Effect of Retinoic Acid on Gene Expression in Human Conjunctival Epithelium: Secretory Phospholipase A_2 Mediates Retinoic Acid Induction of MUC16

Yuichi Hori, Sandra J. Spurr-Michaud, Cindy Leigh Russo, Pablo Argüeso, and Ilene K. Gipson

Purpose. How vitamin A contributes to the maintenance of the wet-surfaced phenotype at the ocular surface is not well understood. This study sought to identify vitamin A–responsive genes in ocular surface epithelia using gene microarray analysis of cultures of a human conjunctival epithelial (HCjE) cell line grown with all-trans-retinoic acid (RA). The analysis showed that secretory phospholipase A_2 group IIA ($sPLA_2$-IIA) was the gene most upregulated by RA, followed by the membrane-associated mucin MUC16 at a later time point. Since eicosanoids, the product of arachidonic acid generated by the PLA_2 family, have been shown to increase mucin production, this study sought to determine whether $sPLA_2$ mediates the RA induction of MUC16.

Methods. HCjE cells were cultured with or without RA for 3, 6, 24, and 48 hours. Complementary RNA prepared from RNA of the HCjE cells was hybridized to human gene chips and analyzed using commercial software. Microarray data on mucin expression were validated by real-time PCR. To investigate whether $sPLA_2$ is associated with RA-induced MUC16 upregulation, HCjE cells were incubated with RA and the broad-spectrum PLA_2 inhibitor aristolochic acid (ArA) or the specific $sPLA_2$-IIA inhibitor LY315920, followed by analysis of MUC16 mRNA and protein by real-time PCR and Western blot analysis.

Results. After RA addition, 28 transcripts were upregulated and 6 downregulated by more than twofold ($P < 0.01$) at both 3 and 6 hours (early phase). Eighty gene transcripts were upregulated and 45 downregulated at both 24 and 48 hours (late phase). Group IIA $sPLA_2$, significantly upregulated by 24 hours, and MUC16 were the most upregulated RNAs by RA at 48 hours. $sPLA_2$ upregulation by RA was confirmed by Western blot analysis. When HCjE cells were incubated with RA plus ArA or specific inhibitor of $sPLA_2$-IIA, LY315920, the RA-induced MUC16 mRNA was significantly reduced ($P < 0.01$).

Conclusions. The RA-associated upregulation of membrane-associated mucin MUC16 at late phase appears to be through $sPLA_2$-IIA. Upregulation of this hydrophilic membrane-associated mucin may be one of the important mechanisms by which vitamin A facilitates maintenance of the wet-surfaced phenotype on the ocular surface. (*Invest Ophthalmol Vis Sci.* 2005; 46:4050–4061) DOI:10.1167/iovs.05-0627

Vitamin A and its derivatives, retinoids, are required for normal growth and development of vertebrates. They are absorbed in the small intestine, stored in the liver, and oxidized to all-trans-retinoic acid (RA) in many target cells.[1,2] According to a survey by Balmer and Blomhoff,[3] >500 genes have been reported as regulatory targets of RA. The regulatory effect of RA can be direct or indirect.[3,4] Direct regulation of RA is driven by a heterodimer of a retinoic acid receptor (RAR) and a retinoid X receptor bound to an RA response element on the promoter region of the gene.[5,6] Indirect regulation reflects the actions of intermediate transcription factors, such as the POU transcription factor, Brn-2, through which corticotrophin-releasing hormone is upregulated.[7]

It is well known that ocular surface epithelia have an absolute requirement for vitamin A to maintain their wet-surfaced phenotype, and topical vitamin A has been reported to be effective as a treatment for severe squamous metaplasia.[8–10] Vitamin A deficiency leads to abnormal differentiation of the ocular surface epithelia, resulting in keratinization of both conjunctival and corneal epithelia.[11] In a rabbit model, vitamin A deficiency caused conjunctival goblet cell loss and increased epithelial cell stratification in addition to reduced paracellular permeability of the ocular surface.[12] Tei et al.[13] demonstrated that vitamin A deficiency in rats results in a decreased expression of mRNA for the membrane-associated mucin rMuc4 and the goblet cell mucin rMuc5AC in conjunctival epithelium, and hypothesized that lack of these hydrophilic mucins contributed to dryness and keratinization of the ocular surface.

Mucins are high-molecular-weight and highly O-glycosylated glycoproteins present at the interface between wet-surfaced epithelia and their extracellular environments. At the ocular surface, mucins are believed to attract and hold water due to their hydrophilic character, thus preventing desiccation of the epithelial surface.[14] Mucins have been classified as either membrane-associated, including the mucins MUC1, -3A, -3B, -4, -11, -13, -15, -16, -17, and -20,[15–21] or secreted. The latter include the gel-forming mucins secreted by goblet cells of various epithelia, MUC2, -5AC, -5B, -6, and -19,[15,22] and the small soluble mucins, MUC7 and -9.[15,23] Ocular surface epithelia produce and place at the epithelia–tear film interface at least three membrane-associated mucins, MUC1, -4, and -16. Goblet cells of the conjunctiva produce and secrete MUC5AC, and a small soluble mucin, MUC7, is expressed in the lacrimal gland.[24–27]

Little is known regarding regulation of mucin gene expression by ocular surface epithelia. Dexamethasome has been reported to upregulate MUC1, and serum is a potent upregulator of MUC4 and -16.[28] Several studies provide evidence suggesting that eicosanoid metabolites, products of arachidonic acid generated by the PLA_2 family, can stimulate mucin production in airway epithelia[29,30] and in ocular surface tis-

From the Schepens Eye Research Institute and the Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts.

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Corresponding author: Ilene K Gipson, Schepens Eye Research Institute, Harvard Medical School, 20 Staniford St., Boston, MA 02114; gipson@vision.eri.harvard.edu.

sue.[31–33] The specific PLA_2 involved in this regulation is unknown.

Only a few studies have examined specific effects of RA in terms of gene expression in ocular surface tissues. Bossenbroek et al.[34] demonstrated that RA induces the β-subunit of the RAR-β mRNA in rabbit corneal and conjunctival fibroblasts, and Hori et al.[35] reported that RA upregulates the membrane-associated mucins MUC4 and -16 at both the mRNA and protein level in a telomerase-immortalized human conjunctival epithelial (HCjE) cell line. Undoubtedly the action of RA on ocular surface epithelia is complex, involving many genes that maintain the wet-surfaced phenotype. The molecular mechanisms or group of genes through which vitamin A acts to maintain a wet-surfaced phenotype on the ocular surface are, however, unknown.

DNA microarray analyses offer a powerful method to identify potential sets of genes induced by RA, by simultaneously screening expression changes in thousands of transcripts in a single experiment.[36,37] This technology has the potential to elucidate biological processes that depend on the interaction of multiple genes and cellular pathways. Recently, several reports analyzed gene expression in corneal tissues using the microarray technique.[38–41] These studies compared gene expression patterns in corneal fibroblasts after treatment with interleukin 1,[38] in rodent corneas healing after wounding,[39,41] and in human donor corneas.[40] To our knowledge, there has been no microarray analysis of the effect of RA on human ocular surface epithelial cells.

We recently reported that HCjE cells express two subtypes of RAR mRNA, RAR-α and RAR-γ, and that RA induced MUC4 and -16 in the cell line.[35] The purpose of this study was to determine, using microarray technology, which genes in the HCjE conjunctival epithelial cells were regulated by RA over time. On learning that RA upregulates secretory phospholipase A_2 group IIA ($sPLA_2$-IIA) and, at a later time point, the membrane-associated mucin MUC16, and in light of previous data indicating that eicosanoids stimulate mucin production,[29,30] we sought to determine whether the MUC16 induction was mediated by $sPLA_2$-IIA.

METHODS

Cell Culture

The telomerase-immortalized HCjE cell line was used in this study. The derivation and character of the cell line was previously reported.[28,42] Culture of HCjE cells with all-trans-RA was based on our previous report.[35] Briefly, HCjE cells were cultured in medium (Gibco Keratinocyte-Serum Free Medium [K-sfm]; Gibco-Invitrogen Corp., Rockville, MD) at 37°C in a 5% carbon dioxide atmosphere, followed by culture in a 1:1 mixture of K-sfm and low-calcium DMEM/F12 (Gibco-Invitrogen) to confluence. At confluence, the cells were cultured in DMEM/F12 for 24 hours (baseline control), and then changed to DMEM/F12 with 100 nM RA dissolved in DMSO (Sigma-Aldrich, St. Louis, MO) for 3, 6, 24, or 48 hours. Experiments were performed in duplicate for each time point. Cell cultures were examined by phase-contrast microscopy (Nikon TS100; Nikon, Melville, NY).

