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Molecular and metabolic retinoid pathways in the human ocular surface

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Purpose: To maintain its integrity, the human ocular surface (cornea and conjunctiva) has an absolute requirement for vitamin A and its active derivatives, the retinoic acids. These retinoids regulate transcriptional levels of target genes through the activation of members of a super-family of ligand-dependant nuclear receptors that feature retinoic acid receptors (RAR) α, β, and γ as well as retinoid X receptors (RXR) α, β, and γ. The expression patterns of these receptors have been partial characterized in rabbit, mouse, and human cornea and conjunctiva, but systematic tissue and cellular expression of the three RARs and three RXRs had to be completed at the adult human ocular surface. The first objective of our work was to define their expression patterns in term of genes and proteins for total human conjunctiva, cornea, and the major cell types comprising the adult human ocular surface. The second objective was to demonstrate the presence of different enzymes transforming vitamin A to retinoic acid and the functionality of this metabolic pathway in the corneal epithelium.

Methods: Total mRNA was extracted from human total cornea, conjunctiva, corneal epithelial cells (primary culture and established cell line), corneal keratocytes (primary culture), corneal endothelial cells (established cell line), and conjunctival epithelial cells (established cell line) and was submitted to reverse transcription-polymerase chain reaction (RT-PCR) analysis to determine the expression patterns of the RARs and RXRs using specific primers. Immunological staining (via histochemistry and cellular chemistry) experiments were performed to better localize RAR and RXR proteins in the ocular surface at tissue and cellular levels. We also checked mRNA expression of cellular retinol binding proteins (CRBPs) and cellular retinoic acid binding proteins (CRABPs) with the enzymes involved in retinoic acid generation, i.e., alcohol dehydrogenases (ADHs) and retinal dehydrogenases (RALDHs) or degradation (Cyp26 family members). The enzymatic generation of functional retinoids was confirmed using epithelial corneal cells treated with specific inhibitors of retinol metabolism.

Results: RAR α, RAR γ, and RXR α are expressed in the cornea, conjunctiva, and all of their constitutive cells, whereas RXR γ and RXR β were never detected in the cornea or conjunctiva. RAR β was absent in primary cultures of corneal keratinocytes. ADH3, ADH4, dehydrogenase/reductase (SDR family) 4 (DHRS4), dehydrogenase/reductase (SDR family) 9 (DHRS9), RALDH1, and RALDH3 are expressed in the ocular surface, as were the retinoid-binding proteins CRBP1, CRABP1, and CRABP2. Retinol dehydrogenase 4 (RODH4) was only detected in the conjunctiva. Corneal epithelial cells convert retinol into retinoic acid using an enzymatic pathway.

Conclusions: For the first time, we have established an exhaustive description of the expressions patterns of RARs, RXRs, ADHs, RALDHs, CRBP, and CRABPs in the human ocular surface. Our results for the human ocular surface demonstrated the presence of all the metabolic and molecular actors of the retinoic acid signaling pathway. We also demonstrated the enzymatic conversion of retinol into active retinoids in the corneal environment.

It is well established that the cornea and the conjunctiva have an absolute requirement for vitamin A (retinol), which acts through its metabolites such as retinaldehyde, which forms the visual chromophore, and retinoic acids, which regulate gene expression. Early studies dealing with the effects of dietary vitamin A deficiency (VAD) or mouse gene targeting strategies on embryonic ocular development and malformations have demonstrated that vitamin A is required for eye morphogenesis [1,2]. Lack of vitamin A causes abnormal differentiation of the ocular surface that results in keratinization, ulceration, epithelial squamous metaplasia, and a deficiency of conjunctival goblet cells [3,4]. When xerophthalmia and keratomalacia due to vitamin A deficiency was recognized as an important public health problem in many developing countries, it triggered many studies on the use of retinoids on the ocular surface [5]. Due to these implications, topical retinoids have been tested as pharmacological agents for the treatment of ocular surface diseases involving wound healing [6], squamous metaplasia [7] or dry eye [8].

These pleiotropic effects of retinoids are mediated by retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are ligand-activated transcription factors. Two families of nuclear receptors for retinoic acid (RA) have been

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characterized. Members of the RAR family (α, β, and γ) are activated by most of the physiologically-occurring retinoids (*all-trans* RA, 9-*cis* RA, 4-oxo RA, and 3,4di-hydro RA). In contrast, members of the RXR family (α, β, and γ) are only activated by 9-*cis* RA [9,10]. The transcriptional regulation of the target genes relies on the recognition and cooperative assembly of RAR-RXR heterodimers in short DNA sequences in their promoter regions that are known as retinoic acid response elements (RARE). Several corneal and conjunctival target genes of retinoids have already been described, including proteases and their inhibitors [11], epidermal growth factor (EGF) [12], mucine [13] or AP-2 transcription factor [14]. To better understand the transcriptional regulating properties involved, partial characterization of the RARs and RXRs have been realized in rabbit, mouse, and human conjunctiva and cornea [2,15,16], but the cellular and systematic tissue expressions of these receptors had to be completed at the human ocular surface.

To be biologically active (see Figure 1), retinol must first be oxidized to retinaldehyde then to retinoic acid. A large number of enzymes catalyze the oxidation of retinol to retinaldehyde including alcohol dehydrogenases (ADH) and retinol dehydrogenase of the microsomal fraction (DHRS) as well as several enzymes able to catalyze the oxidation of retinaldehyde to retinoic acid, i.e., retinal dehydrogenase (RALDH 1, 2, 3, and 4) [17]. Retinoic acid catabolism is also an important mechanism for controlling RA levels in cells and tissues, and it is performed by three specific cytochrome P450s: CYP26A, CYP26B, and CYP26C [18]. Since retinol, retinaldehyde, and retinoic acid are lipids, they lack appreciable water solubility and consequently must be bound to proteins within cells. Several intracellular binding proteins for retinol, retinaldehyde, and retinoic acid have been identified and extensively characterized. They include cellular retinol-binding proteins type 1 and 2 (CRBP 1 and 2) and cellular retinoic acid-binding proteins type 1 and 2 (CRABP 1 and 2).

