Characterization of a Spontaneously Immortalized Cell Line (IOBA-NHC) from Normal Human Conjunctiva

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Purpose. To characterize a new nontransfected, spontaneously immortalized epithelial cell line from normal human conjunctiva (IOBA-NHC), both morphologically and functionally, to determine whether the differentiated phenotype of conjunctival epithelial cells is preserved.

Methods. Outgrowing cells from explanted conjunctival tissue were successively passaged and preliminarily characterized at passage 3 to assess epithelial origin. The cells were further characterized at passages 15 to 20, 40, 60, and 100 by analyzing (1) proliferation and in vitro behavior (viability, plating efficiency, colony forming efficiency and colony size, and Ki-67 protein expression), (2) karyotype and G-banding, (3) epithelial marker expression (cytokeratins, desmoplakins, EGF receptor), (4) absence of contaminating cell types, (5) expression of conjunctival differentiation markers (mucin gene expression), and (6) functional capability in response to proinflammatory stimuli. IOBA-NHC cells were analyzed by light and electron (transmission and scanning) microscopy, immunohistochemistry, electrophoresis and Western blot analysis, flow cytometry, and reverse transcription–polymerase chain reaction (RT-PCR).

Results. IOBA-NHC cells showed high proliferative ability in vitro and typical epithelial morphology. Cytokeratins and GalNAc, GluNAc, mannose, and sialic acid residues were immunodetected in these cells. No contaminating cell types were found. MUC1, -2, and -4, but not -5AC or -7 mucin genes were expressed in every cell passage tested. Exposure of cells to inflammatory mediators (IFNγ and/or TNFα) resulted in increased expression of intercellular adhesion molecule (ICAM)-1 and HLA-DR.

Conclusions. Morphologic and functional characterization of the nontransfected, spontaneously immortalized IOBA-NHC cell line shows that this new cell line may be a useful experimental tool in the field of ocular surface cell biology. (*Invest Ophthalmol Vis Sci.* 2003;44:4263–4274) DOI:10.1167/iovs.03-0560

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Conjunctival mucosa is involved in many processes that help to maintain a healthy ocular surface. The epithelium (both goblet[1] and non-goblet[2] cells) contributes to the mucous layer of the tear film and secretes cytokines and growth factors that participate in immune-mediated processes at the ocular surface.[3] There is increasing evidence of a relation between altered physiology of the conjunctival epithelium and development of inflammatory diseases of the ocular surface[4] (i.e., dry eye syndrome). It would be useful to know more about the conjunctival epithelium to gain a better understanding of ocular surface disease.

There are several ways to obtain human conjunctival epithelium. Human biopsy specimens provide both epithelial and stromal cells; however, the information obtained from these is limited. Moreover, human tissue is not always available. Conjunctival impression cytology is an alternative for obtaining human conjunctival epithelial cells, but the information is also limited. In vitro systems offer the possibility of studying the influence of metabolites, mediators, or drugs on the behavior of living cells in a controlled environment. Primary cultures of human conjunctival epithelial cells show several epithelial cell layers and the ability to produce and secrete mucin-type glycoproteins. However, primary cultures are prepared from human conjunctiva biopsy specimens, and eventually the tissue availability reduces primary culture preparation. Cell lines offer a better approach to in vitro studies, because cells multiply quickly and easily. Nevertheless, there is currently only one continuous, untransfected epithelial cell line from human conjunctiva available, the Wong-Kilbourne derivative of Chang cells[5] (American Type Culture Collection [CCL] 20.2 clone 1-5c-4; Manassas, VA), and this cell line is contaminated by HeLa cells.[6] Also, few transformed cell lines from conjunctival epithelium have been reported[7] (Ward SL, et al. *IOVS* 1998; 39:ARVO Abstract 397; Smit EE, et al. *IOVS* 2001;42:ARVO Abstract 4938).

In our laboratory, we characterized a cell line spontaneously arising from a primary culture of human conjunctival epithelium, showing continuous proliferation. The purpose was to determine whether the conjunctival epithelial morphologic and functional characteristics of this conjunctival cell line (IOBA-NHC) were maintained in vitro so that it might be used as a model to study the physiopathology of human conjunctiva.

Materials and Methods

All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Culture plastic material was from Nunc (Roskilde, Denmark), and reagents for cell culture were from Invitrogen-Gibco (Inchinnan, UK). All antibodies were monoclonal with the exception of anti-desmoplakins-1 and -2 (Table 1) and were purchased from Dako (Glostrup, Denmark), ICN (Costa Mesa, CA), Oncogene Research Products (Boston, MA), Serotec, Ltd. (Oxford, UK), and Sigma-Aldrich. Anti-K7 antibody was a generous gift of Darlene A. Dartt, PhD, (Schepens Eye Research Institute, Harvard Medical School, Boston, MA). Secondary antibodies were fluorescein isothiocyanate

TABLE 1. Antibodies Used for Immunocytochemistry or Western Blot Analyses

Antigen (Name of Antibody)	Source	Dilution		Specificity
		IMC	WB	
Vimentin (V9)	Dako	1:200	1:200	Vimentin
CK 19 (RCK108)	Dako	1:25	NU	Conjunctival differentiation
CK 7 (OV-TL12/30)	D. Dartt	1:200	1:200	Secretory epithelium
CKs 11, 14, and 19 (AE-1)	ICN	1:100	1:500	Acidic cytokeratins†
CKs 1, 2, and 10 (AE-2)	ICN	1:100	1:500	Skin type differentiation
CKs 5, 7, and 8 (AE-3)	ICN	1:100	1:500	Basic cytokeratins†
CK 3 (AE-5)	ICN	1:100	1:500	Corneal type differentiation
CK 13 (AE-8)	ICN	1:100	1:500	Nonkeratinized epithelium
Desmoplakin-1, -2 (AHP320)	Serotec	1:200	1:200	Desmosomal proteins
EGF receptor (F4)	Sigma	1:1000	1:2000	Epithelial cells
Ki-67 nuclear protein (Ki-67)	Dako	1:100	NU	Proliferative status
vW factor (F8/86)*	Dako	1:50	NU	Endothelial cells
CD1a (NA1/34)	Dako	1:100	NU	Langerhans' cells
Fibroblast ag Ab-1 (AS02)	Oncogene	1:100	NU	Fibroblasts

See the Methods section for locations of sources. IMC, immunocytochemistry; WB, Western blot; NU, not used.

* Previous factor VIII-related antigen.

† All epithelial cells express at least one acidic and one basic keratin.

(FITC)–conjugated (immunofluorescence), or horseradish peroxidase (HRP)–conjugated anti-IgG (Western blot analysis) and were purchased from Jackson ImmunoResearch (West Grove, PA). Lectins were FITC-, Texas red (TxR)–, or tetramethylrhodamine-isothiocyanate (TRITC)–conjugated and were obtained from EY Laboratories, Inc. (San Mateo, CA) or were the kind gift of J. Mario Wolosin, PhD (Mount Sinai School of Medicine, New York, NY). Antibodies for flow cytometry were obtained from BD PharMingen (San Diego, CA). Propidium iodide (PI) and antifade fluorescent mounting medium (Vectashield) were from Molecular Probes Europe BV (Leiden, The Netherlands) and Vector Laboratories (Burlingame, CA), respectively. All the reagents for Western blot analysis were purchased from Bio-Rad Laboratories (Hercules, CA). Reagents for reverse transcription–polymerase chain reaction (RT-PCR) were purchased from Life Technologies (primers and One-Step RT-PCR System) and Clontech (Palo Alto, CA; control RNAs).

Conjunctival Epithelial Cell Isolation and Culture

Three 4 × 4-mm conjunctival biopsy specimens (superior, bulbar) from healthy donors who were undergoing cataract surgery were obtained. Informed consent from patients was obtained in accordance with the recommendations of the Declaration of Helsinki, The IOBA (University Institute of Applied Ophthalmobiology) Research Committee approved the experiments. Specimens were placed in sterile DMEM/F12 culture medium supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, and 2.5 μg/mL amphotericin B and transported to the cell culture laboratory. A specimen from a 49-year-old white man was the only one that produced continuously growing cells and these gave rise to the IOBA-NHC cell line.

