expression at this stage. If the duration of Le\textsuperscript{x} expression were no more than several hours, it would be expected that, at the time the corneas were collected, while some animals may not have yet begun to express, others may have already ceased to express the antigen.

In brief, the present study provides evidence that a 145-kD Le\textsuperscript{x}-bearing glycoprotein plays a role through the Le\textsuperscript{x} determinant in corneal epithelial cell-cell adhesion. We propose that Le\textsuperscript{x}-mediated cell-cell interactions most likely contribute to the mechanisms which mediate corneal epithelial cell differentiation and that they may play a role in the induction of connexins and formation of gap junctions in corneal epithelium.

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FIGURE LEGENDS

Figure 1: Phase contrast micrographs of sparse/exponentially growing (top) and confluent/contact-inhibited (bottom) cultures of corneal epithelium. Bar = 450 µM.

Figure 2: Western blot analysis showing that contact-inhibited but not sparse/exponentially growing cultures of corneal epithelium express a glycoprotein bearing the Le\(^x\) determinant chains whereas sparse cultures express a glycoprotein lacking Le\(^x\) but containing Le\(^x\)-precursor side chains. To detect glycoproteins containing Le\(^x\) determinant, protein extracts of confluent (C) and sparse (S) cultures were incubated with *Aleuria aurantia* lectin (AAL)-agarose, AAL-bound glycoproteins were electrophoresed on SDS-polyacrylamide gels and the protein blots were processed for immunostaining with an anti-Le\(^x\) mAb, MMA. To detect glycoproteins bearing terminal lactosamine units, protein extracts were incubated with *Ricinus communis* agglutinin-I (RCA-I)-agarose and RCA-I-bound glycoproteins were electrophoresed and immunostained with mAb 1B2 which binds to unfucosylated lactosamine precursor of Le\(^*\). Note that confluent cultures (panel MMA, lane C) contained a major mAb MMA-reactive glycoprotein (145-kD) which was not detected in the sparse cultures (panel MMA, lane S). In contrast, sparse cultures contained a major mAb 1B2-reactive component (135-kD) (panel 1B2, lane S). Samples derived from equal amount of original cell protein were electrophoresed in both S and C lanes. Right panel shows the Coomassie blue staining pattern of the total protein extracts of confluent and sparse cultures, 16 µg protein each. Bovine serum albumin-Le\(^x\) (BSA-Le\(^x\), 0.5 µg) served as a positive control for mAb MMA. The bottom panel shows direct correlation between the contact-inhibition of cell growth as measured by \(^3\)H-thymidine incorporation and the expression of the Le\(^x\)-GP. S, sparse; C1, early confluent; C2, tightly packed.
fully confluent; C3, postconfluent cultures beginning to lift from culture wells in some areas; ND, not determined.

**Figure 3:** **A:** Le<sup>+</sup>-antigen is expressed at sites of corneal epithelial cell-cell adhesion. Confluent cultures of corneal epithelium grown on coverslips were stained with the anti-Le<sup>+</sup> mAb anti-SSEA-1 (*i* and *ii*) or MMA (*iii* and *iv*). The primary antibody was detected using an anti-mouse secondary antibody coupled to FITC. Confocal microscopy images demonstrating localization of the antigen at sites of cell-cell adhesion are shown. Two different fields are shown for each antibody staining. Cells stained with mAb MMA are shown at higher magnification compared to those stained with anti-SSEA-1 (Bar=18 μM). **B:** Antibodies to Le<sup>+</sup> inhibit the formation of cell-cell adhesion contacts. Primary cultures of rabbit corneal epithelial cells were plated in 24-well culture plates (5 x 10<sup>5</sup> cells/ml, 0.6 ml/well). After allowing the cells to adhere to the wells (3h), the cells were incubated overnight with medium alone (*i*) or medium containing 0.8 mg/ml of mAb CSLEX-1 (*ii*), mAb MMA (*iii*) or mAb anti-SSEA-1 (*iv*). At the end of the incubation periods, cells were fixed with glutaraldehyde and evaluated under a phase-contrast microscope. Note that cultures incubated in medium alone (*i*) or in medium containing mAb CSLEX-1 (*ii*) and purified IgM (not shown) successfully formed tightly packed contact-inhibited cultures. In contrast, cultures incubated in the presence of anti-Le<sup>+</sup> antibodies, MMA (*iii*) and anti-SSEA-1 (*iv*), remained round presumably due to the inhibition of the formation of cell-cell adhesion contacts. Prior to glutaraldehyde fixation, the cell borders of cultures shown in panels (*i*) and (*ii*) were markedly distinct and were similar to those shown for cultures in Fig. 1, bottom panel. **C:** The effect of mAb MMA on the formation of cell-cell adhesion contacts and disruption of the monolayers is dose-dependent. (*i*) cells were allowed to adhere to culture wells for 3 h and were then incubated overnight with the mAb MMA. (*ii*) tightly packed monolayer cultures...
were incubated overnight with mAb MMA. Cell rounding: -, <5%; +/-, 5-10%; +, >10 to <25%; ++, >25 to <50%; ++++, >50%.