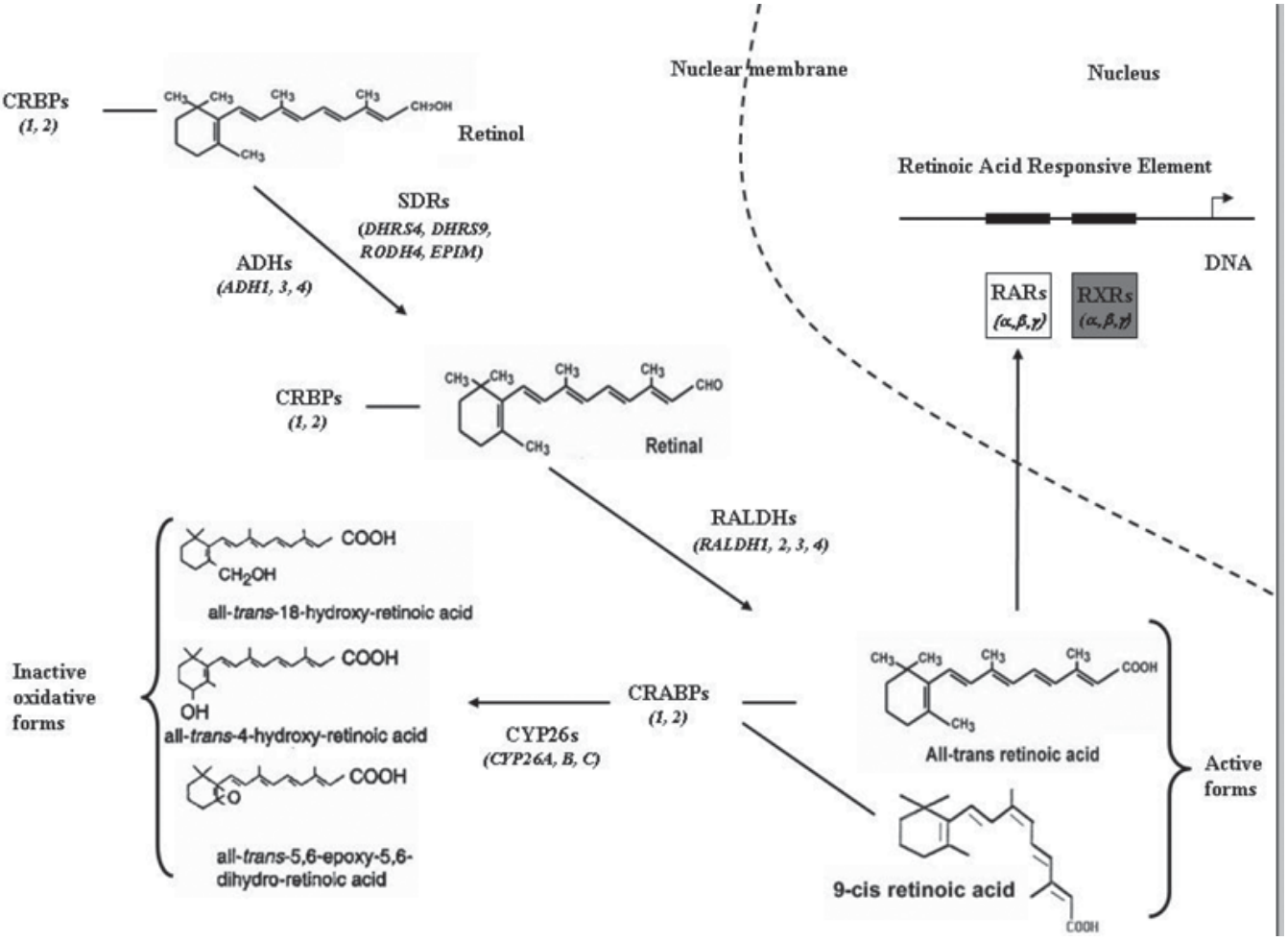

Figure 1. Schematic representation of the intracellular retinoid signaling pathway. Retinol is converted into retinal and retinoic acids, before either it acts through nuclear retinoid receptors or is inactivated by hydroxylation. Abbreviations are: ADH: alcohol dehydrogenase; CRABP: cellular retinoic acid binding protein; CRBP: cellular retinol binding protein; CYP26: cytochrome p450 retinoic acid inducible; RAR: retinoic acid receptor; RALDH: retinaldehyde dehydrogenase; RXR: retinoid X receptor, SDR: short-chain dehydrogenase/reductase.

Fragmental data have established the presence of retinoid-binding capacities [19] and some global retinoid metabolic pathways in the mammalian ocular surface [20]. It has also been established that tears contain detectable levels of certain retinoids [21]. Curiously, the human ocular surface metabolism of retinoids and, more particularly, the ability of human corneal epithelial cells to metabolize the retinol has not yet been documented.

The aim of this work was (1) to identify the molecular and metabolic actors involved in the retinoid pathway in the human ocular surface and (2) to evaluate retinol conversion into retinoic acid in the human corneal epithelial cells.