Connective tissue was carefully removed from the specimen under the microscope with surgical scissors. The explant was then plated epithelial side up on a 35-mm culture dish and incubated at 37°C in a 5% CO_2. The culture medium was DMEM/F12 supplemented with 1 μg/mL bovine pancreas insulin, 2 ng/mL mouse epidermal growth factor (EGF), 0.1 μg/mL cholera toxin, 5 μg/mL hydrocortisone, 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. The medium was changed every 2 to 3 days, and cell growing was assessed daily by phase-contrast microscopy.

Confluence was reached after 6 weeks. Cells were successfully subcultured; the split ratio was 1:3 for the first 30 passages, 1:4 from 30 to 60 passages, 1:8 from 60 to 80 passages, and 1:16 thereafter. Cells were never transfected or induced in any way other than subculturing for propagation. The cell line was named IOBA-NHC (normal human conjunctival tissue) and currently has reached up to 100 passages.

Exclusion of Microbial Contamination

IOBA-NHC cells were grown in antibiotic-free culture medium for at least 4 weeks to check for mycoplasmal, bacterial, and viral infection. Latent bacterial or viral infection of cells was further excluded by repeating tests after growing the cells for at least 8 weeks in antibiotic-free culture medium. A *Mycoplasma* detection kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to detect the most common *Mycoplasma* species contaminating mammalian cell cultures. Although cells usually showed no contamination, some cell lots were contaminated with *Mycoplasma arginini.* However, cells were treated with BM-cyclin-1 and -2 (Roche Diagnostics GmbH) for 3 weeks, and no further *Mycoplasma* contamination was detected in later routine controls. The presence of adenovirus and herpes simplex virus-1 and -2 was excluded by RT-PCR.[8,9]

Karyotype Analysis and G-Banding

High-resolution banding[10] facilitated the identification of IOBA-NHC cell chromosomes and major structural abnormalities. Giemsa-banded karyotypes[11] were examined under a microscope (model BX50; Olympus, Tokyo, Japan) and their digital images analyzed with a commercial system (Cytovision Karyotyper ver. 4.1 for UNIX; Applied Imaging Corp., Santa Clara, CA). Images were compared to references.[11] Chromosome analysis was performed 10 times in cells from different passages. An average of 30 metaphase spreads was screened for each analysis.

Assays for Testing In Vitro Proliferation

Cell viability was measured periodically using the trypan blue dye exclusion method.[12] Cells always displayed viability higher than 96%, and only cells with at least 98% were used in each assay. Four combined methods were used to determine the ratio of proliferating cells in the IOBA-NHC cell line: plating efficiency, colony-forming efficiency (CFE), colony size (in passages 20–30) and immunostaining with anti-Ki-67 nuclear protein monoclonal antibody (in passages 20 and 60). All assays were performed in triplicate, and each experiment was performed on a different day.

Plating Efficiency. Cells were enzymatically detached from the culture surface, counted in a hemocytometer, and plated in amounts on the order of 10^4 cells/plate. Unattached cells in the culture medium

TABLE 2. Lectins Used and Their Carbohydrate Specificity

Plant Source	Abbreviation	Source	Carbohydrate Specificity
Arachis hypogaea	PNA	EY Labs	Gal(β1,3)GalNAc>Galactosamine>Gal
Artocarpus integrifolia	AIA	EY Labs	Gal(β1,3)GalNAc
Datura stramonium	DSA	JM Wolosin	Glu(β1,4)GluNAc>LacNAc
Galanthus nivalis	GNA	JM Wolosin	Manα(1,3)Man
Helix pomatia	HPA	Sigma-Aldrich	α-GalNAc>Gal(β1,4)GalNAc>α-GluNAc
Limax flavus	LFA	JM Wolosin	Sialic acid*
Maackia amurensis	MAA	JM Wolosin	α2,3-Linked sialic acid
Ulex europaeus	UEA-I	JM Wolosin	Fuc(α1,2)Gal(β1,4)-GluNAc
Vicia villosa	VVA	JM Wolosin	α- or β-GalNAc

See the Methods section for locations of sources. Gal, galactose; GalNAc, *N*-acetylgalactose; Man, Mannose; GluNAc, *N*-acetylglucose; Fuc, Fucose; LacNAc, *N*-acetyllactosamine.

* Regardless of the linkage.

withdrawn during the first and second replacements of medium were also counted. Plating efficiency was calculated as the percentage of attached cells (difference in the number of plated cells and detached cells) versus the total number of plated cells.[13]

Colony-Forming Efficiency. This method measures of the ability of seeded cells to form colonies (group of at least four cells derived from a single cell) and to proliferate.[14] Five culture dishes were counted for each experiment. Colony-forming efficiency (CFE) was calculated as follows: number of colonies on day 5/number of viable cells seeded on day 1 $\times$ 100.

Colony Size. Defined as the total number of cells in a given colony, colony size was calculated by counting cell numbers from 25 to 30 randomly selected colonies from each culture dish in the CFE experiment.

Immunostaining against Anti-Ki-67. This nuclear protein is expressed in all human proliferating cells during late G_1, S, M, and G_2 phases of the cell cycle.[15] Cells were incubated in phosphate-buffered saline (PBS) containing 1.0% bovine serum albumin (BSA) plus 0.05% Tween-20 for 1 hour at room temperature. Cells were then incubated in 1:100 anti-Ki-67 for 1 hour, washed in buffer, and incubated in FITC-conjugated goat anti-mouse IgG (1:100) for 1 hour. An incubation in PI was performed before mounting, to facilitate cell body and nucleus identification. The number of cycling cells was calculated as the percentage of Ki-67–positive cells versus the total number of cells. Counts were made in at least five digitalized images taken from randomly selected fields, using the confocal microscope $\times$40 objective by two different masked observers. Experiments were repeated at least twice.

IOBA-NHC Cell Line Morphologic Characterization

An early characterization of IOBA-NHC cells at passage 3 was performed. According to Freshney,[16] by the third passage primary cultured cells become more stable and proliferate rapidly. Also, cells in later passages (15–20, 40, 60, and 100) were studied to investigate potential differences in marker expression along the lifespan of the cell line.

Light and Electron Microscopy. IOBA-NHC cells were fixed in cold methanol for 10 minutes, permeabilized in cold acetone, rehydrated, and stained with hematoxylin/eosin (H/E) and Giemsa. For transmission electron microscopy (TEM), cells were fixed in 1.0% glutaraldehyde in 0.1 M sodium cacodylate-HCl (pH 7.4) for 10 minutes at 37°C, washed in 0.1 M sodium cacodylate-HCl, postfixed in 1.0% osmium tetroxide and 4% tannic acid and embedded in Spurr medium. The remainder of the procedure was performed as previously described.[17] Sections were stained with uranyl acetate and lead citrate[18] and viewed with an electron microscope (model JEM-1200; JEOL, Tokyo, Japan).

For scanning electron microscopy (SEM), fixed cells were washed in 0.2 M sucrose solution, dehydrated in a graded ethanol series, critical point dried,[19] and gold sputter coated (15–20 nm) in vacuum evaporation under argon gas at a conducting amperage of 20 mA. A microscope with a Maiya Rolf Holder CS-I photographic system (model T300; JEOL) was used to examine the cells.

Immunocytochemistry and Confocal Microscopy. Human conjunctival epithelium identity was confirmed by means of immunofluorescent staining against specific markers. Table 1 lists the monoclonal antibodies used and their specificity and dilution. Epithelial marker expression was studied by using antibodies against several cytokeratins (CK), desmosomal proteins (desmoplakin-1 and -2), vimentin, and epithelial growth factor receptor (EGFR). Specific conjunctival markers, such as CK3, CK7, and CK19, were also studied. The absence of contaminating cell types from conjunctival tissues, such as fibroblasts, endothelial cells, and Langerhans' cells, was evaluated.

TABLE 3. Primer Sequence and Transcript Size for Each Gene Analyzed by RT-PCR

Genes	Primer Sequences	Size (bp)	Reference
β2 MG	5′-TCCAACATCAACATCTTGGTCAGA		
	3′-AAACCAGATAACCACAACCATGG	250	AN*:AF072097
MUC1	5′-AGGCTCAGCTTCTACTCTGG		
	3′-GACAGACAGCCAAGGCAATG	656	24
MUC2	5′-TGCCTGGCCCTGTCTTTG		
	3′-CAGCTCCAGCATGAGTGC	438	26
MUC4	5′-TGAAACAGCTACCTCATCCCTCTG		
	3′-AAGTTGCTGGTGATTGTCCTTCTG	200	AN:NM_004532
MUC5AC	5′-GTTCTCCGGCCTCATCTTCTCC		
	3′-GCTCAAAGACCTTGCTCAGAATCAG	350	AN:AJ001402
MUC7	5′-GCTAAAAGCAAGCAACTGGATTGA		AN:L13283
	3′-AAGTGAGATTTGGGTGATTGGTGA	199	26

β2 MG, β2 microglobulin; bp, base pair.