Isolation of RNA

After culture with RA, total RNA was isolated from the cells using a commercial reagant (TRIzol; Invitrogen, Rockville, MD), following the manufacturer's protocol. Further purification of total RNA was done using a commercial kit (RNeasy Mini Kit; Qiagen, Valencia, CA). The 260:280-nm absorbance ratio of RNA samples used in this experiment ranged consistently from 1.8 to 2.1. The integrity and concentration of total RNA were measured using a bioanalysis unit (Agilent 2100 Bioanalyser; Agilent Technologies, Palo Alto, CA).

Microarray

The microarray experiments were performed at the Bauer Center for Genomics Research of Harvard University (Cambridge, MA). Five μg total RNA was converted to double-stranded cDNA with T7-$(dT)_{24}$ oligomer primers (Superscript Choice System; Invitrogen). The cDNA was purified with phase-lock gels (Eppendorf's Phase Lock Gels; Brinkmann, Westbury, NY), followed by extraction with phenol-chloroform, and then ethanol precipitation. Biotin-labeled complementary RNA (cRNA) was produced by in vitro transcription using a commercial kit (Bioarray High Yield RNA Transcription Labeling Kit; Enzo Diagnostics, Inc., Farmingdale, NY). The biotinylated cRNA was purified with a kit column (RNeasy Mini Kit; Qiagen) and fragmented in 40 mM Tris acetate, pH 8.1; 100 mM KOAc; and 30 mM MgOAc (approximately 35 to 200 bases). After confirmation of the quality of the cRNA by hybridizing an aliquot to the test array (Affymetrix Test3 Array; Affymetrix, Inc., Santa Clara, CA), 10 μg biotinylated cRNA was hybridized for 16 hours at 45°C to a human microarray chip (Affymetrix GeneChip, HG-U133A; Affymetrix; details on the probe design and sequence information for each gene on the chip are available on the manufacturer's website, http://www.affymetrix.com/index.affx). The chip was washed and stained with streptavidin-phycoerythrin in a fluidics unit (Affymetrix Fluidics Station 400; Affymetrix). Two microarray chips were probed for each time point with cRNA from two different experiments (control, 3, 6, 24, and 48 hours).

Microarray Data Analysis

The microarrays were scanned with the manufacturer's scanner and software suite (Affymetrix Gene Array Scanner and Affymetrix Microarray Suite 5.0 software; Affymetrix). The corresponding scanned data was deposited into an enterprise gene expression data analysis system (Rosetta Resolver; Rosetta Biosoftware, Kirkland, WA). The data analysis system uses the microarray chip's error model to create an intensity profile for each chip with data deposition after preprocessing (background correction and intrachip normalization). Array data from two individual experiments were combined for each time point (data from replicates are combined by computing group averages, taking into account certain measurement error calculations; http://www.rosettabio.com/tech/; see "Data processing and analysis methods in the Rosetta Resolver System" pdf), and the system's ratio-building tool (Rosetta Resolver Ratio Builder; Rosetta Biosoftware) was used to calculate fold changes and ratio *P*-values for the differential expression of RA treated samples versus control. Those genes with values of $P \leq 0.01$ and fold difference ≥ 2 were considered to be significantly differentially expressed.

Real-Time PCR

Real-time PCR experiments were performed to confirm data of mucin gene expression obtained by microarray analysis. Levels of MUC1, -4, and -16 mRNA were determined over the time course by real-time PCR (TaqMan chemistry with the ABI Prism 7900HT Sequence Detection System; Applied Biosystems, Foster City, CA). The same RNA samples that were used to prepare probes for microarray hybridizations were used for the PCR analysis. Total RNA (2.0 μg) from the HCjE cells was reverse transcribed using first-strand synthesis (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen), as previously described.[43,44] The primers and double-labeled fluorogenic probes (TaqMan; Applied Biosystems) used for MUC1, -4, -16, and GAPDH amplification in this study have been previously reported.[26,43,45] In addition, new primers and a probe for MUC4 (MUC4 C-term) were designed with the assistance of computer software (Primer Express; Applied Biosystems) to amplify the target sequence of the C terminus of MUC4 used in the gene chip analysis (Affymetrix Probe Set ID 217110_s_at). The sequences of MUC4 C-term primers and probe were: sense, 5′-TAGGCTACCTCAAGACTCACCTCAT-3′; antisense, 5′-TCCCTTTTCCAGTCTCCCAAA-3′; and probe, 5′-TACCGCACATTTAAGGCGCCATTGC-3′. Nucleotide database searches were performed to confirm the sequence specificity of the MUC4 sequence (BLAST; National Center for Biotech-

nology Information, Bethesda, MD; public domain download available at http://www.ncbi.nlm.nih.gov/BLAST/). Conventional RT-PCR experiments were performed to confirm that only a single band is obtained when amplifying conjunctival cDNA with the MUC4 C-term primers. To verify the identity of the MUC4 PCR product, the band in the agarose gel was excised, and the extracted DNA was sequenced (DNA Sequence Center for Vision Research of Massachusetts Eye and Ear Infirmary, Boston, MA).

For relative quantitation in real-time PCR experiments, we used the delta C_T method (Applied Biosystems) reported previously.[28,35] Samples were assayed in duplicate ($n = 4$), using thermal cycling conditions consisting of 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Controls lacking a cDNA template were run in each assay to confirm the lack of DNA contamination in reagents used for amplification.

PLA_2 Inhibitor Treatment

To investigate whether RA regulation of MUC16 is associated with $sPLA_2$, the effect of the broad-spectrum PLA_2 inhibitor aristolochic acid (ArA; Sigma-Aldrich)[46] on MUC16 mRNA levels was determined in HCjE cells cultured as above with 100 nM RA plus 100 μM ArA, the inhibitor alone, or vehicle (DMSO) alone for 24 and 48 hours. These experiments were followed up by testing the effect of an inhibitor specific for group IIA $sPLA_2$, LY315920[47] (gift of David W. Snyder and James H. Prather, Lilly Research Laboratories, Eli Lilly and Co., Greenfield, IN). HCjE cells were treated with 100 nM RA, 100 nM RA plus 10 μm LY315920, the inhibitor alone, or vehicle (DMSO) alone for 24 and 48 hours. MUC16 mRNA and protein levels were determined by real-time PCR and Western blot analysis, respectively. The experiments were performed twice for both inhibitors, each experiment being done in duplicate.

SDS-PAGE and Western Blot Analysis

Protein from cells cultured with or without RA and/or PLA_2 inhibitors was extracted with RIPA buffer (50 nM Tris, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 150 nM NaCl) plus complete protease inhibitor cocktail (Roche Biochemical; Indianapolis, IN). Culture media were also harvested and centrifuged at 3500 rpm for 4 minutes. Supernatants were collected and concentrated by centrifugal separation (Nanosep 10K Omega Centrifugal Devices; Pall Life Sciences, Ann Arbor, MI). Protein concentration was determined with a commercial assay kit (BCA Protein Assay Reagent Kit; Pierce, Rockford, IL). Five micrograms of total protein from cell lysate or 10 μg from conditioned culture medium was diluted in Laemmli sample buffer[48] and loaded in each lane. SDS-PAGE was performed under reducing conditions on 4% stacking and 7.5% (for MUC16 and GAPDH) or 15% (for $sPLA_2$) separating gels. Proteins were transferred onto nitrocellulose membranes by conventional methods.[49] Primary antibodies against MUC16 (OC125, mouse monoclonal; DAKO, Carpinteria, CA), $sPLA_2$ ($sPLA_2$, mouse monoclonal; Upstate, Lake Placid, NY), and GAPDH (rabbit polyclonal; Abcam, Cambridge, MA) were used. The conditions for immunoblotting with these antibodies have been reported previously.[35,50] Protein bands were detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate; Pierce) after exposure to film (Hyperfilm; Amersham Biosciences, Buckinghamshire, UK). Band intensities were quantified with imaging software (NIH Image, Version 1.62; National Institutes of Health, Bethesda, MD; public domain download available at http://rsb.info.nih.gov/nih-image/; and 1D Image Analysis Software, Version 2.02; Eastman Kodak, Co., Rochester, NY).

Statistical Analysis

Statistical comparisons of results obtained by real-time PCR and Western blot analysis were performed with the Fisher protected, least significant difference (PLSD) test (Statview 5.0 for Macintosh; SAS Institute, Inc., Cary, NC). Values of $P < 0.05$ were considered significant.