METHODS

Collection of human tissues: Pieces of normal human bulbar conjunctiva were removed from 10 patients during ocular surgery (retina detachment, strabismus, ectropion or entropion) once informed consent was obtained, according to the tenets of the Declaration of Helsinki. The research was approved by the institutional human experimentation committee (regional ethics committee, CCP Sud-Est VI). Ten normal human corneas unsuitable for graft for serological reasons were provided by the regional corneal graft bank. All tissues appeared normal after classical histological examination. They were rinsed using cold sterile phosphate buffer saline (PBS), embedded with Tissue-Teck OCT (Sakura, Zoeterwounde, Netherlands) or put in trizol (Invitrogen, Cergy-Pontoise, France), and stored at -80 °C until use. For immunohistological experiments, 10 µm-thick cryosections were cut and mounted on Super Frost slides (Fischer Scientific, Pittsburgh, PA).

Cell cultures: A human corneal epithelium (HCE) cell line transformed with SV 40 (ATCC/CRL11135) was cultured under standard conditions (5% CO_2, 95% humidified air, 37 °C) in Dulbecco's Modified Eagle's Medium plus Ham's nutrient mixture F-12 (DMEM-F12) supplemented with 5% fetal calf serum, 5 µg/ml of insulin, 0.1 µg/ml of cholera toxin, 50 mg/ml of streptomycin, 50 IU/ml of penicillin, 0.5 mg/ml of epithelium growth factor, and 0.5% DMSO.

Primary cell cultures of human corneal epithelium were established from donor corneas obtained from the local eye bank. Briefly, the limbus was dissected from five human corneas and cut into small pieces. Explants were transferred to

Table 1. Sequences of synthetic oligonucleotides used for the reverse transcription-polymerase chain reaction analysis

HUMAN GENE	SEQUENCE (5'-3')		Product Size (Pb)	Gene Number
	Forward	Reverse		
Retinoid Acid Receptor Alpha (RARA)	agtcctcaggctaccactat	cctccttcttcttcttgttt	225	NM_000964
Retinoid Acid Receptor Beta (RARB)	atggatgttctgtcagtgag	catagtggtaccctgatgat	268	NM_000965
Retinoid Acid Receptor Gamma (RARG)	accaataaggagcgactct	atctcctctgagctggtg	212	M24857
Retinoid X Receptor Alpha (RXRA)	ggatcccacacttctcag	gagtcagggttaaagaggac	286	NM_002957
Retinoid X Receptor Beta (RXRB)	agtactgccgctatcagaa	gttagtcacagggtcatttg	242	NM_021976
Retinoid X Receptor Gamma (RXRG)	ctacacagataccccagtga	gggtagttcatgtttccaat	249	NM_006917
Aldehyde Dehydrogenase 1A (ADH1)	tcttggtggctttaaaagta	gtctcaaaacatcagaatgg	175	NM_000667
Aldehyde Dehydrogenase 3 (ADH3)	atgaagttcgcattaagatg	tttcaagcagtagttgctct	239	NM_000669
Aldehyde Dehydrogenase 4 (ADH4)	aacctgcctggttaatttat	actttcaacttcccagaact	231	NM_000670
Dehydrogenase/reductase 4 (DHRS4)	tatcctagtctccaatgctg	caaggctgttttactgacat	235	NM_021004
Dehydrogenase/reductase 9 (DHRS9)	aaacctcagagagacttcgt	tctagtgtcagccagtcagt	169	NM_005771
Microsomal retinol dehydrogenase 4 (RODH4)	catactggacgtgaacttgt	ccaggaagctctttaagaat	278	NM_003708
3-hydroxysteroid epimerase (EPIM)	tgaacactgaggactctatga	ttcaactatgctgattttcac	242	NM_003725
Retinal Dehydrogenase 1 (RALDH1)	tactcaccgatttgaagatt	ttgtcaacatcctccttatc	151	NM_000689
Retinal Dehydrogenase 2 (RALDH2)	tgacttccagcaagatagag	tggattatagacagggaaca	179	NM_003888

For each gene, the nucleotide sequences of the two specific primers were detailed. The size of the amplification product (pairs of bases/pb) was noted. The gene accession number used to find both primers is indicated.

25 ml culture flasks, left for 2 h to allow the epithelial cells to adhere to the support, and cultured as previously described for HCE.

Human corneal keratocyte cell cultures were established from five donor corneas. Briefly, an 8 mm diameter corneal button was punched out and the Descemet's membrane with attached endothelium was stripped off. The epithelium was also totally removed by alcohol treatment and scraping. The naked residual stroma was then cut into small pieces and treated with 0.2% collagenase B (Roche Diagnostics, Meylan, France) for 3 h at 37 °C. After centrifugation, the pellet of keratocytes was resuspended in DMEM-F12, supplemented with 10% FCS and 1% each of glutamine, penicillin, streptomycin and amphotericin B, and then further subcultured. During subculture, keratocyte-derived fibroblasts were detached using a 0.1% trypsin/EDTA solution.

A human corneal endothelial cell line (HTCE) was kindly provided by Pr. K. Engelmann and Dr. J. Bednarz. This cell line was obtained by transfection with the coding gene for the large T proTCECEN and reproduced the morphological and functional characteristics of normal endothelium. It was cultured under standard conditions (5% CO_2, 95% humidified air, 37 °C) in 50% M199 and 50% Ham's F12 supplemented with 10% FCS, 2% glutamine, 50 mg/ml of streptomycin, 50 IU/ml of penicillin, 20 μg/ml ascorbic acid, and 20 μg/ml of insulin.

A human epithelial conjunctival cell line (Wong-Kilbourne derivative of Chang conjunctiva, clone 1-5c-4, ATCC CCL-20.2) was cultured under standard conditions (5% CO_2-95% humidified air, 37 °C) in Eagle's Minimal Essential Medium supplemented with 10% FCS, 2 mmol/l L-glutamine, 50 mg/ml streptomycin, and 50 IU/ml penicillin.