* AN, Genebank accession no.

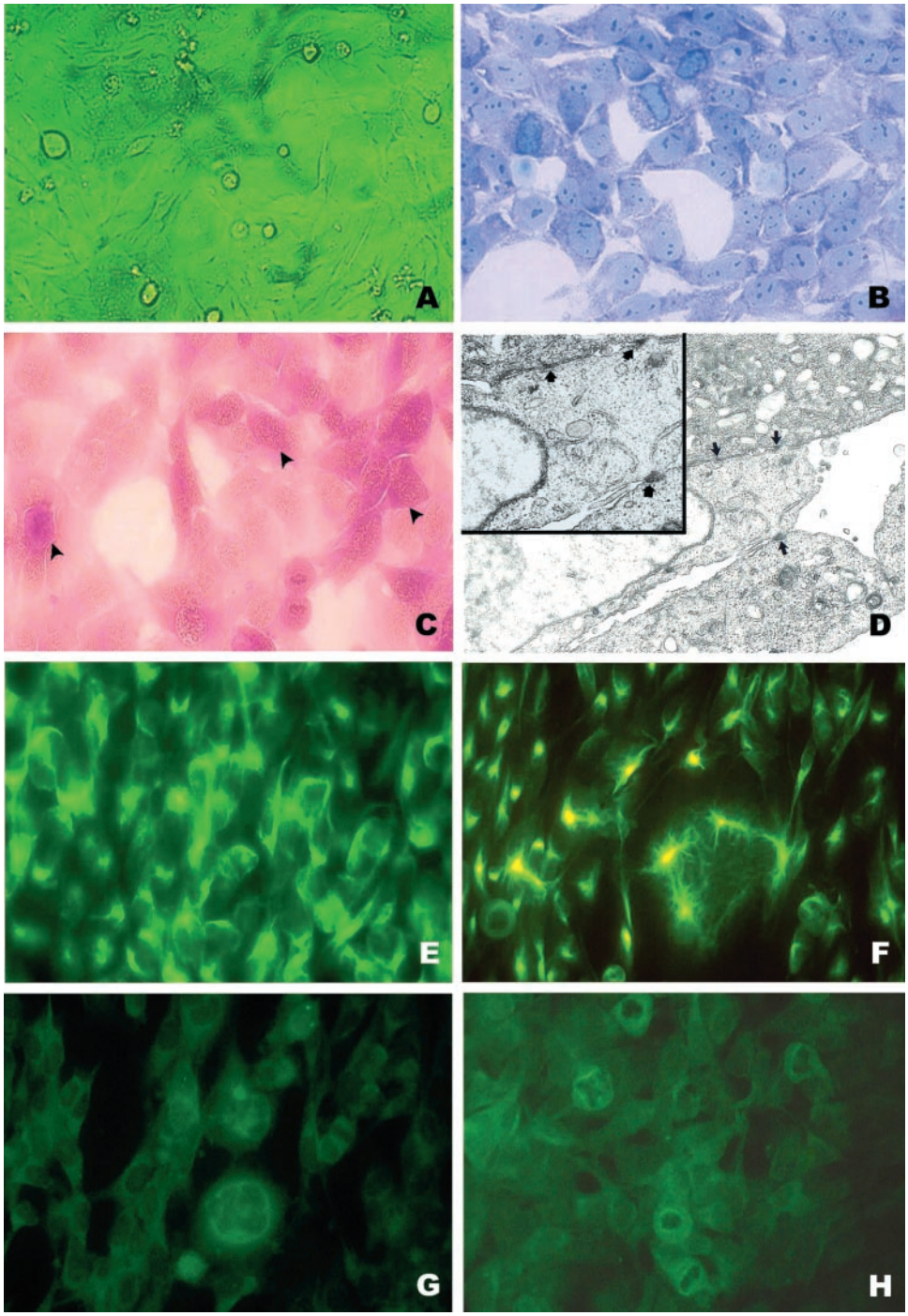

FIGURE 1. Early characterization of IOBA-NHC cells in passage 3. (**A**) Phase-contrast photomicrograph showing cobblestone-like appearance of a cultured cell monolayer. (**B**) Giemsa and (**C**) PAS staining. *Arrowheads*: PAS^+ cells. (**D**) TEM photomicrograph (transverse section) showing desmosomes (*arrows*). *Inset*: higher magnification of the cytoplasmic area depicting the organelles and desmosomes in more detail (*arrows*). Immunofluorescence photomicrographs showing positive reaction against (**E**) AE-3 and (**G**) anti-vimentin antibodies, and negative reaction against (**F**) AE-2 and (**H**) AE-5 antibodies. Magnification: (**A–C**, **E–H**) ×40; (**D**) ×9,800; inset ×18,000.

Cells were fixed in cold methanol and stored at −20°C until use. Cells were rehydrated and incubated in blocking buffer (PBS containing 1.0% BSA with or without 0.03% Tween-20) for 1 hour at room temperature and were then exposed to primary antibodies (Table 1) for 1 hour at room temperature. After washing, cells were incubated in FITC-conjugated goat anti-mouse IgG (1:100) for 1 hour, washed, incubated in PI, and mounted. The specificity of every primary antibody had been tested in our laboratory. Primary antibodies were omitted in control studies. Experiments were repeated at least three times.

Preparations were examined with a confocal laser scanning microscope (model LSM310; Carl Zeiss Meditec, Jena, Germany) equipped with a krypton-argon laser. FITC and PI were excited with a 488- and 543-nm emission laser beam, respectively, and detected with a band-pass emission barrier filter. Digital images were stored from each slide, and some were converted to black-and-white images (Photoshop, ver. 5.0; Adobe Systems, Mountain View, CA).

Electrophoresis and Western Blot Analysis. IOBA-NHC cells and human and rat conjunctival tissues (used as positive controls) were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer plus proteinase inhibitors (10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1% deoxycholic acid, 1% Triton X-100, 1 mM EDTA, 0.1% SDS, 1 mM sodium orthovanadate, 0.57 mM phenylmethylsulfonyl fluoride (PMSF), and 1 U/mL aprotinin). After homogenization, samples were incubated at 4°C for 30 minutes and centrifuged at 3200*g* for 20 minutes at 4°C.

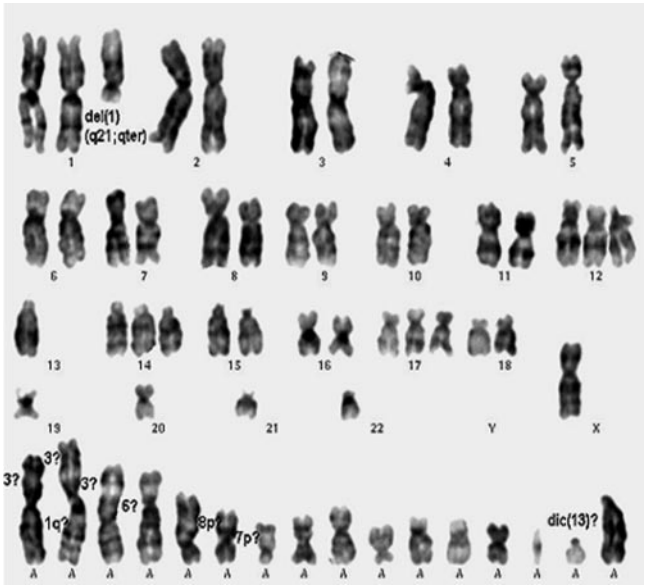

FIGURE 2. Representative G-banded karyotype of IOBA-NHC cells showing marker chromosomes (**A**) (chromosome number = 60): 60, X0,+del(1)(q21;qter), +der(6)(?), +12, −13, +14, +17, −19, −20, −21, −22, +MAR, +MAR, +MAR, +mar, +mar, +mar, +mar, +mar, +mar, +mar, +mar, +mar, +mar, +mar (for details, see the International System for Human Cytogenetics Nomenclature [ISCN][11]).