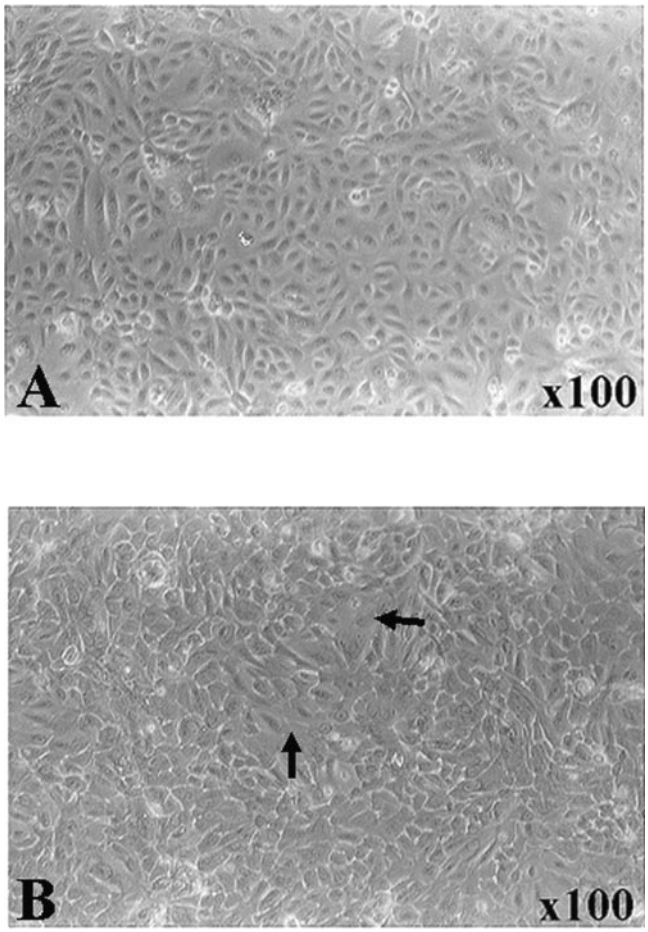

FIGURE 1. Phase-contrast micrographs of cultures of HCjE cells. (**A**) Confluent cells before the addition of RA (control). (**B**) Cells after 48 hours of RA treatment (100 nM). Note the presence of large, flattened cells (*arrows*); these are the apical cells of islands of stratification.[28]

RESULTS

Characteristics of HCjE Cells

The growth and mucin characteristics of the HCjE cell line have been described previously.[28] When grown to confluence in K-sfm, then in high-calcium media DMEM/F12 plus 10% calf serum for 7 days, the levels of MUC16 mRNA are comparable to those seen in native tissues and primary cultures.

The microscopic appearance of HCjE cells[28] grown to confluence, then cultured for 24 hours in DMEM/F12, is shown in Figure 1A. Figure 1B shows the microscopic appearance of HCjE cells cultured as in Figure 1A, then cultured for an additional 48 hours with RA. The cells cultured with RA formed islands with large, flattened apical cells (compare Figs. 1A and 1B), indicating further differentiation of the cells. The appearance of cells at 48 hours of culture with RA were similar to those shown in a previous report, which demonstrated that MUC16 was present on the apical cells of stratified islands of HCjE cells (see Fig. 7D in Ref. 36).

Microarray Data

The microarray chip used in this study (HG-U133A; Affymetrix) contains 22,383 genes. Forty-three percent of the genes represented on the chip, 9516, were detected in HCjE control samples. In response to culture with RA, 114 transcripts were

TABLE 1. Changes in Gene Expression in HCjE Cells Treated with RA for 3 and 6 Hours ($P < 0.01$)

Gene Function and Name	Accession Number	Fold Change 3 h	Fold Change 6 h
Upregulated			
Blood coagulation			
Thrombomodulin	NM000361	3.7	2.0
Cell proliferation/differentiation			
Epithelial membrane protein 1	BF445047	4.5	6.6
Sciellin	NM003843	3.3	4.6
S100 calcium binding protein A7	NM002963	3.3	3.3
S100 calcium binding protein P	NM005980	2.5	3.4
Metabolism			
Short-chain dehydrogenase/reductase 1	NM004753	10.9	22.6
Cytochrome P450, subfamily XXIV	NM000782	7.1	7.5
Serine protease inhibitor, Kazal type, 5	NM006846	2.6	2.7
Kallikrein 13	NM015596	2.5	2.8
Kallikrein 10	AK026045	2.5	2.1
Oncogenesis			
Serine proteinase inhibitor, clade B, member 3	U19556	2.5	2.1
Signal transduction/cell signaling			
Leukemia inhibitory factor	NM002309	6.1	2.5
Diphtheria toxin receptor	M60278	5.8	2.5
Putative chemokine receptor	NM006018	5.8	3.5
CD14 antigen	NM000519	5.5	6.3
Retinoic acid induced 3 (RAI3)	NM003979	4.5	8.4
FYN binding protein	AF198052	3.4	3.1
Neuroepithelial cell transforming gene 1	NM005863	2.8	2.4
A kinase (PRKA) anchor protein 12	AB003476	2.3	3.7
Upregulated (*continued*)			
Structural molecule			
Keratin 23	NM015515	3.7	5.9
Involucrin	NM005547	3.7	6.0
Keratin 4	X07695	3.1	5.9
Transcription factor			
Inhibitor of DNA binding 1	D13889	4.9	6.3
Nuclear receptor interacting protein 1	NM003489	3.4	2.1
SRY (sex determining region Y)-box 9	AI382146	2.1	2.0
Transport			
Carbonic anhydrase II	M36532	3.2	2.8
Others			
Hypothetical protein FLJ22671	NM024861	3.4	4.8
Homo sapiens EST from clone 898903	BG177920	2.1	2.0
Downregulated			
Cell cycle/cell death			
Ubiquitin specific protease 18	NM017414	−12.5	−12.5
M-phase phosphoprotein 9	NM022782	−9.9	−3.7
Protein phosphatase 1	N26005	−2.6	−3.2
Response to stimulus/immunity			
Interferon-induced protein 44	BE049439	−3.4	−3.4
Signal transduction/cell signaling			
Frizzled homolog 2	L37822	−12.7	−6.2
Transcription factor			
Promyelocytic leukemia	AF230401	−3.0	−3.0

upregulated and 84 downregulated after 3 hours; 102 were upregulated and 212 downregulated after 6 hours; 275 were upregulated and 180 downregulated after 24 hours; and 277 were upregulated and 384 downregulated after 48 hours ($P < 0.01$). (For a complete list of results on each individual gene on the microarray, see the Gene Expression Omnibus database at http://www.ncbi.nlm.nih.gov/geo; accession number GSE2835.)

For further analysis, we categorized time points into two phases, early (3 and 6 hours) and late (24 and 48 hours). In this analysis, "regulated gene" is defined as a transcript that displayed greater than twofold change, with $P < 0.01$, at both time points in each phase after RA treatment. In the early phase, we identified 28 genes that were upregulated by 100 nM RA and 6 genes that were downregulated (Table 1). Among the upregulated genes, short-chain dehydrogenase/reductase 1 (GenBank Accession No. NM004753) was the most upregulated gene (10.9-fold increase at 3 hours and 22.6-fold increase at 6 hours). Others included structural molecules keratin 23 (NM015515), keratin 4 (X07695), and involucrin (NM005547). Among the downregulated genes were the cell cycle/cell death genes ubiquitin-specific protease 18 (NM017414), M-phase phosphoprotein 9 (NM022782), and protein phosphatase 1 (N26005).

In the late phase, 80 genes were upregulated by 100 nM RA (Table 2), while 45 genes were downregulated (Table 3). Among the upregulated genes, a number associated with metabolism were identified, including group IIA PLA_2 (NM000300); matrix metalloproteinases MMP7 (NM002423), MMP3 (NM002422), and MMP2 (NM004530); and cytochrome P450 subfamily 4B1 (J02871). Structural molecules such as MUC16 (NM024690), chitinase 3–like 1 (M80927), keratin 23, and keratin 4 were also upregulated (Table 2). Among the downregulated genes, a number of cell cycle/cell death genes were identified, including cell-division-cycle-associated 3 (NM031299), G-2– and S-phase–expressed 1 (BF973178), and minichromosomal-maintenance-deficient (MCM)-2 (NM004526), MCM-5 (NM006739), MCM-7 (D55716), and MCM-10 (NM018518) (Table 3).