Figure 4: Rabbit corneal epithelial cells express Fut4 and Fut3/5/6. A: Poly(A)+ RNA (0.7 to 1.0 µg) from confluent cultures of rabbit corneal epithelial cells (RCE) was subjected to RT-PCR as described in Methods. The expected fragments of 213bp and 258bp were amplified using Fut4 and Fut3/5/6 gene-specific primers respectively. Poly(A)+ RNA isolated from CHO cell mutants LEC30 and LEC11 served as positive controls for Fut4 and Fut3/5/6 respectively. In each case, no components were amplified when reaction mixtures lacked reverse transcriptase (not shown). B: (i) Clustal W analysis of deduced amino sequence of the rabbit Fut4 PCR fragment compared with human, mouse and hamster Fuc-TIV. First amino acid represents residue no. 294, 323 and 290 of human, mouse and hamster Fuc-TIV respectively. (ii) Rabbit Fut3/5/6 fragment compared with human, bovine and hamster Fuc-TIII/V/VI. First amino acid represents residue no. 178, 177, 191 of human Fuc-TIII, Fuc-TV and Fuc-TVII respectively. In bovine and hamster, first amino acid represents residue no. 182 and 179 respectively. Solid boxes indicate identity; shaded boxes indicate similarity.

Figure 5: Cell-cell contact-regulated expression of Fut4 and Fut3/5/6 in corneal epithelium. Poly(A)+ RNA (3 µg) from confluent (C) and sparse (S) cultures of corneal epithelium was electrophoresed on 1% agarose-formaldehyde gel, blotted onto Hybond nylon membrane and probed with a 213 bp 32P-labeled rabbit corneal epithelial Fut4 cDNA fragment. A duplicate blot was probed with a 258 bp Fut3/5/6 probe. Blots were subsequently stripped and hybridized to a GAPDH probe. Note that confluent cultures contain higher levels of both Fut4 and Fut3/5/6 mRNA transcripts compared to the
sparse cultures. C:S ratio based on the density of each band is shown under each panel.

**Figure 6:** Region-specific expression of Le^x^-glycoconjugates in an adult rabbit cornea. Unfixed frozen sections of corneas were immunostained with mAb MMA as described in Experimental Procedures. **A:** A composite photograph showing the staining pattern of the epithelium across the cornea. The region between the red arrows was constructed into a composite using the first 25% (2 cm) width of each of the 17 consecutive prints. Note that the anti-Le^x^ mAb did not react with limbal epithelium (L), the transitional zone between the cornea (the region between the limbus) and conjunctiva (the region on either side of the limbus) but reacted intensely with peripheral corneal epithelium. The immunoreactivity of corneal epithelium decreased towards the center of the cornea with the increasing degree of maturation of cells. Also, the superficial cell layers of conjunctival epithelium stained intensely with the antibody. **B:** Photographs showing representative regions from limbal (i and ii), peripheral (iii and iv), midperipheral (v and vi), and central (vii and viii) epithelium of the cornea shown in Panel A. Tissue sections stained with mAb MMA (i, iii, v and vii) and Mayer's hematoxylin (ii, iv, vi and viii) are shown. Limbal epithelium (L) is shown with flanking conjunctival (black arrow) and peripheral (arrowhead) corneal epithelium. Note the characteristic difference in the Le^x^-distribution pattern of corneal and conjunctival epithelium; while predominantly basal and middle cell layers of corneal epithelium (iii and v) were stained with mAb MMA, largely superficial cell layers of conjunctival epithelium (i, black arrow) reacted positively with the antibody. (Bar = 50µm).