To extract insoluble cytokeratins, pellets were washed in 10 mM Tris-HCl (pH 7.2) containing 5 mM EDTA and 0.4 mM PMSF and resuspended in urea buffer (8 M urea, 40 mM Tris-HCl; pH 7.2), and 5% β-mercaptoethanol).[20] After a 1-hour incubation at 4°C, samples were centrifuged at 3200*g* for 5 minutes at 4°C, and the cytokeratin-enriched fraction collected.

Proteins in the homogenate were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gels.[21] Proteins were then transferred to nitrocellulose membranes, blocked in 5% dried milk in TBST (10 mM Tris-HCl [pH 8.0]), 150 mM NaCl, 4% FBS, and 0.05% Tween-20), and incubated with primary antibodies (Table 1) for 1 hour at room temperature. Membranes were washed three times in TBST and incubated with HRP-conjugated donkey anti-mouse (1:10,000) or anti-rabbit (1:2,500) IgG for 1 hour at room temperature. Immunoreactive bands were visualized by the enhanced chemiluminescence method. Images were acquired with a commercial system (ChemiDOC; Bio-Rad Laboratories, Inc., Hercules, CA) and analyzed on computer (Quantity One software; Bio-Rad Laboratories, Inc.). Results are representative of three independent experiments.

Functional Characterization

Mucin Production Study. Because differentiated conjunctival goblet and non-goblet epithelial cells synthesize and secrete mucin-type glycoproteins, mucin production ability was studied in IOBA-NHC cells. Cells from different passages (15–20, 40, 60, 100) were studied using the following techniques.

Alcian Blue/PAS Staining. Combined staining with alcian blue (AB)/PAS allows identification of acidic and sialylated glycoproteins.[22] Cells were fixed in cold methanol for 10 minutes, permeabilized in cold acetone, rehydrated, and stained. Secretory cells were identified by color as AB^+ (blue) or PAS^+ (magenta). Experiments were repeated at least three times.

Lectin-Binding Site Analysis. Lectins are proteins mainly of plant origin that specifically bind to glycosidic residues of glycoconjugates, which allows the presence of a given sugar residue in cell membranes to be detected.[23] A panel of nine FITC-, TxR-, or TRITC-conjugated lectins (Table 2) was used to identify glycoconjugates on the IOBA-NHC cell surface. Fixed cells were blocked for 30 minutes in PBS containing 1% BSA at room temperature. Cells were incubated in a 1:40 solution of each lectin in PBS plus 0.1% BSA for 30 minutes at room temperature. To reveal the glucidic structure attached to terminal sialic acid, cells were incubated with 1 mU/mL neuraminidase at 37°C for 1 hour before incubation with *Arachis hypogaea* lectin. After incubation, preparations were washed and mounted. Controls included lectin preabsorption with specific 0.2 or 0.3 M carbohydrate solution in PBS and incubation with blocking buffer instead of lectin. Experiments were repeated at least three times.

RT-PCR Analysis. The expression of five mucin genes identified in conjunctiva epithelial cells (MUC1, -2, -4, -5AC, and -7)[24–27] was analyzed by RT-PCR. Total RNA was isolated from IOBA-NHC cells and human conjunctival biopsy specimens using a modified acid guanidinium-thiocyanate protocol.[28] The resultant preparation was analyzed spectrophotometrically (260 nm) to determine the RNA concentration. Total RNA (0.5 μg) was reverse-transcribed and used in PCR in a total reaction volume of 25 μL with a commercial system (SuperScript One-Step RT-PCR System; Invitrogen-Life Technologies) under conditions described previously (Corrales RM, et al. *IOVS* 2001;42:ARVO Abstract 2613). Briefly, RT-PCR parameters consisted of cDNA synthesis at 50°C for 30 minutes and predenaturation at 94°C for 2 minutes for all mucin genes. This was followed by 40 cycles of denaturation at 94°C for 1 minute (35 cycles for MUC4 and -7), annealing at 62°C for 30 seconds for MUC1 (59°C for MUC2, 65°C for MUC4, and 60°C for MUC5AC and -7), and extension at 72°C for 1 minute (30 seconds for MUC7) with a final extension at 72°C for 7 minutes in all cases.

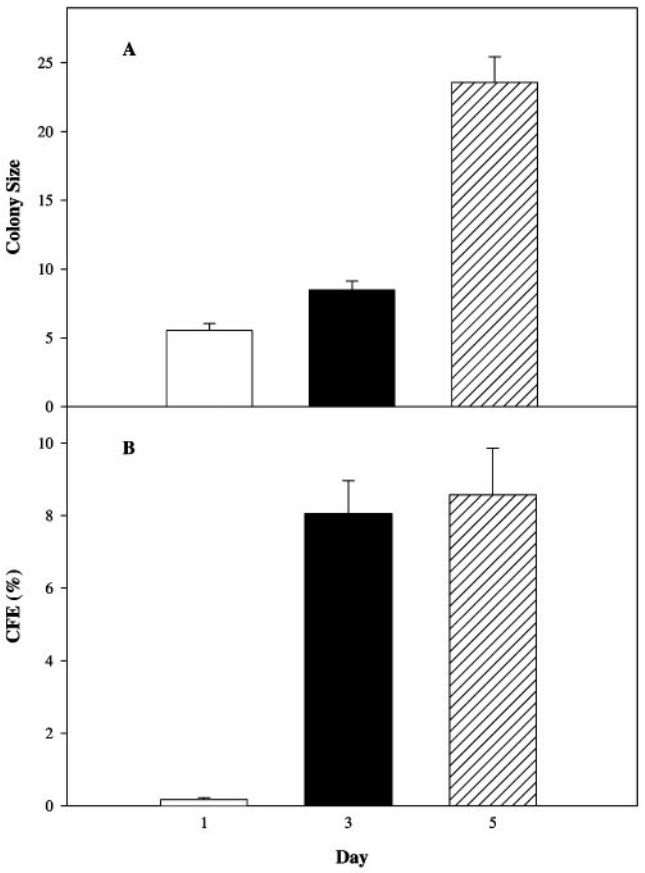

FIGURE 3. (**A**) Colony size and (**B**) CFE for IOBA-NHC cells in passages 20 to 25. Colony size and percentage of relative CFE was calculated for days 1, 3, and 5 after a known number of cells were plated. Data are representative of at least five measurements per day. The experiment was performed in duplicate.