Nine upregulated genes overlapped both the early and late phases: sciellin (NM003843); S100 calcium-binding protein A7 (NM002963); S100 calcium-binding protein P (NM005980); serine protease inhibitor, Kazal type, 5 (NM006846); serine protease inhibitor, clade B, member 3 (U19556); A kinase (PRKA) anchor protein 12 (AB003476); keratin 23; keratin 4; and carbonic anhydrase II (J03037). No downregulated genes overlapped both early and late phases.

For each time point of culture of the HCjE cells with RA, we used two individual microarray chips. The correlation coefficients of intensities of all genes between the two chips at each time point (control, 3, 6, 24, and 48 hours) were at least 0.96 (0.972, 0.961, 0.980, 0.966, and 0.965, respectively). These coefficients indicate that the data from duplicate experiments for each time point were highly reproducible. Therefore, the data from two individual experiments for each time point were combined using analysis software (Rosetta Resolver; Rosetta Biosoftware), and the ratios for RA treatment at each time point versus control were generated.[51]

$sPLA_2$ Protein and MUC16 Expression

From the microarray data, we found that the expression of the group IIA $sPLA_2$ gene was the most upregulated gene by RA at both 24 (28.6-fold change) and 48 hours (15.4-fold change) (Table 2). The expression of the membrane-associated mucin

TABLE 2. Upregulated Gene Expression (80 Genes) in HCjE Cells Treated with RA for 24 and 48 Hours ($P < 0.01$)

Gene Function and Name	Accession Number	Fold Change 24 h	Fold Change 48 h
Cell motility			
Crystallin, α B	AF007162	5.0	5.0
Nebulette	NM006393	5.1	3.8
Cell proliferation/differentiation			
Kallikrein 7 (chymotryptic, stratum corneum)	NM005046	6.7	7.1
S100 calcium binding protein P	NM005980	9.2	6.4
p8 Protein (candidate of metastasis 1)	AF135266	2.5	5.6
Glycoprotein (transmembrane) nmb	NM002510	4.6	5.3
S100 calcium binding protein A7	NM002963	3.7	5.0
Insulin-like growth factor binding protein 6	NM002178	4.5	4.1
Sciellin	NM003843	3.5	2.2
Kruppel-like factor 4	BF514079	2.0	2.0
Metabolism			
Phospholipase A2, group IIA*	NM000300	28.6	15.3
Matrix metalloproteinase 7	NM002423	7.6	8.6
Matrix metalloproteinase 3	NM002422	4.2	8.4
WAP 4-disulfide core domain 2	NM006103	4.0	6.1
Cytochrome P450, subfamily IVB1	J02871	12.1	5.3
Myo-inositol 1-phosphate synthase A1	AL137749	2.9	4.6
Spermidine/spermine *N*1-acetyltransferase	BE971383	2.7	3.8
Serine protease inhibitor, Kazal type, 5	NM006846	3.1	3.6
Aldehyde dehydrogenase 2 family	NM000690	2.5	3.2
Hydroxysteroid (17-β) dehydrogenase 1	NM000413	2.4	3.2
Death-associated protein kinase 1	NM004938	4.0	3.0
Sialyltransferase	NM006456	2.2	3.0
Cystatin E/M	NM001323	3.4	2.8
Spermidine/spermine *N*1-acetyltransferase	M55580	2.0	2.7
Butyrobetaine, 2-oxoglutarate dioxygenase 1	NM003986	2.7	2.6
Hydroxysteroid (17-β) dehydrogenase 2	NM002153	2.3	2.6
Biliverdin reductase B	NM000713	2.4	2.6
Kynureninase (L-kynurenine hydrolase)	BC000879	3.1	2.4
Dual oxidase 1	NM017434	2.0	2.3
Cytochrome b5 reductase b5R.2	NM016229	2.5	2.3
Protein phosphatase 2, regulatory Subunit B	AA974416	4.0	2.1
Matrix metalloproteinase 2	NM004530	2.0	2.0
Oncogenesis			
Serine proteinase inhibitor, clade B, member 3	U19556	4.1	5.2
Serine proteinase inhibitor, clade B, member 4	U19557	3.9	4.9
Homeodomain-only protein	AB059408	3.3	3.9
TBC1 domain family, member 3	AL136860	2.8	2.7
Lysosomal-associated membrane protein 2	NM002294	2.2	2.2
Serine proteinase inhibitor, clade B, member 1	NM030666	2.3	2.1
Response to stimulus/Immunity			
Immune costimulatory protein B7-H4	NM024626	12.8	6.2
Guanylate binding protein 2	NM004120	3.8	3.3
Superoxide dismutase 2, mitochondrial	BF575213	2.0	3.1
Dual specificity phosphatase 1	NM004417	2.8	2.6
Superoxide dismutase 2, mitochondrial	W46388	4.4	2.6
Neutrophil cytosolic factor 2	BC001606	2.5	2.5
TP53 activated protein 1	BC002709	4.4	2.4
B-cell linker	NM013314	3.8	2.4
Signal transduction/cell signaling			
GABA A receptor, pi	NM014211	10.0	6.2
C-type lectin, superfamily member 12	AF313468	3.7	3.3
TNFRSFIA-associated via death domain	L41690	2.1	2.8
Decay accelerating factor for complement	BC001288	2.6	2.8
Phospholipase D1	U38545	2.7	2.7
B-factor, properdin	NM001710	2.1	2.6
GABA-A receptor associated protein 3	AF180519	2.9	2.4
A kinase (PRKA) anchor protein 12	AB003476	2.6	2.3
Structural molecule			
Mucin 16 (MUC16)*	NM024690	4.4	11.5
Chitinase 3-like 1	M80927	5.0	7.7
Keratin 23	NM015515	9.2	6.5
Keratin 4	X07695	7.5	6.2
Spondin 2, extracellular matrix protein	NM012445	3.6	4.3
Histone 1, H1c	BC002649	3.4	3.8
Transmembrane 7 superfamily member 1	NM003272	3.6	3.6
Matrilin 2	NM002380	2.7	2.4
Histone 2, H2be	NM003528	2.2	2.0
Transgelin	AA150165	2.1	2.0
Transcription factor			
Insulin receptor substrate 2	AF073310	4.8	2.9
Transport			
ATP-binding cassette, subfamily C	NM020037	9.8	9.4
Lipocalin 2	NM005564	12.7	7.7
Solute carrier family 16, member 4	NM004696	6.8	4.7
Retinol binding protein 1, cellular	NM002899	3.0	3.9
Solute carrier family 16, member 2	NM006517	3.6	2.9
Carbonic anhydrase II	M36532	4.2	2.1
Unknown			
Adipose specific 2	NM006829	8.1	4.6
HGFL gene	AL540260	3.4	3.8
Chromosome 14 open reading frame 138	AI628605	2.7	2.0
Others			
Hypothetical protein FLJ21511	NM025087	11.9	5.6
DKFZP586H2123 protein	AI671186	5.8	5.4
Hypothetical protein FLJ14675	BG036668	2.4	3.1
Hypothetical protein LOC54103	AK026747	3.2	2.9
Hypothetical protein MGC14376	AF070569	2.2	2.4
Hypothetical protein FLJ23309	NM024896	2.4	2.2

* These genes were further investigated.

MUC16 gene was the second most upregulated by RA after 48 hours (11.5-fold change) (Table 2). The latter data confirmed our previous report that 100 nM RA upregulated MUC16 expression both at the mRNA and protein levels.[35] Since previous reports suggest a role of arachidonic acid metabolites in mucus regulation, we focused on these two genes for further analysis by designing experiments to investigate whether the RA-induced MUC16 upregulation was mediated by $sPLA_2$.

Western blot followed by densitometric analysis of $sPLA_2$-IIA in HCjE cells and their culture media after 24- and 48-hour exposures to RA was compared to control cells and media to confirm the experimental results of the microarray data at the protein level. Cell lysate and conditioned culture medium were examined to look for evidence of synthesis and secretion of $sPLA_2$-IIA by these cells. As shown in Figure 2, $sPLA_2$ protein was not detected in the HCjE cells or their culture media in the control cultures. However, after 24 hours of culture with RA, $sPLA_2$-IIA protein was found in the cells, and the amount dramatically increased at 48 hours (Fig. 2A), indicating translation of the $sPLA_2$ mRNA detected at 24 and 48 hours by microarray analysis. $sPLA_2$-IIA was not detected in the culture media until 48 hours after addition of RA (Fig. 2B).