**Figure 7:** Transient expression of Le^x^-antigen in central corneal epithelium of a 12-day-old rabbit. Unfixed frozen sections of whole eyes were immunostained with mAb MMA as described in
Experimental Procedures. Representative regions from central (A), midperipheral (B), and peripheral (C and D) corneal epithelium are shown. Panels A-C, sections stained with mAb MMA; D, section stained with Mayer’s hematoxylin. Note that unlike corneal epithelium of the adult animals (Fig. 6) which showed intense Le^+ immunoreactivity in the peripheral region with decreasing staining intensity towards the center of the cornea, that of a 12-day-old cornea showed intense immunoreactivity in the central corneal epithelium with decreasing intensity towards the periphery of the cornea. (Bar = 50µm). Basal cell layer in C is indicated by arrows.
Table 1: Le<sup>x</sup>-Expression Pattern in Developing Rabbit Corneal Epithelium

| Age (days) | Epithelium |   |   |
|-----------|------------|---|---|
|           | Limbal     | Peripheral | Central |
| 1         | -          | -           | -        |
| 7         | -          | -           | -        |
| 10        | -          | -           | -        |
| 12        | -          | -           | + +(3/7); -(4/7)<sup>a</sup> |
| 14        | -          | -           | -        |
| 30        | -          | +/-         | -        |
| 60        | -          | ++          | +/-      |
| 90        | -          | ++ +        | +/-      |
| one yr.   | -          | ++ +        | +/-      |

+++ intense, ++ moderate, +/- trace, - negative

<sup>a</sup> The heterogeneity amongst rabbits of the same age group suggests transient Le<sup>x</sup> expression.
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Figure 2

| Days in culture | 4   | 10  | 15  | 18  |
|-----------------|-----|-----|-----|-----|
| Morphology      | S   | C1  | C2  | C3  |
| Lex-GP          |     |     |     |     |
| Cell Growth     | 1.3 | 0.65| 0.24| ND  |

- mAb MMA
- mAb 1B2
- Coomassie Blue
Figure-3

A

B

C

| MAb conc. (mg/ml) | Cell rounding |
|------------------|--------------|
| 0                | (i)          |
| 0.05             | (ii)         |
| 0.10             | (+/-)        |
| 0.20             | (+)          |
| 0.40             | (++)         |
| 0.80             | (+++)        |
Figure 4

(A) Gel electrophoresis of Fut4 and Fut3/5/6

(B(i): Fuc-TIV

| Rabbit  | LDYLTEKLURNAFLAGAVPVWLPDNRSNYERFVPRCAFIHVDDFPSASALATYLLFLDRNPVTVYRRYFQ |
| CHO     | VDYITEKLURNAFLAGAVPVWLPDNRSNYERFVPRCAFIHVDDFPSASALAYLLFLDRNLAVYRRYFQ |
| Mouse   | VDYITEKLURNAFLAGAVPVWLPDNRSNYERFVPRCAFIHVDDFPSASALAYLLFLDRNVAVYRRYFR |
| Human   | LDLYTEKLURNAFLAGAVPVWLPDNRSNYERFVPRCAFIHVDDFPSASLSLYLLFLDRNPAYYRRYFQ |

(ii): Fuc-TIII/V/VI

| Rabbit Fuc-T | QPTAQLNLSAKTELVADWVNQNASARCVYYHCLRCGLPGHFPFVASHCQPPLRASMMEVLHSRYKYLAFENSLLYPDYIT |
| CHO Fuc-TV1  | PPSVTVNMSAKTDLVAADWVNQNASARCVYYKLQSHLHDVYCRGHPLRSRGDEMGTLHARYKYLAFENSLLYPDYIT |
| Bovine Fuc-T | QFVEELNLSAKTELVADWVNQNASARCVYYKLQHLPQHYPFVASHCQPPLRHAKLQLSRYKYLAFENSLLYPDYIT |
| Human Fuc-TIII | QPARYPLLNLSAKTELVADWVNQNASARCVYYQSLQAHLLKORDVYCRSRPLPKGMETLRSRYKYLAFENSLLYPDYIT |
| Human Fuc-TV1 | QPAHPPNLSAKTELVADWVNQNASARCVYYQSLQAHLLKORDVYCRSRPLPKGMETLRSRYKYLAFENSLLYPDYIT |
| Human Fuc-TV1 | QPAHPPNLSAKTELVADWVNQNASARCVYYQSLQAHLLKORDVYCRSRPLPKGMETLRSRYKYLAFENSLLYPDYIT |
Figure-5

**kb**

|   | C | S |
|---|---|---|
| 9.49 |   |   |
| 7.46 |   |   |
| 4.40 |   |   |
| 3.3  |   |   |
| 2.37 |   |   |
| 1.35 |   |   |
| 0.24 |   |   |

**Fut4**

- C:S 6.6:1

**Fut3/5/6**

- C:S 3.5:1

**GAPDH**

- C:S 1.2:1
Role of the Lewisx glycan determinant in corneal epithelial cell adhesion and differentiation

Zhiyi Cao, Zheng Zhao, Royce Mohan, Joseph Alroy, Pamela Stanley and Noorjahan Panjwani

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