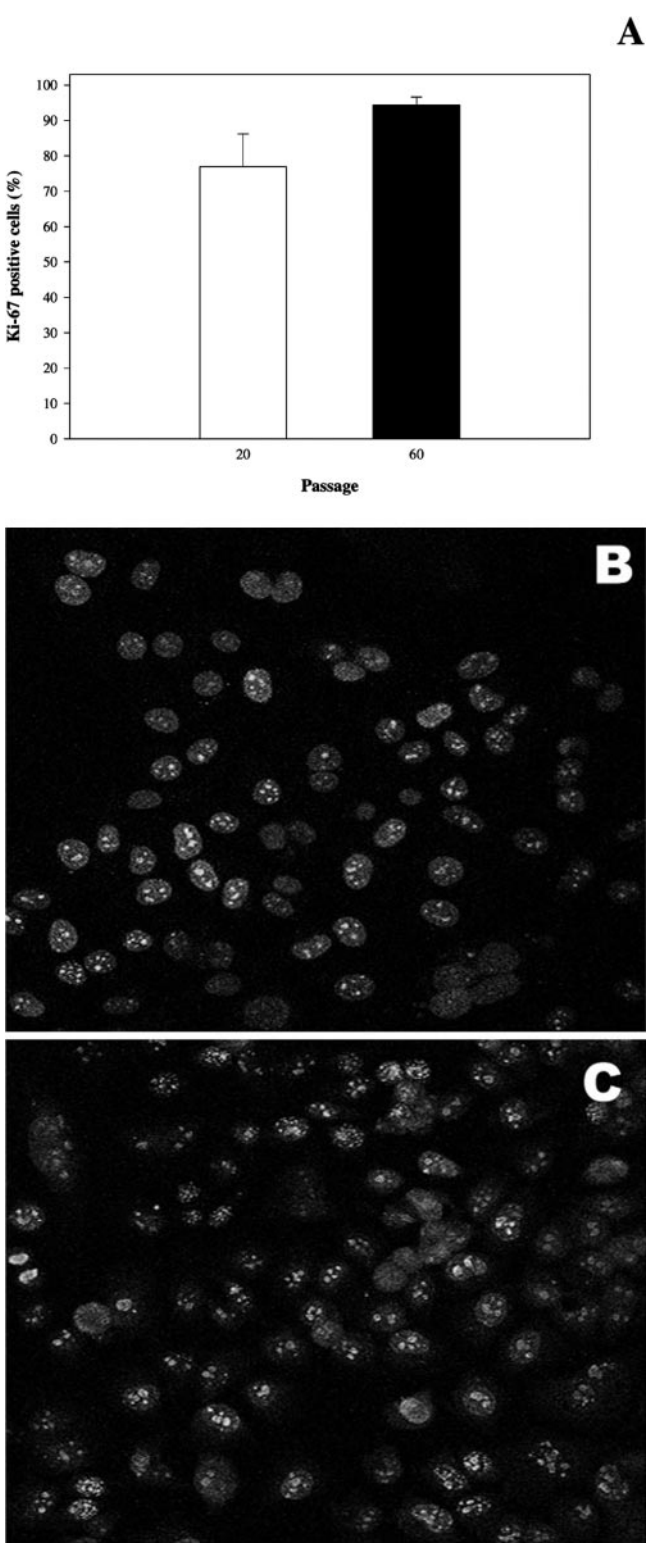

FIGURE 4. Histogram (**A**) showing number of K-67–positive cells, and black-and-white converted confocal microscopic digital images of immunofluorescence experiments with anti-Ki-67 antibody in IOBA-NHC cells in passages 20 (**B**) and 60 (**C**).

Oligonucleotide primers to the non–tandem-repeat region were designed from published[24,26] data or GenBank sequences (http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD; Table 3). Total RNAs from mammary gland (for MUC1) and trachea (for MUC2, -4, -5AC, and -7 and β2-microglobulin) tissues were used as positive controls, and water was used as a negative control for the amplification. PCR on RNA that was not reverse transcribed served as a negative control for the reverse transcription to confirm the absence of amplification from genomic DNA in RNA preparations. After amplification, the PCR mixture (10 μL) was electrophoresed on 1.4% agarose gel and stained with ethidium bromide, and the results were photographed (GelCam; Polaroid, Cambridge, MA). Results are representative of at least three independent experiments.

Inflammatory Response. Conjunctival epithelial cells have been shown to play a relevant role in inflammatory processes at the

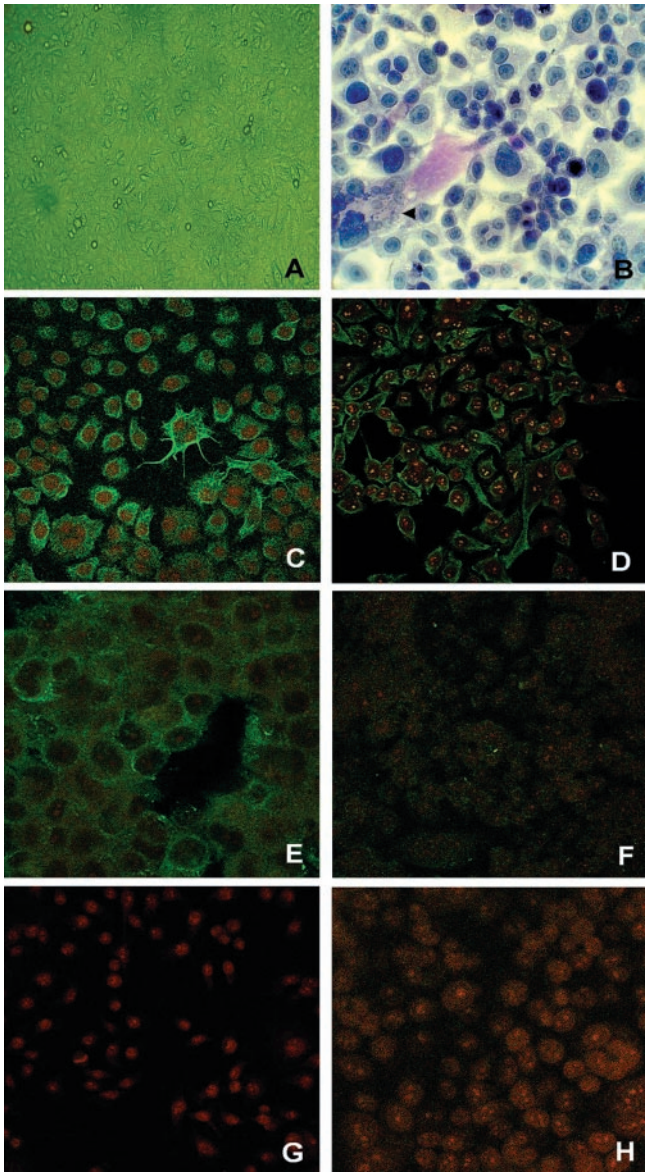

FIGURE 5. Characterization of IOBA-NHC cells in later passages. (**A**) Phase-contrast photomicrograph showing the maintenance of typical epithelial-like appearance. (**B**) PAS staining shows PAS^+ cells and the presence of some giant, multinuclear cells (*arrowhead*). Representative immunofluorescence photomicrographs show positive reaction against (**C**) anti-CK3, (**D**) anti-CK-7, and (**E**) anti-desmoplakin-1 and -2 antibodies, and negative reaction against (**F**) Langerhans' cells, (**G**) endothelial cells, and (**H**) fibroblast markers. Magnification: (**A**) ×20; (**B**, **C**, **D**, **F**, **G**) ×40; (**E**, **H**) ×63.

ocular surface.[3,29,30] The response of IOBA-NHC cells on stimulation with several proinflammatory mediators was studied.

Flow Cytometry. IOBA-NHC cells were stimulated with human recombinant IFN-γ (100, 500, and 1000 U/mL; R&D Systems, Minneapolis, MN) or human recombinant TNF-α (1, 10, or 100 ng/mL) (R&D Systems) for 24, 48, or 72 hours. Untreated or stimulated cells were harvested at the indicated time points, resuspended in flow cytometry buffer (1% BSA, 0.02% azide, PBS, ice cold), and stained with phycoerythrin (PE)-mouse anti-human CD54 (ICAM-1; HA58) monoclonal antibody, PE-conjugated mouse IgG_1 κ isotype control (MOPC-21), PE-conjugated mouse anti-human HLA-DR monoclonal antibody (G46-6), or PE-conjugated mouse IgG_{2a} κ isotype control monoclonal antibody (G155-178), according to the manufacturer's instructions and incubated at 4°C for 30 minutes. Surface staining was measured using the software that accompanied the flow cytometry system (FACSCalibur Flow cytometer and Cell Quest software; BD Biosciences, Mountain View, CA). Results are representative of two independent experiments.

Statistical Analysis

Data from the cell viability, plating-efficiency, CFE, and colony-size experiments were analyzed with a biostatistical program (Sigma-Aldrich). Student's *t*-test was used for comparisons. Differences were considered statistically significant when $P < 0.05$. All results are expressed as the mean ± SE.

Results

Primary cultures showed a mixed-shape population of polygonal and more elongated cells growing slowly but uniformly in colonies. By day 20 after plating, cells started to grow faster, forming a cobblestone-like cell monolayer. Confluence was reached by the sixth week in culture, and cells were successfully subcultured at an initial split ratio of 1 to 3.

To evaluate their epithelial nature, we characterized IOBA-NHC cells at an early stage (passage 3). Cells had a polygonal morphology (Fig. 1A) with nuclei showing several intensely stained nucleoli (Fig. 1B). Abundant mitotic figures were observed. No giant, multinuclear cells were present. Sparse PAS^+ cells were observed (Fig. 1C). TEM of transverse sections from cultured cells showed bundles of filaments (presumably CKs), desmosomes, and microvilli (Fig 1D). The presence of CKs was further confirmed by immunocytochemistry (see Table 1). CK-5, -7, and -8 (AE-3+) (Fig. 1E) were immunodetected, whereas the remainder of the keratins analyzed were not. Figures 1G and 1H show the negative reaction against AE-2 and -5 antibodies. Vimentin filaments were also detected (Fig. 1F), as described in most cells in culture. Sections of conjunctival epithelium from a control biopsy showed immunoreactive cells for AE-1, AE-3, and AE-8, but no for vimentin, as expected (data not shown).