TABLE 3. Downregulated Gene Expression (45 Genes) in HCjE Cells Treated with RA for 24 and 48 Hours ($P < 0.01$)

Gene Function and Name	Accession Number	Fold Change 24 h	Fold Change 48 h
Cell cycle/cell death			
Cell division cycle associated 3	NM031299	−2.2	−33.8
G-2 and S-phase expressed 1	BF973178	−4.8	−15.6
High-mobility group box 2	BC000903	−2.1	−11.4
Aurora kinase B	AB011446	−3.3	−7.5
BUB1	AL137654	−6.3	−7.2
MCM10	NM018518	−2.1	−6.7
Kinesin family member 2C	AY026505	−3.3	−6.1
Antigen identified by antibody Ki-67	AU132185	−2.0	−5.8
CDC6 cell division cycle 6 homolog	NM001254	−2.0	−5.5
DNA replication factor	AF321125	−3.0	−3.0
MCM2	NM004526	−2.1	−3.0
Kinesin family member 22	AC002301	−2.9	−2.9
MCM7	D55716	−2.0	−2.9
MCM5	NM006739	−3.6	−2.7
Cell proliferation/differentiation			
Polo-like kinase (Drosophila)	NM005030	−3.2	−8.7
TPX2	AF098158	−2.0	−6.5
Nucleolar protein ANKT	NM018454	−2.3	−6.1
Replication factor C (activator 1) 2, 40kDa	M87338	−2.4	−3.0
Fusion (FUS)	NM004960	−4.0	−2.9
Inhibin, β C	NM005538	−2.1	−2.2
DEAD box polypeptide 39	NM005804	−2.2	−2.2
Metabolism			
Thymidylate synthetase	NM001071	−2.0	−8.6
Ligase 1, DNA, ATP-dependent	NM000234	−2.1	−5.4
Thymidine kinase 1, soluble	NM003258	−2.3	−5.0
Ubiquitin carrier protein	NM014501	−2.7	−3.6
Ubiquitin specific protease 1	AW499935	−5.3	−3.0
Flap structure–specific endonuclease 1	NM004111	−2.4	−2.9
Phosphoserine aminotransferase 1	NM021154	−2.2	−2.6
Metabolism (*continued*)			
Degenerative spermatocyte homolog, lipid desaturase	NM003676	−2.4	−2.3
Sex determination			
Mago-nashi homolog, proliferation-associated	AF067173	−2.6	−2.7
Signal transduction/cell signaling			
Neurogranin	NM006176	−7.0	−12.8
Spermatogenesis			
ASF1B	NM018154	−2.3	−7.6
Structural molecule			
Lamin B1	NM005573	−5.1	−23.0
H2A histone family, member X	NM002105	−2.0	−4.0
Mitochondrial ribosomal protein L24	NM024540	−2.4	−2.2
Lysosomal-associated multispanning membrane protein-5	NM006762	−2.0	−2.1
Transcription factor			
Topoisomerase (DNA) II alpha 170kDa	AU159942	−3.9	−18.2
Interleukin enhancer binding factor 3	AF147209	−2.0	−2.1
Transport			
Treacher Collins-Franceschetti syndrome 1	NM000356	−2.4	−2.6
Unknown			
Paternally expressed 10	BE858180	−18.5	−4.5
Others			
Hypothetical protein DKFZp762E1312	NM018410	−2.3	−21.8
HSPC037 protein	BC003186	−2.1	−9.1
Hypothetical protein DKFZp762A227	BC003163	−2.7	−5.1
PRO2000 protein	NM014109	−2.0	−3.6
Hypothetical protein MGC:10200	BF038461	−2.2	−2.2

To verify the microarray quantitation of MUC16 mRNA expression, real-time PCR was performed using the same samples that were used to prepare probes for microarray hybridization. Figure 3 shows the expression profile of MUC16 mRNA as measured by these two independent methods. In both methods, the level of the control was set at 1, and all other RA cultures were expressed relative to it. Although the relative values of expression amounts were different, the expression patterns of MUC16 mRNA in the two methods were similar over time (Fig. 3).

Effect of PLA_2 Inhibitors on MUC16 Expression

To determine whether $sPLA_2$ mediates the RA induction of MUC16, the MUC16 expression was examined after treatment of HCjE cells with RA in the presence of the broad-spectrum PLA_2 inhibitor ArA. Figure 4 shows the relative expression of MUC16 mRNA after culture with 100 nM RA plus ArA as determined by real-time PCR. The addition of ArA along with the RA treatment significantly inhibited the RA-induced increase in MUC16 mRNA expression at both 24 and 48 hours ($P < 0.01$ and $P < 0.0001$, respectively). Complete inhibition of the RA-induced MUC16 mRNA expression was observed at both 24 and 48 hours, compared with vehicle. The reduction of MUC16 after addition of ArA was also confirmed at the protein level. Figure 5 shows that RA-induced MUC16 protein synthesis was significantly inhibited by the addition of 100 μM ArA at 24 and 48 hours; 75% less mucin was present at 24 hours and 50% at 48 hours. Thus, the reduction in protein expression follows that of the mRNA.

Having shown inhibition of RA-induced MUC16 upregulation using a broad-spectrum PLA_2 inhibitor, ArA, we sought to determine whether a specific inhibitor of group IIA $sPLA_2$, LY315920,[47] would affect the RA-induced MUC16 expression. We examined MUC16 mRNA expression levels by real-time PCR in HCjE cultures treated for 24 and 48 hours with vehicle (DMSO), 100 nM RA, 100 nM RA plus 10 μM LY315920, or 10 μM LY315920 alone. As shown in Figure 6, addition of 10 μM LY315920 significantly inhibited RA-induced MUC16 expression by 100% at 24 hours and 99% at 48 hours. As shown in Figure 7, the addition of LY315920 resulted in complete inhibition of the RA-induced increase in MUC16 protein detected in cell lysates at both 24 and 48 hours ($P < 0.01$ and $P < 0.0001$, respectively).

MUC1 and MUC4 mRNA Expression

Analysis of the microarray data showed no upregulation by RA of the mRNAs for the other membrane-associated mucins, MUC1 and -4, expressed by the HCjE cells. This is in agreement with our previous data for MUC1, but not MUC4, as determined by real-time PCR.[35] To investigate this difference, real-time PCR analysis was done using previously published primers and probes for MUC1 and -4[28,43] and the same samples that were used to prepare probes for microarray hybridization. Figure 8 shows the independent verification of the microarray quantitation as determined by real-time PCR. MUC1 mRNA quantitation by the two methods yielded similar results (Fig. 8A). However, even though we used aliquots of the same RNA, microarray data for MUC4 mRNA did not reflect the results

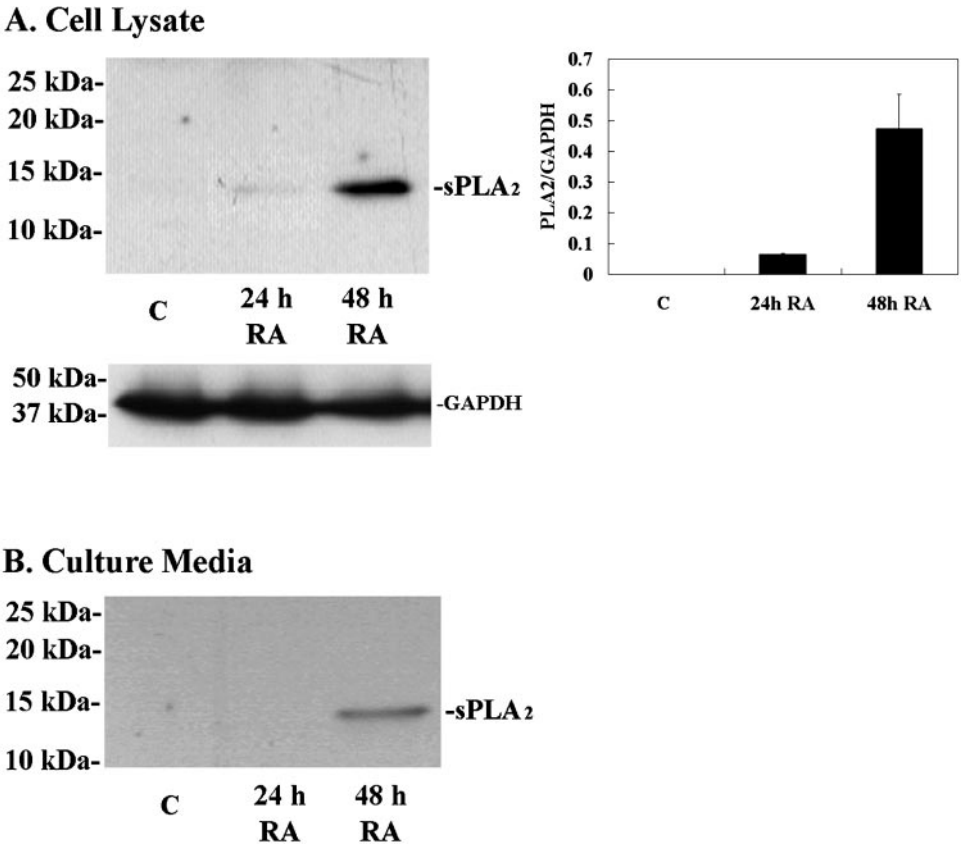

FIGURE 2. Immunoblot and densitometric analysis of group IIA $sPLA_2$ protein in HCjE cells and culture media grown with 100 nM RA for 24 and 48 hours, compared with baseline (C, 0-hour control). (**A**) Five micrograms of total protein was separated by SDS-PAGE. The expression of $sPLA_2$ protein (14 kDa) was not detected in the control, whereas it was induced by 24 hours after addition of RA and upregulated in a time-dependent manner. Densitometric comparisons of $sPLA_2$ normalized to GAPDH were obtained at each time point. (**B**) No $sPLA_2$ was detected in the culture medium from control cultures or those treated with RA for 24 hours. Secretion of $sPLA_2$ was induced by 48 hours after addition of RA. Error bars, SEM.