Each cornea led to independent primary cell cultures. Purity and identity of the established cell lines and primary cultures were confirmed using established markers in immunological or reverse transcriptase-polymerase chain reaction (RT-PCR) assays as previously described [22]. Molecular and immunological assays were always performed at the fifth subculture of all the cultures. Homogeneous state of confluence (80%) was also respected.

Reverse transcriptase-polymerase chain reaction experiments: Total mRNA was extracted from human total cornea, conjunctiva and cell cultures using trizol (Invitrogen, Cergy-Pontoise, France). RNA quality was studied via the RNA-to-

Table 2. mRNA expression patterns of nuclear receptors, enzymes, and binding proteins of the retinoid pathways in human adult cornea and conjunctiva

GENES	RARA	RARB	RARG	RXRA	RXRB	RXRG	ADH1	ADH3	ADH4	DHRS4	DHRS9	RODH4
Total cornea (n=5)	+	+	+	+	-	-	-	+	+	+	+	-
Total conjunctiva (n=10)	+	+	+	+	-	-	-	+	+	+	+	+
GENES	**EPIM**	**RALDH1**	**RALDH2**	**RALDH3**	**RALDH4**	**CYP26A**	**CYP26B**	**CYP26C**	**CRBP1**	**CRBP2**	**CRABP1**	**CRABP2**
Total cornea (n=5)	-	+	-	+	-	-	-	-	+	-	+	+
Total conjunctiva (n=10)	-	+	-	+	-	-	-	-	+	-	+	+

The expression patterns were determined by RT-PCR on total mRNA extracted from corneal and conjunctival tissues using specific primers. Positive controls (36B4) were performed under the same conditions using specific primers for the housekeeping gene 36B4 (219 base pairs). Negative controls (NC) were performed in the absence of an oligonucleotide or a matrix. Abbreviations are: ADH: alcohol dehydrogenase; CRABP: cellular retinoic acid binding protein; CRBP: cellular retinol binding protein; CYP26: inducible cytochrome p450 retinoic acid; DHRS: short chain dehydrogenase/reductase; EPIM: 3-hydroxysteroid dehydrogenase; RARA: retinoic acid receptor α; RARB: retinoic acid receptor β; RARG: retinoic acid receptor γ; RALDH: retinaldehyde dehydrogenase; RODH4: microsomol NAD sup+-dependent retinol dehydrogenase 4; RXRA: retinoid X receptor α; RXRB: retinoid X receptor β; RXRG: retinoid X receptor γ.

protein ratio (260 nm/280 nm) and by gel electrophoresis (2% agarose) to observe the presence of intact 28S and 18S RNA bands. Specific oligonucleotide primers were originally generated using the web program Primer3, based on the published full-length human mRNA sequences of each specific gene and designed to avoid genomic DNA amplification (Table 1). All the primers were first checked for their specific ability to amplify defined mRNA regions using human tissue already reported to express these genes (positive controls).

cDNA was generated using the Superscript First-Strand Synthesis System for RT-PCR (Gibco-BRL, Cergy-Pontoise, France). PCR amplification was performed in an Eppendorf Mastercycler (Eppendorf, Le Pecq, France), using 50 ng of total mRNA per reaction and according to the following program: initial denaturation at 95 °C for 5 min followed by denaturation at 95 °C for 45 s, annealing at 59 °C for 45 s, and extension at 72 °C for 60 s (36 cycles), and a final extension of 72 °C for 7 min. The PCR products were separated on 2% agarose gel and sequenced on both strands to confirm the specificity of the reaction using the same primers on a DNA Dye Terminator Cycle Sequencing Kit (Applied Biosytems, Courtaboeuf, France) and the Applied Biosystems model 377 DNA Sequencer. Amplification of the housekeeping gene, acidic ribosomal phosphoprotein P0 36B4 (219 base pairs), was used as positive control. A negative control for amplimer contamination was set up using a complete PCR reaction mix without cDNA.

Immunohistological and immunocytological experiments: Cryosections of total cornea, conjunctiva, and cells grown in Laboratory-Tek chambers (MC2, Clermont-Ferrand, France) were fixed in 4% paraformaldehyde in PBS (pH 7.4) at 25 °C for 10 min, rinsed three times with PBS, incubated at 25 °C for 10 min in H_2O_2 (quenching of endogenous peroxydases), and incubated in PBS with 3% BSA (Sigma Aldrich, St. Quentin Fallavier, France) at 25 °C for 30 min. Cells and tissues were incubated overnight at 4 °C in the presence of RAR α, β, and γ as well as RXR α, β, and γ (Santa-Cruz 551, 552, 550, 553, 831, 555, respectively) and certified rabbit polyclonal primary antibodies (1/200 in PBS; Tebu, Le Perray-en-Yvelines, France). This step was followed by three PBS washes, a 1 h incubation in the presence of a secondary FITC-labeled anti-rabbit antibody (Interchim, Montluçon, France) at room temperature, and three more washes with PBS. The samples were then examined after DAPI nuclear staining (five min, dilution in PBS 1/500) and after mounting in a Vectashield aqueous mounting fluid (Vector, Burlingame, CA) under a

Table 3. RNA expression patterns of nuclear receptors, enzymes and binding proteins of the retinoid pathways in human adult corneal and conjunctival cells