Karyotype Analysis and G-Banding

Chromosome analysis confirmed the human origin of the IOBA-NHC cell line. Figure 2 shows one of the representative G-banded karyotypes of these cells. Heteroploidy was observed, as described in many other cell lines, as well as some structural abnormalities (Fig. 2) and a near triploid modal chromosome number (65 ± 4). The Y chromosome was not detected, and many marker chromosomes were observed.

In Vitro Proliferation

Plating efficiency was 92.1 ± 2.04 (mean ± SE of three different experiments). Colonies were homogeneously present over the dish culture surface. Small colonies (4–6 cells/colony) were observed the first day. Colony size moderately increased up to 20 to 25 cells per colony by day 5 in culture (Fig. 3A). The percentage of CFE varied from 0.17% ± 0.05% on the first day to 8.58% ± 1.28% on the fifth day (Fig. 3B).

Nearly 80% (77.0% ± 9.2%) of cells in passage 20 were positively stained for Ki-67 protein (Fig. 4A). An increase in the percentage of positive cells (94.3% ± 2.3%) was observed in passage 60, but this increase was not statistically significant. As described,[14] several different mitotic phase-related Ki-67 staining patterns were observed in IOBA-NHC cells: diffuse staining in the entire nucleus, intense staining in the nucleoli, and localized staining in the periphery of chromosomes (Figs. 4B, 4C).

Morphologic Characterization

Cells were characterized in later passages (15–20, 40, 60, and 100). The epithelial-cell–like appearance was maintained in every passage (Fig. 5A). H/E and Giemsa staining always showed the typical polygonal morphology, the presence of nuclei with intensely stained nucleoli, and abundant mitotic figures. Giant, multinuclear cells were observed from the 15th passage onward (Fig. 5B). Abundant PAS-stained but not AB-stained cells were observed (Fig. 5B). This indicates neutral but not acid mucin-like glycoprotein content in the IOBA-NHC cells.

Specific epithelial markers were also evaluated. The positivity against AE3 antibody that was present in early-passage cells was lost from the 15th passage on. CK-3 and -7 and desmoplakin-1 and -2 were immunodetected (Figs. 5C–E). Fluorescence was homogeneously distributed. However, other conjunctival markers such as CK-19 or EGFR were not detected. Negative controls without primary antibodies showed no fluorescent reaction.

Contamination with any cell type usually present in conjunctival tissue (Langerhans' cells, endothelial cells, or fibroblasts) was excluded in every tested passage, as immunofluorescence experiments with anti-CD1, anti-vWF, and anti-fibroblast antigen Ab-1 monoclonal antibodies (see Table 1) showed no fluorescence (Figs. 5F–H).

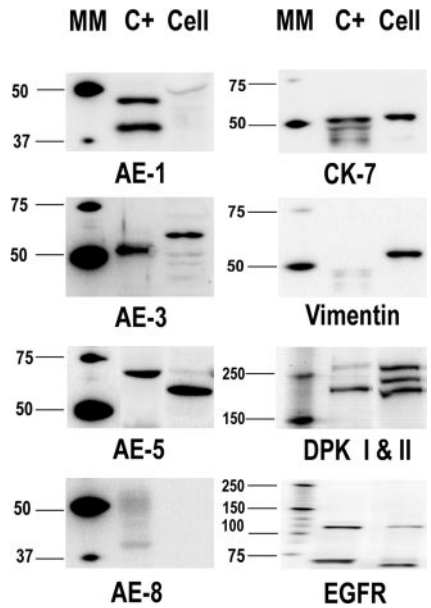

Figure 6. Western blot analysis of cytokeratins and vimentin in urea-extracted fraction and desmoplakin-1 and -2 and EGFR in soluble fraction from IOBA-NHC cell lysates (Cell) and control human or rat conjunctival homogenates (C+). MM, molecular size markers in kilodaltons.

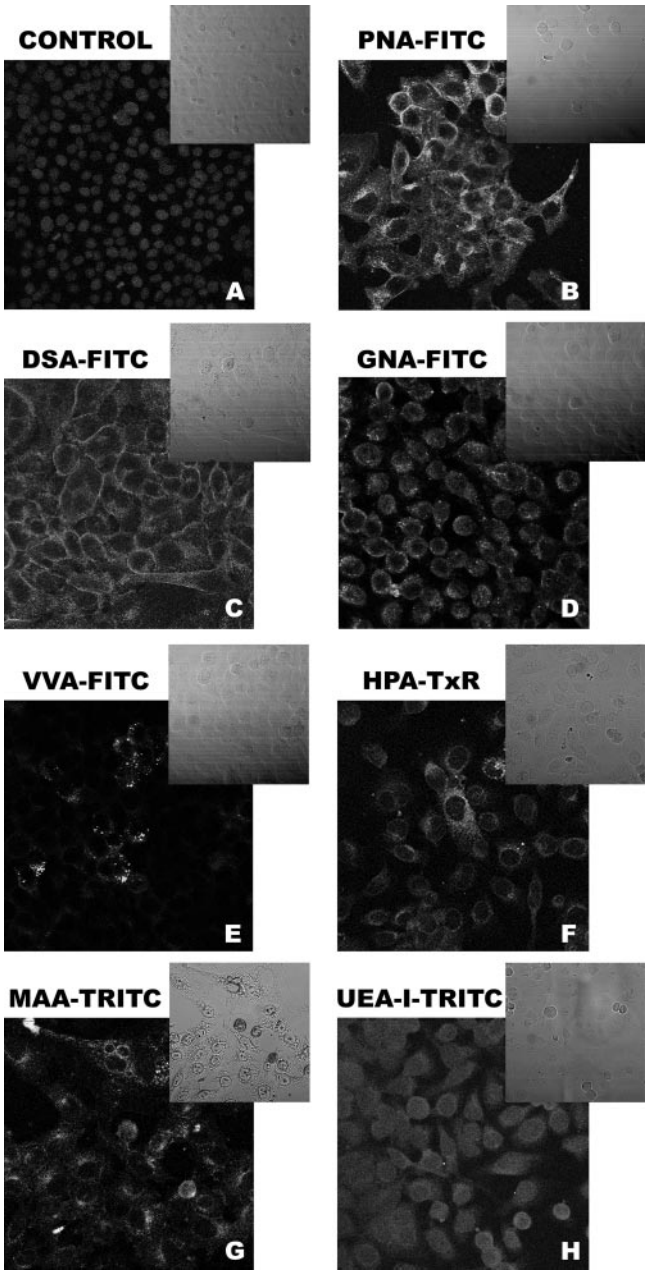

FIGURE 7. Black-and-white converted confocal microscopic digital photomicrographs showing the presence of LBSs in IOBA-NHC cells with different distribution patterns. (**A**) Negative control, (**B**) fluorescein isothiocyanate (FITC)-conjugated *Arachis hypogaea* lectin (PNA), (**C**) FITC-conjugated *Datura stramonium* lectin (DSA), (**D**) FITC-conjugated *Galanthus nivalis* lectin (GNA), (**E**) FITC-conjugated *Vicia villosa* lectin (VVA), (**F**) Texas red (TxR)-conjugated *Helix pomatia* lectin (HPA), (**G**) Tetramethylrhodamine isothiocyanate (TRITC)-conjugated *Maackia amurensis* lectin (MAA), (**H**) TRITC-conjugated *Ulex europaeus* lectin (UEA-I). *Insets*: transmitted-light control images. Magnification: ×63.

The presence of intermediate filaments (cytokeratins and vimentin) was confirmed by Western blot analysis. The urea-extracted cytokeratin fraction (insoluble) revealed the presence of several cytokeratins. Immunoreactive bands were detected using AE-1, -3, and -5 and CK-7, but not AE-8, anti-cytokeratin antibodies in IOBA-NHC cell lysates (Fig. 6). A single 50-kDa band (revealed by the AE-1 antibody) appeared in cell lysates, indicating the presence of CK-14/15.[31] A major 59-kDa band (revealed by AE-3 antibody) appeared, corresponding to CK-4 and or -5. Three weakly stained 69-, and close to 53-kDa bands could correspond to CK-1 and K-7 or -8.