obtained with real-time PCR (Fig. 8B). In an attempt to resolve this discrepancy between the two methods, we designed new MUC4 primers and probe for real-time PCR (MUC4 C-term), to amplify the sequence from the same region of the C terminus of MUC4 as used in the gene chip. As shown in Figure 8C, MUC4 C-term mRNA was upregulated by RA over the time course. This agrees with the real-time PCR data obtained with the other published MUC4 primers and probe, and differed from the microarray data (Fig. 8B and 8C), suggesting a false negative result for MUC4 using microarray analysis. Figure 8D shows that a unique band, corresponding to the predicted size for the MUC4 C-term (75 bp), was obtained after 40 cycles of cDNA amplification. Sequencing of the PCR product verified that MUC4 was the amplified product.

DISCUSSION

This study demonstrates the effect of RA on the gene expression profile of human conjunctival epithelia using an immortalized conjunctival epithelial cell line and microarray analysis. In searching through the upregulated genes at both early and late phases for proteins or glycoproteins that may facilitate maintenance of a wet-surfaced phenotype and prevent the keratinization characteristic of keratomileusis, we noted that $sPLA_2$-IIA and MUC16 were the two most highly upregulated mRNAs after RA treatment in the late phase. We therefore focused on the relationship between $sPLA_2$ and RA-associated MUC16 induction in further studies. The major conclusions of these experiments are that RA upregulates both $sPLA_2$-IIA and

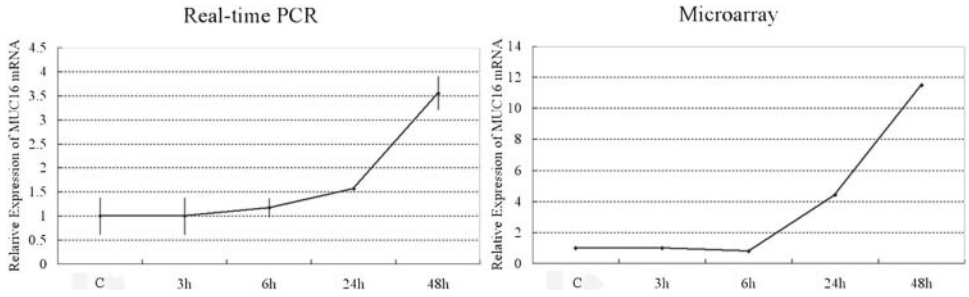

FIGURE 3. Relative mRNA level of MUC16 in HCjE cells treated with 100 nM RA for 0 to 48 hours. To verify the microarray quantitation of MUC16 mRNA expression, relative mRNA levels of MUC16 were measured by real-time PCR (*left; n* = 2) in the same samples that were used to prepare probe for microarray hybridizations (*right*). In both methods, the level of control (C) was set at 1, and RA-cultured cells at 3, 6, 24, and 48 hours were expressed relative to it. Expression amounts differed between these two techniques, but quantitation with the two methods showed a similar trend.

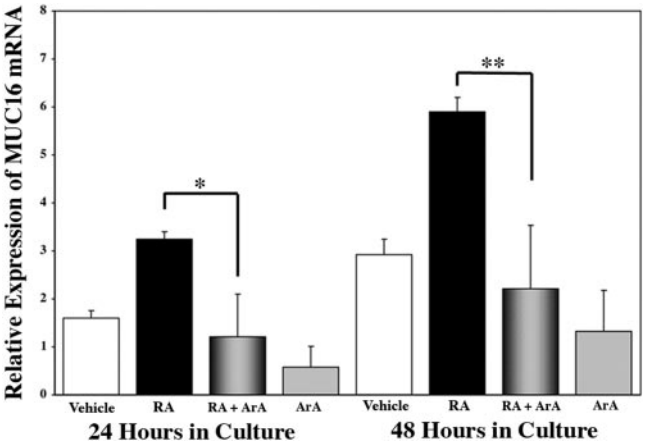

FIGURE 4. Real-time PCR analysis of MUC16 mRNA expression in HCjE cells grown for 24 or 48 hours in the presence of DMSO alone (vehicle), 100 nM RA alone, 100 nM RA + 100 nM ArA, or 100 nM ArA alone. MUC16 expression is determined relative to the 0-hour baseline value. Culture with RA significantly upregulated MUC16 compared to vehicle control at both 24 and 48 hours ($P < 0.02$ and $P < 0.0001$, respectively). The addition of ArA along with RA significantly inhibited the RA-induced MUC16 mRNA expression at both 24 and 48 hours. ArA alone had no significant effect. Error bars, SEM; $^{*}P < 0.01$; $^{**}P < 0.0001$.

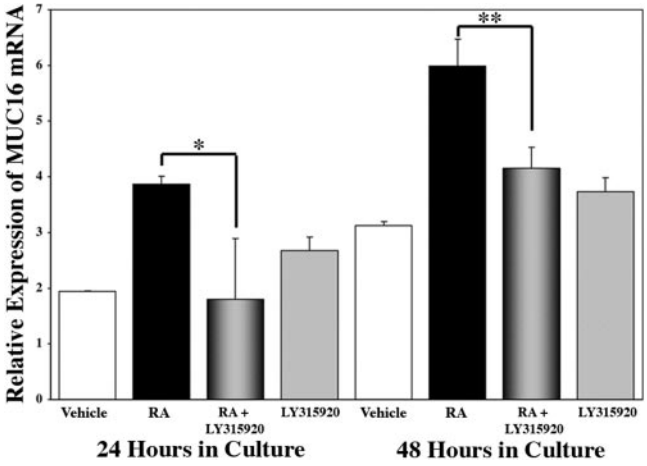

FIGURE 6. Real-time PCR analysis of MUC16 mRNA expression in conjunctival cells cultured with a specific inhibitor of $sPLA_2$-IIA, LY315920. HCjE cells were grown for 24 or 48 hours with DMSO alone (vehicle), 100 nM RA, 100 nM RA + 10 μM LY315920, or 10 μM LY315920 alone. MUC16 expression is determined relative to the 0-hour baseline value. The addition of LY315920 along with RA significantly inhibited the RA-induced MUC16 expression at both 24 and 48 hours. Culture with the inhibitor alone had no significant effect on MUC16 expression. Error bars, SEM; $^{*}P < 0.01$; $^{**}P < 0.05$.

MUC16, and that the MUC16 induction is mediated by $sPLA_2$-IIA.

Both molecules are involved in defense of the ocular surface. MUC16 is one of the class of membrane-associated mucins that appear to be major constituents of the glycocalyx of all wet-surfaced epithelial cells,[52] where they are hypothesized to facilitate maintenance of fluid on the apical surface and prevent pathogen invasion.[53] Group IIA PLA_2, a member of the extracellular (secreted) $sPLA_2$ family, is a low-molecular-weight (14 kDa) enzyme.[54] All members of the $sPLA_2$ family catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release fatty acids (usually arachidonic acid) and lysophospholipids, important in biosynthesis of lipid mediators (Fig. 9).[55,56] $sPLA_2$s also bind a variety of membrane and soluble proteins and can serve as high-affinity ligands. The latter include the proteoglycans and the M receptor.[55] Both the enzymatic activity and ligand binding appear to mediate a large range of cellular activities (for review, see Ref. 57). $sPLA_2$-IIA has been reported to be secreted by the lacrimal gland and has been recognized as an antibacterial molecule in tear fluid,[58–60] where it acts by cleaving arachidonic acid from the bacterial phospholipid membrane.