Gene	RARA	RARB	RARG	RXRA	RXRB	RXRG	ADH1	ADH3	ADH4	DHRS4	DHRS9	RODH4
Corneal Epithelial Cells (PC)	+	+	+	+	-	-	-	+	+	+	+	-
Corneal Epithelial Cells (CL)	+	+	+	+	-	-	-	+	+	+	+	-
Corneal Keratinocytes (PC)	+	-	+	+	-	-	-	-	-	+	-	-
Corneal Endothelial Cells (CL)	+	+	+	+	-	-	-	-	-	+	-	-
Conjunctival Epithelial Cells (CL)	+	+	+	+	-	-	-	+	+	+	+	+
Gene	**EPIM**	**RALDH1**	**RALDH2**	**RALDH3**	**RALDH4**	**CYP26A**	**CYP26B**	**CYP26C**	**CRBP1**	**CRBP2**	**CRABP1**	**CRABP2**
Corneal Epithelial Cells (PC)	-	+	-	+	-	-	-	-	+	-	+	+
Corneal Epithelial Cells (CL)	-	+	-	+	-	-	-	-	+	-	+	+
Corneal Keratinocytes (PC)	-	+	-	+	-	-	-	-	+	-	+	+
Corneal Endothelial Cells (CL)	-	+	-	+	-	-	-	-	+	-	+	+
Conjunctival Epithelial Cells (CL)	-	+	-	+	-	-	-	-	+	-	+	+

The expression patterns were determined by RT-PCR on total mRNA extracted from corneal and conjunctival cells using specific primers. Positive controls (36B4) were performed under the same conditions using specific primers for the housekeeping gene 36B4 (219 base pairs). Negative controls (NC) were performed in the absence of an oligonucleotide or a matrix. Abbreviations are: ADH: alcohol dehydrogenase; CRABP: cellular retinoic acid binding protein; CRBP: cellular retinol binding protein; CYP26: inducible cytochrome p450 retinoic acid; DHRS: short-chain dehydrogenase/reductase; EPIM: 3-hydroxysteroid dehydrogenase; RARA: retinoic acid receptor α; RARB: retinoic acid receptor β; RARG: retinoic acid receptor γ; RALDH: retinaldehyde dehydrogenase; RODH4: microsomol NAD sup+-dependent retinol dehydrogenase 4; RXRA: retinoid X receptor α; RXRB: retinoid X receptor β; RXRG: retinoid X receptor γ.

Zeiss Axiophot microscope. For negative controls, sections were incubated with normal rabbit IgG in place of the anti-RARs and anti-RXRs.

Transfection of cultured cells: Plasmid DR5-*tk*-CAT contains one copy of the retinoic acid-responsive element DR5 (direct repeat 5) bound to a herpes simplex thymidine kinase promoter upstream of a chimeric chloramphenicol acetyl transferase (CAT) reporter gene [23]. The human corneal epithelial cells were trypsinized 16 h before transfection. A total of $3x10^5$ cells in six well plates were transfected using GeneJammer with 1 μg of reporter DR5-*tk*-CAT plasmid and 1 μg of cytomegalovirus (CMV)-β-galactosidase vector serving as internal control to normalize variations in transfection efficiency. The CMV-β-galactosidase plasmid contains CMV promoter and enhancer sequences that drive a β-galactosidase (*βGAL*) gene. Parallel transfections of the corresponding vectors CAT and β-GAL at equivalent concentrations were performed in all experiments. After overnight incubation with DNA, the cells were washed and incubated with fresh medium for a further 12 h. They were treated for another 24 h with retinoids (retinol or all-trans retinoic acid [ATRA]) in 1000X stock solutions in DMSO. For this step, the maximal DMSO concentration to which the cells were exposed was <0.1%. In parallel experiments, ethanol, bisdiamine (Acros organics, Noisy-le-Grand, France), 4-methylpyrazole and DMSO were added at concentrations of 100 mmol/l, 100 μmol/l, 2 mmol/l, and 500 mmol/l, respectively. Ethanol, bisdiamine, and 4-methylpyrazole were chosen because they are well known to inhibit retinol conversion into retinoic acid. Cell viability assays were performed for each treatment (ethanol, bisdiamine, 4-methylpyrazole, and DMSO) using 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]bis(4-methoxy-6-nitro) benzensulfonic acid hydrate (XTT) assays (Roche Diagnostics, Meylan, France). For this purpose, cells were seeded