A major band of ~60-kDa was detected with the AE-5 antibody that could correspond to CK-3. The other band may correspond to a proteolytic breakdown product of the polypeptide recognized by AE-5, as previously reported.[32] Also, a single 55kDa-band was revealed with the anti-CK7 antibody. No bands were detected in the urea-extracted fraction using the AE-8 antibody. Immunoblot analysis with the anti-vimentin antibody revealed a single 55-kDa band in cell lysates but not in conjunctival homogenates, as expected.

Immunoreactive bands for desmoplakin-1 and -2 and EGFR were detected in the soluble fraction of cell lysates and conjunctival homogenates (Fig. 6). Three bands (250-, 241-, and 215-kDa) were revealed using the anti-desmoplakin-1 and -2 antibody, whereas only the 250- and 215-kDa bands appeared in conjunctival homogenates. The 250-kDa band corresponds to desmoplakin-1, the 215-kDa band corresponds to desmoplakin-2, and the 241-kDa band may correspond to a phosphorylated form of desmoplakin-2, as reported elsewhere.[33] Also, two 110- and 64-kDa bands were revealed in both cell lysates and conjunctival homogenates by the EGFR antibody. These truncated isoforms of the EGFR have been reported recently in human tissue.[34] Because EGFR was detected by Western blot analysis but not by immunohistochemistry, its presence in IOBA-NHC cells was further confirmed by flow cytometry analysis (data not shown).

Functional Characterization

Several lectins (Table 2) were assayed in cells from the different passages to identify the secretory cells further. Lectin-binding sites were detected for all lectin tested, with the exception of LFA and UEA-I (Fig. 7). Thus, we identified the carbohydrate residues GalNAc, GluNAc, and mannose and sialic acid residues in IOBA-NHC cells, previously reported in conjunctival epithelial cells.[35–37]

Mucin Gene Expression in IOBA-NHC Cells

Cell extracts from several passages (20, 40, and 60) were subjected to RT-PCR analysis to study the expression of conjunctiva-related mucin genes (MUC1, -2, -4 -5AC, and -7).

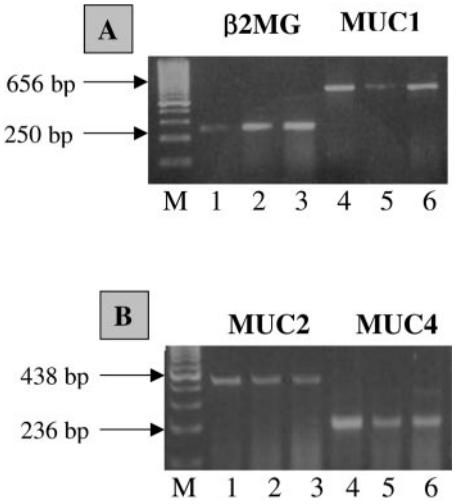

FIGURE 8. Ethidium bromide–stained 1.4% agarose gels showing amplified specific products for (**A**) β2-microglobulin (β2MG) (*lanes 1–3*) and MUC1 genes (*lanes 4–6*); (**B**) MUC2 (*lanes 1–3*) and MUC4 (*lanes 4–6*) genes in IOBA-NHC cells in 20 (*lanes 1–4*), 40 (*lanes 2–5*), and 60 (*lanes 3–6*) passages. *Lane M*: base pair ladder.

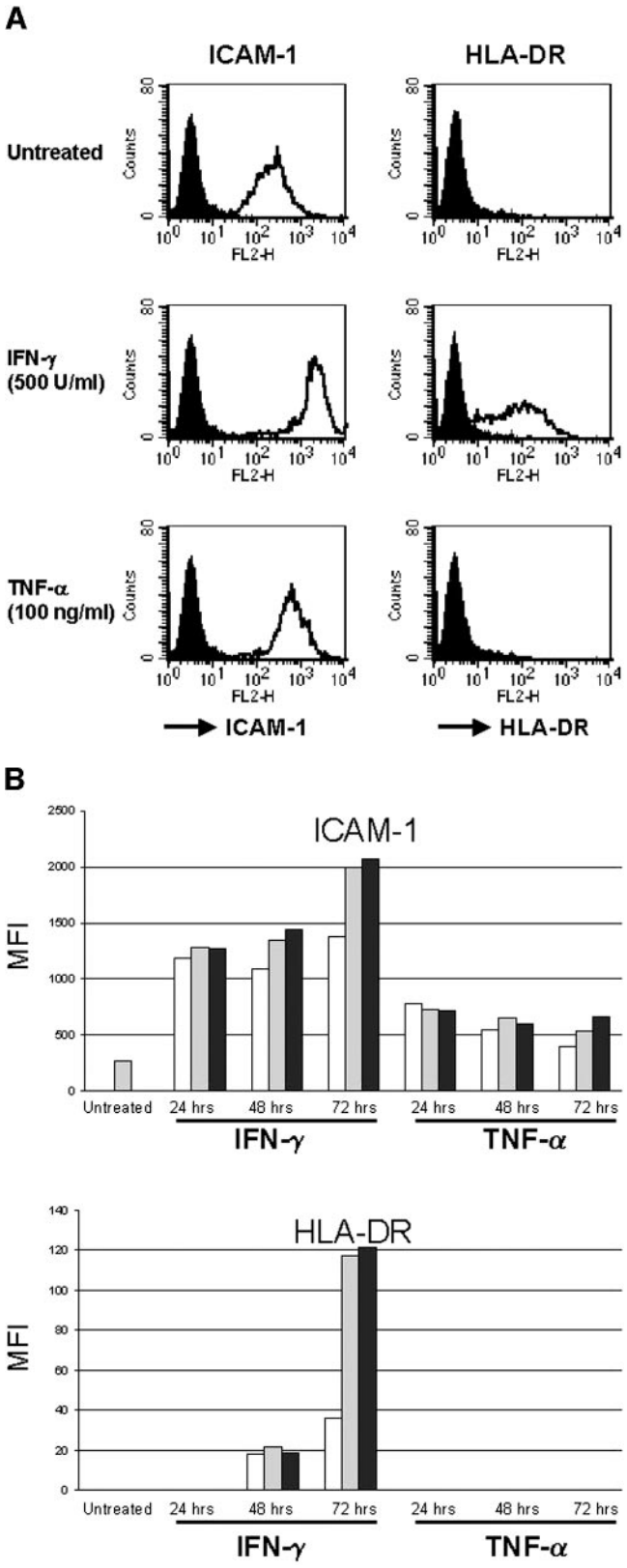

FIGURE 9. Representative flow cytometry histograms depicting ICAM-1 and HLA-DR expression on IOBA-NHC cells in the unstimulated state or after 72 hours of stimulation with human recombinant IFN-γ (500 U/mL) or TNF-α (100 ng/mL). (**A**) *Top:* Unstimulated IOBA-NHC cells constitutively expressed ICAM-1 (*open trace*) but not HLA-DR, compared with the appropriate isotype control antibody (*filled trace*). *Middle:* Stimulation of IOBA-NHC cells with IFN-γ (500 U/mL) for 72 hours resulted in a significant increase in expression of both ICAM-1 and HLA-DR. *Bottom*: TNF-α increased ICAM-1 expression compared with the untreated ICAM-1 levels, but not to the extent detected with IFN-γ treatment. TNF-α did not induce detectable levels of HLA-DR. (**B**) Mean fluorescence intensities for expression of ICAM-1 and HLA-DR, as determined by flow cytometry in the untreated or cytokine-treated IOBA-NHC cells at 24, 48, and 72 hours. (□) 100 U/mL IFN-γ or 1 ng/mL TNF-α; (▨) 500 U/mL IFN-γ or 10 ng/mL TNF-α; (■) 1000 U/mL IFN-γ or 100 ng/mL TNF-α. Results are representative of two independent experiments.

MUC-1, -2, and -4 amplified gene products of the predicted size were detected (Fig. 8) in cells from passages 20 (lanes 1 and 4), 40 (lanes 2 and 5), and 60 (lanes 3 and 6). MUC5AC and -7 gene products were not detected (data not shown).