To date, there has been no report of a link between PLA_2 and mucin gene expression, nor have data been reported on RA induction of $sPLA_2$-IIA. PLA_2 is a key enzyme for eicosanoid metabolism, due to its regulation of the release of arachidonic acid. Arachidonic acid serves as a precursor to eicosanoids, a group of inflammatory mediators. Previous studies suggest that several lipoxygenase pathway eicosanoid metabolites, hydroxyeicosatetraenoic acids (HETEs), can stimulate mucus production in airway epithelium.[29,30] In addition, Jackson et al.[31] reported that topical application of 15(S)-HETE to the rabbit ocular surface increases the thickness of the mucin layer on the surface of the corneal epithelium, and Jumblatt et al.[32,33] demonstrated that 15(S)-HETE increases the amount of MUC1 protein, but not MUC2, -4, or -5AC, in ex vivo human conjunctival tissue. Because the latter study was done before the identification of MUC16 in ocular surface epithelia,[26] MUC16 regulation by 15(S)-HETE was not assayed. We found no change in MUC1 expression in response to RA, but did find significant increases in the membrane-associated mucin MUC16 and the eicosanoid metabolic enzyme $sPLA_2$. The previous studies linking eicosanoid metabolites and mucus production led us to hypothesize that $sPLA_2$ may be associated with RA-induced MUC16 regulation. Our data suggest that upregulation of $sPLA_2$ levels in conjunctival cells may result in increased production of arachidonic acid and its lipoxygenase pathway eicosanoid metabolites, HETEs, resulting in increased biosynthesis of the

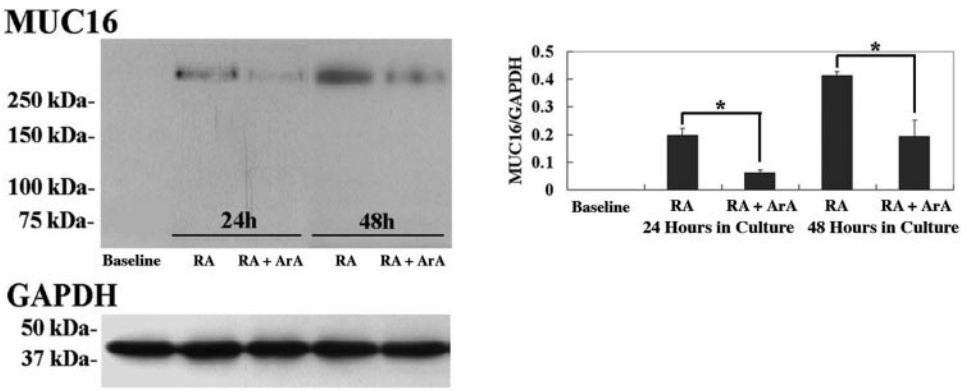

FIGURE 5. Western blot and densitometric analysis of MUC16 protein from HCjE cells grown without (baseline, 0-hour control) and with 100 nM RA ± 100 μM ArA (PLA_2 inhibitor) for 24 and 48 hours. Densitometric comparisons of MUC16 normalized to GAPDH were obtained at each time point. The addition of ArA along with RA significantly inhibited the RA-induced MUC16 expression at both 24 and 48 hours. Error bars, SEM; $^{*}P < 0.05$.

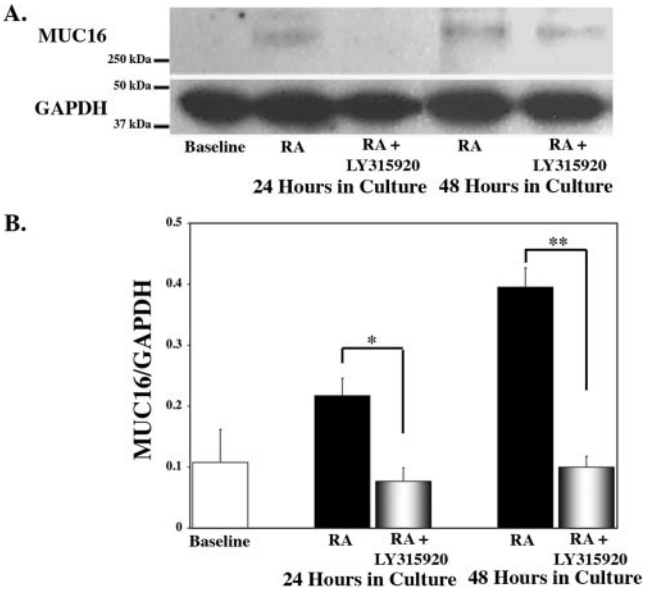

FIGURE 7. Analysis of MUC16 protein from HCjE cells cultured with a specific inhibitor of $sPLA_2$-IIA, LY315920. (**A**) Representative immunoblots of MUC16 mucin and GAPDH protein in HCjE cells at 0-hour baseline and at 24 and 48 hours after treatment with 100 nM RA ± 10 μM LY315920. (**B**) Comparisons of amount of MUC16 normalized to GAPDH were obtained by densitometry for each time point. Culture with RA alone significantly increased MUC16 protein at both 24 and 48 hours ($P < 0.05$ and $P < 0.0001$, respectively). The addition of LY315920 along with RA significantly inhibited the RA-induced MUC16 protein expression at both 24 and 48 hours. Error bars, SEM; $^*P < 0.01$; $^{**}P < 0.0001$.

membrane-associated mucin MUC16. The small increase in MUC16 upregulation in cultures treated with the $sPLA_2$ inhibitor but not with RA, compared to no increase with the broad-spectrum inhibitor of PLA_2, suggests that the mechanism of upregulation of MUC16 is not entirely controlled by $sPLA_2$ and RA induction, and that additional PLA_2 regulators may be active.

The significant inhibition of the RA-induced MUC16 expression by the broad-spectrum PLA_2 inhibitor ArA at 24 and 48 hours, for both mRNA and protein in HCjE cells, suggests that eicosanoids may be involved in the regulation of MUC16. Use of specific inhibitors of the group IIA $sPLA_2$ in RA-induced MUC16 expression also resulted in a highly significant inhibition at both 24 (100%) and 48 hours (99%) after addition of RA. These data indicated that RA induction of MUC16 is mediated either by eicosanoids or by ligand binding by $sPLA_2$-IIA that signals through the cell membrane. Other factors may also be involved in the regulation of MUC16, since at baseline (0-hour controls), low levels of MUC16 mRNA are expressed, and that expression increases over time without RA, albeit at lower levels. Recently Landreville et al.[61] reported that group IIA $sPLA_2$ is not expressed in human corneal epithelial cells isolated from fresh donor corneas. These cells normally express high levels of MUC16[26]; thus, in the corneal epithelium—compared to conjunctival epithelium—either MUC16 gene expression is differentially regulated, or exogenous $sPLA_2$-IIA, secreted by the conjunctival or lacrimal cells into the tear film, may effect upregulation of MUC16 expression in corneal epithelium.

Although microarray analysis is a powerful tool, allowing investigators to examine changes in the expression of thousands of genes simultaneously in a single experiment, there must be caution in interpretation of microarray results without confirmation by other techniques. Microarray results are influenced by array production, RNA extraction, probe labeling, hybridization efficiency, and image analysis.[62] Therefore, to ensure the quality of data obtained from microarray analysis, gene expression patterns are confirmed by other methods—real-time PCR or protein expression. In the present study, in addition to MUC16 upregulation by RA, the microarray data also confirmed the previously reported real-time PCR data that RA did not regulate the membrane-associated mucin MUC1 in HCjE cells.[35] However, the microarray data did not agree with the real-time PCR data for MUC4,[35] even when the same RNA was used to prepare probes for microarray and cDNA for real-time PCR. The primers and probe for gene amplification of MUC4 used in real-time PCR were as previously reported and designed from the area flanking the tandem repeat domains, based on GenBank Accession No. AF058803.[43,63] On the other hand, the target sequences of MUC4 used on the gene chip were designed from the C-terminal region, which included the transmembrane domain, cytoplasmic tail, and 3′ untranslated sequence (http://www.affymetrix.com/index.affx).[64] Then, to investigate whether the difference in expression patterns was a result of the difference in sensitivity between the two methods or represented the expression of splice variants of MUC4, we designed new MUC4 primers and probe for real-time PCR (MUC4 C-term), to amplify the region from the C terminus of MUC4 used in the gene chip, and compared the expression patterns between the two methods. Interestingly, the MUC4 C-term primers and probe data (Fig. 8 B, C) corroborated the real-time PCR data obtained previously and differed from the microarray data found with probes to the same region of the MUC4 gene. Real-time PCR is a sensitive method to detect cDNA and is reported to require 1000-fold less RNA than conventional assays.[62] Since the present real-time PCR data with the new MUC4 C-term primers confirmed our previous data[35] on RA-induced MUC4 expression, it was determined that the real-time PCR data was more reliable, and that the microarray data gave a false negative. This further emphasizes the need to corroborate microarray data.