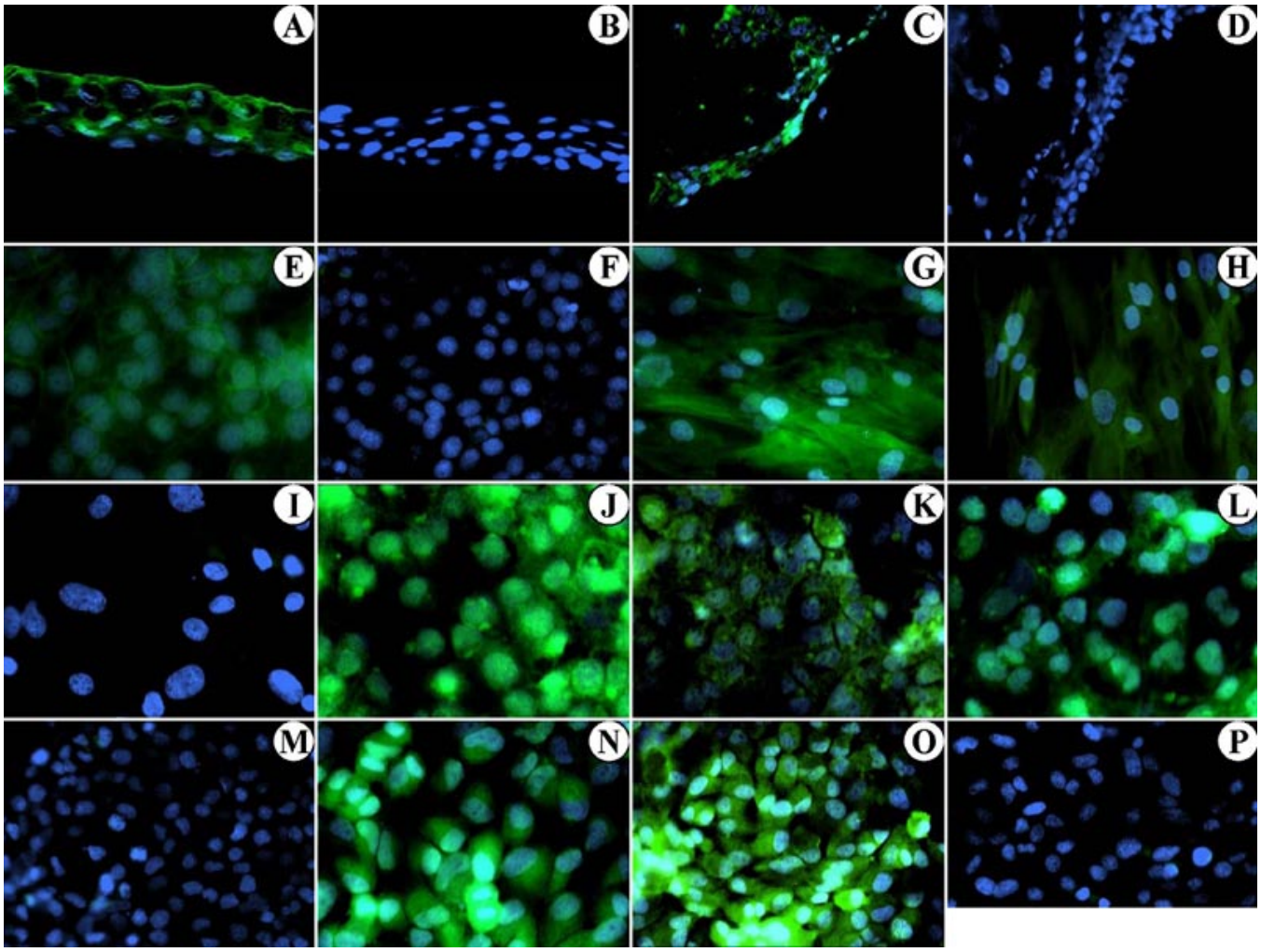

Figure 2. Immunolabeling of nuclear receptor, retinoin acid receptor, and retinoid X receptor proteins in human corneal and conjunctival cells and tissues. RAR α (**A**, **J**, **N**), RAR β (**E**, **L**), RAR γ (**G**, **K**, **O**), and RXR α (**C**, **H**) immunolocalizations (green fluorescence staining) were performed in total cornea (**A**, **B**), total conjunctiva (**C**, **D**), corneal epithelial cells (**E**, **F**), corneal keratinocytes (**G**, **H**, **I**), conjunctival corneal cells (**J**, **K**, **L**, **M**), and conjunctival cells (**N**, **O**, **P**). Cell nuclei were visualized with DAPI staining (blue fluorescence; **A**-**P**). Negative controls for the 4 antibodies used are presented in **B**, **D**, **F**, **I**, **M**, and **P**. Acquisitions were made under a standard Axiophot fluorescence microscope (Zeiss). Magnifications for **A** to **D** were x200, and for **E** to **P** were x400.

on 96 well plates at $5x10^4$ cells/well and treated for 24 h with ethanol, bisdiamine, 4-methylpyrazole, or DMSO at concentrations ranging from 10-200 mmol/l, 10-200 μmol/l, 1-10 mmol/l, and 10-900 mmol/l, respectively. The averages for cells grown in the regular medium were considered as 100% viability, and no toxicity >10% was observed for ethanol between 10 and 100 mmol/l, bisdiamine between 10 and 100 μmol/l, 4-methylpyrazole treatment between 1 and 5 mmol/l, or DMSO treatment between 10 and 500 mmol/l.

Chloramphenicol acetyl transferase and β-galactosidase reporter gene assay: After incubation, human epithelial corneal cells were washed twice with PBS and treated with 700 μl of cell lysis buffer for 1 h at 4 °C. The lysed cells were scraped off and centrifuged at 950 g for five min at 4 °C. CAT was measured by immunoenzymatic assay (Roche Diagnostics, Meylan, France) on 200 μl of supernatant. In all the studies, CAT assays were normalized to β-galactosidase activity, which was determined according to the manufacturer's instructions. For this purpose, 20 μl of the supernatants were incubated with assay reagent based on an original protocol.

Statistical methods: Results, expressed as mean±SD were an average of 10 different experiments per condition. Comparison of means was performed by ANOVA and Fisher's t-test using Statview II 1.03 software (Abacus Concepts Inc., Berkeley, CA). For all the studies conducted, the level of significant difference was set at $p<0.05$.

RESULTS

Identification of the molecular and metabolic factors of the retinoid pathway in adult human ocular surface: To evaluate the expression patterns of nuclear receptors, binding proteins, and enzymes of the retinoid signaling pathways in human adult ocular surface, RT-PCR assays were first performed on total mRNA isolated from five corneas and ten conjunctivas. Reproducible results were obtained in both tissues (Table 2). RAR α, β, γ, and RXR α are expressed in both the cornea and conjunctiva. RXR β and γ were never detected in the cornea and conjunctiva. ADH3, ADH4, RALDH1, RALDH3, DHRS4, DHRS9, CRBP1, CRABP1, and CRABP2 were expressed simultaneously in the cornea and in the conjunctiva. ADH1, RALDH2, RALDH4, CYP26A, CYP26B, CYP26C, CRBP2, and epimerase were never detected in human ocular total cornea and conjunctiva. RODH4 was only expressed in conjunctiva and not detected in the cornea (Table 2).