Inflammatory Cytokine Regulation of ICAM-1 and HLA-DR Expression

IOBA-NHC cells in the untreated state constitutively expressed ICAM-1 (Fig. 9). ICAM-1 constitutive expression was upregulated by human recombinant IFN-γ at all concentrations tested (100, 500, or 1000 U/mL) after 24 hours of stimulation (Fig. 9B). ICAM-1 levels further increased after 72 hours of IFN-γ stimulation (Fig. 9). HLA-DR was not detectable on unstimulated IOBA-NHC. IFN-γ at 500 and 1000 U/mL induced low but detectable HLA-DR levels at 48 hours and significant HLA-DR expression after 72 hours of stimulation (Fig. 9).

Treatment of IOBA-NHC cells with human recombinant TNF-α (1, 10, and 100 ng/mL) induced an increase in ICAM-1 expression compared with the unstimulated ICAM-1 levels at the 24-hour time point, but to a lesser degree than the ICAM-1 increase detected in the presence of IFN-γ (Fig. 9). TNF-α did not induce HLA-DR expression at any of the concentrations or time points tested (Fig. 9).

DISCUSSION

We have described the characterization of an untransfected, spontaneously immortalized, continuous cell line derived from normal human conjunctiva epithelium, the IOBA-NHC cell line.

There has been increasing interest in developing conjunctival systems in vitro during the past two decades. Primary cultures[38–40] and epithelial cells of the first or second passage[41,42] from human conjunctiva have been used for different purposes. Primary cultured epithelial cells exposed to *Chlamydia trachomatis* were used to determine the effect of interferon in a situation that resembled an infection of the ocular surface.[38] Nicolaissen et al.[38] examined the process of cornea re-epithelization in vitro by conjunctival epithelium. Also, conjunctival equivalents were prepared by culturing epithelial cells from conjunctiva on three-dimensional collagen gels containing fibroblasts to study epithelial growth and differentiation.[40] Moreover, our group characterized primary cultured cells from human conjunctiva[41] to use them as an in vitro system to study the conjunctival physiopathology.[43,44] Several other investigators have analyzed the expression of inflammation-related mediators and receptors in early cell passages.[41,42]

A few continuous cell lines derived from human conjunctival epithelium have been reported. The most frequently used is the Wong-Kilbourne derivative of Chang conjunctival cells.[5] However, these cells have HeLa marker chromosomes and the variant A of the enzyme glucose-6-phosphate dehydrogenase,[6] which makes them unsuitable as a conjunctiva epithelium system in vitro. Nevertheless, this cell line is still being used to study the expression of inflammation-related markers or apoptosis.[45–47] Also, two epithelial cell lines (HCO597 and

HC7.08) prepared by transfection of primary cultured human conjunctival epithelial cells with the plasmid RSV-T have been established and used (Ward SL, et al. *IOVS* 1998;39:ARVO Abstract 397; Smit EE, et al. *IOVS* 2001;42:ARVO Abstract 4938). Finally, an immortalized human conjunctival cell line expressing the catalytic subunit of telomerase has been reported.[7] However, to the best of our knowledge, IOBA-NHC cells are the only nontransfected, continuous cell line derived from human conjunctival epithelium. These cells have spontaneously acquired an infinite lifespan and can be considered immortalized.[16]

The cell line characterized in our laboratory demonstrated standard in vitro behavior with the assays used (plating efficiency, CFE, and colony size). These clonogenic assays are reliable methods for analysis of cell proliferation and survival, and they are particularly useful in testing drug sensitivity[48] or the suitability of culture medium components to the growing cell requirements.[14] As expected, IOBA-NHC cells showed a high proliferating ability, as determined by the quantification of Ki-67–positive cells (Fig. 3). Changes in Ki-67 nuclear protein during the cell cycle follow predictable patterns[15] that were observed in cycling IOBA-NHC cells (Fig. 3) as well.

It is not surprising that the chromosome analysis of the IOBA-NHC cell line showed an altered karyotype with marker chromosomes (Fig. 2). Both variations in chromosome numbers and the presence of chromosomal aberrations are often found in cell lines. The capacity of genetic variation is partially responsible for the establishment of a continuous cell line.[49]

The IOBA-NHC cell line showed no contamination, as no other cell type but epithelial was found. Typical epithelial markers were identified in these cells. Ultrastructural details such as desmosomes, the specialized adherens junctions between epithelial-type cells, microvilli on the cell surface, and intermediate filaments in cytosol were observed by TEM and SEM. The desmosomal proteins desmoplakin-1 and -2 are among the major components of desmosomal plaque and participate in the linking of desmosomes to cytoplasmic intermediate filaments.[50] As expected, they were detected in the IOBA-NHC cells by immunofluorescence and Western blot. Although EGFR was not detected by immunofluorescence, Western blot analysis revealed the presence of two 110- and 64-kDa peptidic fragments that may correspond to the 170-kDa EGFR,[51] and flow analysis confirmed the presence of this receptor.

Conjunctival epithelium-related CK-3 and -7, but not CK-19, were detected in passaged IOBA-NHC cells (Figs. 5C, 5D, 6). Paired expression of CK-3 and CK-12 was typically associated with a corneal-type differentiation pattern.[52] However, CK3 is expressed in both corneal and conjunctival epithelial cells, whereas CK-12 is differentially expressed in the corneal epithelium.[53] Also, CK7, a marker for glandular epithelia,[31] was present. This CK was reported to be associated with human conjunctival goblet cell function.[20] Although CK19 is specifically present in conjunctival epithelial cells,[54] this CK was not detected in either young or passaged IOBA-NHC cells. Simple or stratified, nonkeratinized epithelium-related CKs were not detected. As most cell lines lose part of the fully differentiated properties of the living tissue from which they are derived,[55] this may explain the findings in the IOBA-NHC cell line. Similarly, vimentin expression is found in IOBA-NHC cells (Fig. 1G). This mesenchymal cell–related intermediate filament is found in most cells in culture, and keratin–vimentin coexpression in cultured epithelial cells has been considered to indicate proliferating activity.[56]

In the different passages tested, PAS^+ cells were observed (Figs. 1C, 5B), LBSs were present (Fig. 7), and several mucin genes were expressed (Fig. 8). These facts indicate that at least some IOBA-NHC cells produce glycoproteins. Glycoproteins present in the plasma membrane of conjunctival epithelial cells have several roles: to constitute a coating for those cells, to participate in cell-to-cell communication, and to interact with the mucous layer of the tear film to help stabilize it. Mucins, the main constituents of the mucous layer of the tear film, are glycosylated proteins with a high molecular weight. They possess a specific glycosylation pattern that distinguishes mucins from other glycoproteins present in the cell surface. Lectins have helped to identify certain carbohydrates in the conjunctiva and the mucous layer covering its surface.[35,36] In addition, an in vitro study on lectin binding to primary cultured cells from human conjunctival epithelium was reported by our group.[57] GalNAc, GluNAc, mannose, and sialic acid residues were identified in IOBA-NHC cells. This LBS pattern closely resembles that previously reported for conjunctiva epithelial cells, with the exception of the presence of mannose.

Conjunctival mucins are produced by goblet and non-goblet epithelial cells.[1,2] In principle, the MUC1, -2, and -4 mucin genes are those expressed in non-goblet epithelial cells from conjunctiva, although recent studies have also reported some expression of MUC2[58] and -4[59] genes in goblet cells. IOBA-NHC cells express three mucin genes, MUC1, -2, and -4, that would make this cell line useful in testing in vitro mucus secretagogues.

The IOBA-NHC cell line has also been demonstrated to respond to proinflammatory stimuli. These cells upregulate expression of ICAM-1 after cell exposure to the proinflammatory cytokines IFN-γ and TNF-α. IFN-γ induced expression of HLA-DR after 48 hours. ICAM-1 and HLA-DR were chosen because of their reported overexpression in some inflammatory diseases of the ocular surface.[4,29,30] The fact that IOBA-NHC cells showed a positive response in an inflammatory milieu further supports the resemblance of this cell line to the in vivo conjunctival epithelium.

We conclude that the continuous, spontaneously immortalized IOBA-NHC cell line retains morphologic and functional conjunctival epithelial characteristics in vitro. This cell line may be a useful experimental tool in the study of in vitro aspects of conjunctival cells and in investigations of new therapies for ocular surface diseases.

Acknowledgments

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