Analyzing RA-treated HCjE cells at early and late phases provided insight into the magnitude and time course of changes in gene expression at different stages of RA treatment of HCjE cells. Microarray analysis yielded more transcripts that were up- or downregulated by RA in the late phase than in the early phase. We also found that nine genes were upregulated in both the early and late phases. Keratin 4, which is present in nonkeratinized epithelia and is a nonkeratinization marker of epithelial cells, was one of these genes. Expression of the keratin 4 gene increased between 3 and 24 hours (3.1-, 5.9-, and 7.5-fold increases at 3, 6, and 24 hours, respectively) and returned to the 6-hour level at 48 hours (6.2-fold change) (Tables 1 and 2). Since corneal and conjunctival epithelial cells are keratinized in patients with vitamin A deficiency, the continued upregulation of the nonkeratinized marker keratin 4 by RA in HCjE cells is reasonable. Curiously, however, involucrin, which is expressed in normal epithelium,[65,66] but upregulated in keratinized epithelium,[65] was also upregulated in the early phase (3.7- and 6.0-fold increases) (Table 2). Involucrin is reported to be expressed in superficial layers in normal human conjunctival[65] and corneal epithelial cells,[66] and to be upregulated in superficial and intermediate layers of conjunctiva in patients with Stevens-Johnson Syndrome.[65] As described above, the RA-treated HCjE cultures had islands of stratified cells with large apical cells. It is possible that a certain level of upregulation of both proteins is required for the normal differentiation of the HCjE cells from subconfluent to confluent, stratified cultures. Development of an in vitro model for keratinization of these cells may provide new information on the function of both proteins that could be correlated to that of pathologic states, such as Stevens-Johnson Syndrome.

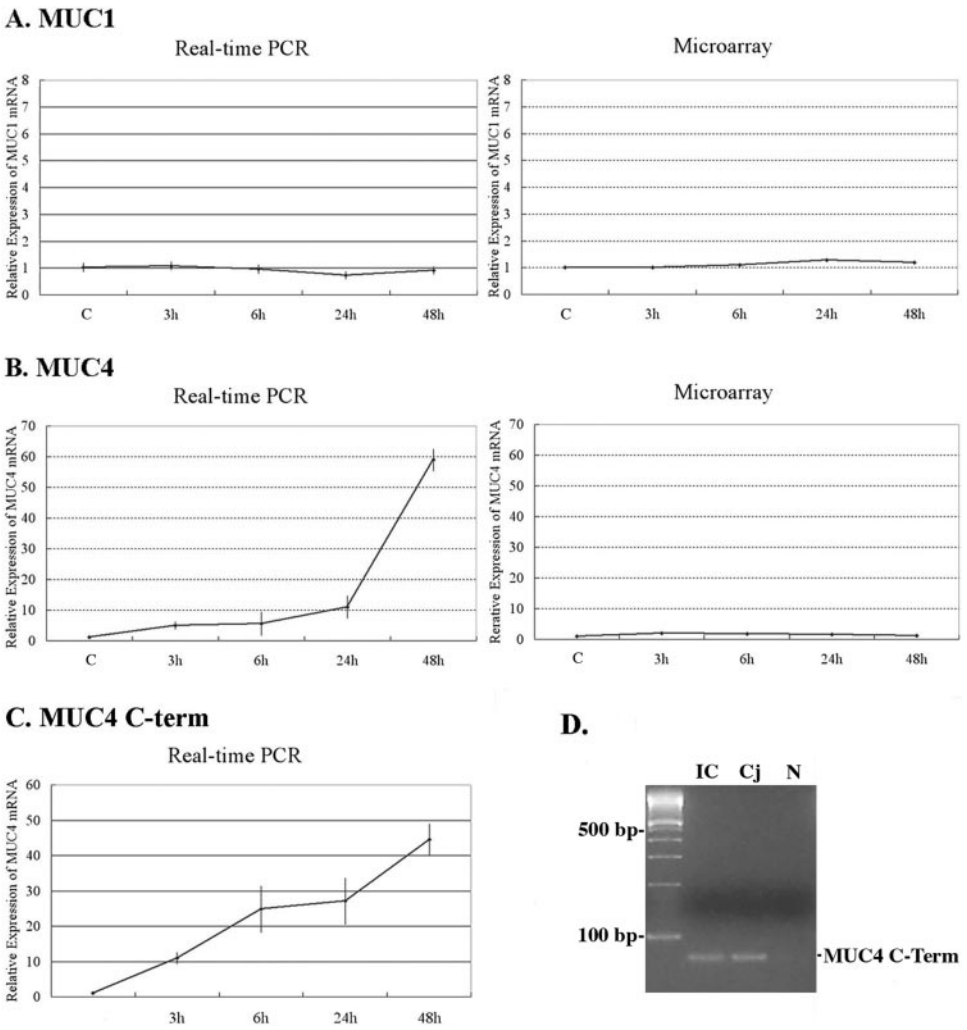

FIGURE 8. Relative mRNA level of MUC1 and MUC4 in HCjE cells in control (C, baseline) cultures and cultures treated with 100 nM RA for 3 to 48 hours. To verify the microarray data, relative mRNA levels of MUC1 and MUC4 mucins were measured with real-time PCR (*left*; $n = 2$) in the same samples that were used to prepare probe for microarray hybridizations (*right*). (**A**) MUC1 mRNA was not upregulated by 100 nM RA, based on both microarray and real-time PCR data. (**B**) Although real-time PCR data demonstrated that RA upregulated MUC4 mRNA, there was no change in the microarray data. (**C**) New primers and probe were designed for real-time PCR of a sequence selected from the MUC4 C-terminus region used in the gene chip. MUC4 C-term mRNA was upregulated by RA over the time course. This expression pattern again differed from that seen by microarray analysis. (**D**) Conventional RT-PCR analysis demonstrates that the MUC4 C-term primers used in real-time PCR produced the expected amplicon size (75 bp) in native conjunctival epithelial and HCjE cells. Samples were electrophoresed on a 4% agarose gel and visualized with ethidium bromide. Sequencing verified that MUC4 was the amplified product. Cj, HCjE culture in presence of serum for 72 hours; IC, native human conjunctival epithelium collected by impression cytology; N, negative control (sterile water).

In summary, this study identified a number of genes that are up- and downregulated by treatment with RA in a cultured HCjE cell line. The membrane-associated mucin MUC16 is highly responsive to RA in the late phase of treatment. This is indicative of an indirect effect of RA on MUC16 gene expression. Group IIA $sPLA_2$ is also highly responsive to treatment with RA, being upregulated by 6 hours post-treatment, peaking at 24 hours, but remaining highly expressed at 48 hours. Use of the specific group IIA $sPLA_2$ inhibitor LY315920 demonstrated that $sPLA_2$ (the gene most upregulated by RA) mediates RA upregulation of MUC16 expression.

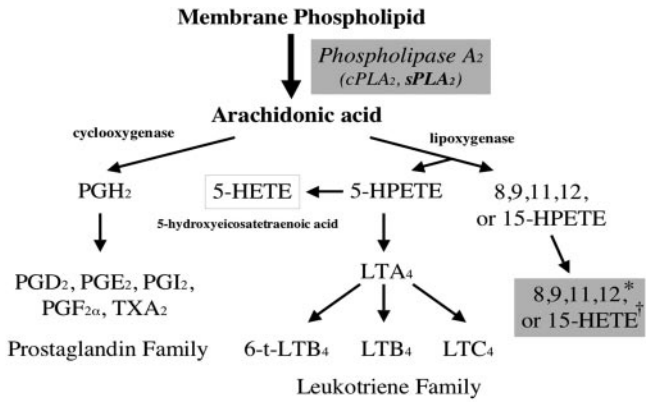

FIGURE 9. Metabolism of arachidonic acid by cyclooxygenase and 5-lipoxigenase pathways. HPTE, hydroperoxyeicosatetraenoic acid; LT, leukotriene; PG, prostaglandin. Modified from *Prostaglandins Leukotrienes and Essential Fatty Acids,* 69, Diaz BL and Arm JP, Phospholipase A(2), 87–97, Copyright 2003, with permission from Elsevier.

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