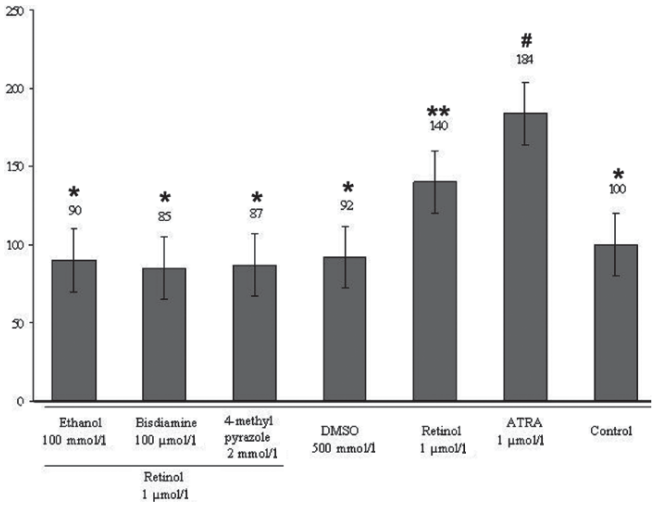

Figure 3. Retinol activation into retinoic acid in corneal epithelial cells. Corneal epithelial cells were transiently transfected with a retinoic-responsive CAT reporter gene (DR5-*tk*-CAT). CAT production was determined after 24 h incubation and normalized to β-galactosidase activity. The value of CAT induction was arbitrarily set at 100 for controls. Each value represents the mean±SD of 10 experiments. The left panel indicates inhibition by different toxins for activation of retinol into retinoic acid. Asterisks, the double asterisk, and the sharp (hash mark) indicate statistical differences between the different incubation conditions tested ($p<0.05$).

These initial data led us to better determine the expression patterns of the nuclear receptors, binding proteins, and enzymes of the retinoid signaling pathways in human corneal and conjunctival compartments at the cellular level. The major cells constituting the cornea and conjunctiva were analyzed using primary cultures or established cell lines such as the primary cell cultures of human corneal epithelium and corneal keratocytes, human corneal endothelial (HTCE) and epithelial (HCE) cell lines, and human epithelial conjunctival cell line (Chang). RAR α, RAR γ, RXR α, RALDH1, RALDH3, DHRS4, CRBP1, CRABP1, and CRABP2 were commonly expressed in corneal epithelial and endothelial cells as well as in keratocytes (Table 3). RAR β was never detected in corneal keratocytes. ADH3, ADH4, and DHRS9 were only detected in epithelial cells. Reproducible results were obtained for each analyzed type of cells. Both epithelial primary cell cultures and established cell lines (HCE) expressed the same nuclear receptors, enzymes, and binding proteins, suggesting that the HCE cell line could be used as a model for studying the biological roles of retinoids in the corneal epithelium. The molecular and metabolic actors previously detected in total conjunctiva (RAR α, RAR β, RAR γ, RXR α, ADH3, ADH4, RODH4, DHRS4, DHRS9, RALDH1, RALDH3, CRBP1, CRABP1, and CRABP2) were all found in human conjunctival epithelial cells.

Using frozen sections, we checked the protein expression and localization of RAR α, β, γ, and RXR α. The transcripts of these had been previously described. We found the four nuclear receptors expressed in the cornea and the conjunctiva (Figure 2A,C, and data not shown). The presence of RAR α, β, γ, and RXR α immunolabeling was also confirmed by analysis of the primary cultures of corneal epithelial and stromal cells and of corneal endothelial and conjunctival established cell lines (Figure 2E,G,H,J,K,L,N,O, and data not shown). Taken together, all the immunocytological and immunohistochemical assays confirmed the presence of the four nuclear receptor proteins, RAR α, β, γ, and RXR α, previously identified by RT-PCR.

Study of retinoic acid generation from retinol by corneal epithelial cells: The production of active retinoids by corneal epithelial cells after incubation with retinol was studied using transient transfections of a retinoid-sensitive reporter gene based on CAT expression and using the HCE cell line (Figure

3). First, we checked that the naive corneal epithelial cells (or corneal epithelial cells transfected with an empty vector) did not produce CAT. CAT production by cells transfected with the DR5-*tk*-CAT plasmid and not treated was considered as the baseline for detection of and comparison with recorded induction levels following retinoid exposure. *All-trans* RA treatments (1 μmol/l) for 24 h increased CAT response (1.8±0.2 fold induction), demonstrating the cellular functionality of retinoic acid signaling at the cellular level. CAT production was also significantly enhanced (1.4±0.1) after retinol (1 μmol/l) treatment but not as much as after stimulation with 1 μmol/l of RA. In addition, we found that epithelial corneal cells only generated the *all-trans* isomer of the retinoic acid from retinol (unpublished data obtained from HPLC experiments). To confirm that active retinoids were generated and catabolized by enzymatic conversion from retinol, we tested potential impairment of these reactions by inhibiting ADH and RALDH, respectively, using ethanol, bisdiamine, and 4-methylpyrazole (at nontoxic concentrations; see Methods). The absolute values of the internal standard (not dependent upon the enzymatic conversion caused by ADH and RALDH) did not change in response to ethanol, bisdiamine, or 4-methylpyrazole treatments (data not shown). This result shows the specific effect of ethanol, bisdiamine, and 4-methylpyrazole on the inhibition of retinoic acid generation and degradation. Ethanol (100 mmol/l), bisdiamine (100 μmol/l), and 4-methylpyrazole (2 mmol/l) all caused a statistically significant decrease in retinoic acid production by primary epithelial corneal cells as illustrated by the insignificant amount of CAT production between control cells and cells treated with retinol and one of the three inhibitors.

DISCUSSION

It has been extensively demonstrated that the cornea and the conjunctiva have an absolute requirement of vitamin A (retinol) thus establishing vitamin A deficiency as an important public health problem in many developing countries and triggering a spate of studies on the clinical uses of retinoids in ocular surface pathologies [5]. Curiously, partial characterization of the retinoid molecular and metabolic actors (RARs and RXRs) have been realized in rabbit, mouse, and human conjunctiva and cornea [2,15,16]. However, there has not yet been reports of systematic tissue and cellular expression patterns of these receptors nor have the metabolic activities on retinoids at the human ocular surface been demonstrated.

Therefore, for the first time, we established a complete expression pattern of RARs and RXRs in human cornea and conjunctiva and in the main cell types. Our data could be put to use to choose specific agonists and antagonists for RARs and RXR α types which could fine-tune a more selective pharmacological approach for treating ocular surface pathologies [24,25]. All three RARs and RXR α were colocalized in conjunctival and corneal tissues and cells, creating an opportunity for the active heterodimer RARs/RXR α to form in the human ocular surface. The establishment of this active heterodimer provides the rationale explaining how retinoic acid regulates previously studied target genes in the ocular surface environment: collagenase [26], α1-proteinase inhibitor [11], interleukin-1α [27], cytochrome P450 (CYP)4B1 [28], and mucins [13,15]. This functional heterodimer also underlies more global physiological effects of the retinoids already described in the cornea such as reduced apoptosis and oxidative stress [29,30] as well as in the conjunctiva (trans-differentiation of epithelial cells [31]). RXR β was present in rabbit and mice eyes but absent from the human ocular surface. This differential expression between the species had to be linked with the absence of phenotype in RXR β null mutant mice, suggesting a weak implication of this nuclear receptor in mammalian ocular physiology. By contrast, the systematic expression of RAR α, RAR γ, and RXR α in all three species (mouse, human, and rabbit) strongly suggests their roles in eye physiology and development, as previously illustrated by the ocular phenotype of the RAR α-, RAR γ-, and RXR α-null mutant mice [32]. Our results concerning RAR β could be considered contradictory to those obtained on human ocular surface by Gipson's group [15]. However, this receptor was found to be expressed in the rabbit cornea and conjunctiva [16]. Furthermore, while certain conflictual results have been found between both conjunctival cell lines, it should be underlined that the concerned studies employed two essentially different cell lines: the telomerase-immortalized human conjunctival epithelial cell line (HCjE) for Hori et al. [13] and the Wong-Kilbourne derivative of Chang conjunctiva, (clone 1-5c-4/ATCC CCL-20.2) for our work, thus establishing the relative heterogeneity of conjunctival cell lines in terms of global mRNA expression patterns.

We have shown for the first time that the corneal epithelial cells are able to produce retinoids at functional levels from retinol, which has already been reported to be present in tears fluid [21]. We established the presence of the cytoplasmic binding proteins (CRBP and CRABP) implicated in this retinoid metabolic pathway. Our results are in agreement with those previously obtained results using radiolabeled retinoid assays, which formed the basis of cytosolic retinoid binding capacities [19,33]. Three other mammalian ocular surface structures have also been shown to produce retinoic acid from retinol: the rabbit [34], the rat [35], and the mouse [36]. For each determination, the induction obtained from retinol was always less than from exposure to *all-trans* retinoic acid. This could illustrate and explain (1) why Gipson's group did not find the same induction of mucin genes when they used calf serum (containing almost retinol) or retinoic acid diluted in DMSO [15], and (2) why retinoic acid is more efficient than retinol in treating xerophthalmia [37]. Our study also clearly demonstrates that the generation of retinoic acid from retinol can be impaired by a toxin like ethanol. This point will need to be emphasized if retinol is to be used in eye droplets to treat ocular surface pathologies such as xerophthalmia or dry eye. If the galenic form contains ethanol to solubilize the retinol, this could impair its transformation into retinoic acids. The same reflection could also apply if these ocular pathologies were treated by systemic administration of retinol [38,39] as the treatment could be made inefficient by the absence of retinol activation against a background where ethanol is present, such

as chronic alcohol addiction. In this scenario, local retinoic treatment (correctly supported [40]) should be preferred. Nevertheless, this topical administration should be strongly followed up. Indeed, we have established an absence of members of the Cyp26 family, which represent one of the main degradation pathways of the retinoic acids thus excluding a physiological capacity to metabolize part of the excess retinoic acid delivery. The absence of such members of the Cyp26 family could also be correlated to potential defects induced by excessive systemic retinoic acid administration during development [41] as well as in adult life [42]. The expression patterns of RA metabolism-associated binding proteins and enzymes found in the corneal endothelial cells suggests that this cellular compartment is also able to produce retinoic acid from retinol. The in situ controlled generation of retinoic acid could modulate the mitogenic effect of epidermal growth factor (EGF) [12] underlining the importance of retinoids produced endogenously in the maintenance of endothelial integrity. These endogenous retinoids could also protect against the free-radical-induced oxidation injury, lipid peroxidation, and consequent endothelial apoptosis [29].

In conclusion, we have identified the metabolic and molecular actors of the retinoid signaling pathway in the adult human ocular surface. Alterations of this metabolic and molecular pathway may be determinant factors in ocular surface physiology and may offer a molecular basis for the explanation and treatment of eye pathologies.

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