Universidad de Valladolid

FACULTAD DE MEDICINA

INSTITUTO DE OFTALMOBIOLOGÍA APLICADA

TESIS DOCTORAL

Desarrollo de un modelo tridimensional de conjuntiva humana con matrices biocompatibles para el estudio de enfermedades inflamatorias

(Development of a three-dimensional model of human conjunctiva with biocompatible matrices to study inflammatory diseases)

Presentada por LAURA GARCÍA POSADAS para optar al grado de doctora por la Universidad de Valladolid

Dirigida por:

Dra. YOLANDA DIEBOLD LUQUE

2015

Universidad de Valladolid

Impreso 2T

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Valladolid, 30 de junio de 2015

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AGRADECIMIENTOS / *ACKNOWLEDGMENTS*

Decía el gran escritor José Saramago que "*El viaje no acaba nunca. Solo los viajeros acaban. E incluso estos pueden prolongarse en memoria, en recuerdo, en relatos*." Esta tesis ha sido un largo viaje que he tenido el placer de hacer acompañada de gente maravillosa. Y todos esos viajeros que hoy dejo atrás, y que por supuesto "se prolongarán en mi memoria", merecen ser reconocidos aquí.

La primera persona a la que debo dar las gracias es a mi directora de tesis. Gracias, Yolanda, por darme la primera oportunidad, y por permitirme equivocarme. Solo así se puede avanzar, crecer y mejorar. Que alguien apueste por ti cuando aún no has podido demostrar nada es lo más difícil. Y por eso quiero darte las gracias. Por eso, y por mantener esa confianza en el momento que más lo necesitaba. Gracias por tu paciencia, y por tener siempre "un minuto" cuando llamaba a tu puerta.

Otra persona muy especial a la debo y quiero agradecer muchas cosas es la profesora Calonge. Marga, gracias por tu infinita paciencia con versiones y revisiones de lo mismo, y por enseñarme que esa "sensación" que tenía en los ojos no era normal. Pero sobre todo, gracias por ser como eres, porque en innumerables ocasiones me has recordado a mi madre, y ese es el mayor piropo que le puedo dedicar a nadie.

Gracias también al profesor Pastor. Porque sin él el IOBA no existiría, y sin el IOBA yo no sería quien soy. ¡Gracias, jefe!

Gracias a todos los "pequeños", que en realidad sois los más grandes. Vosotros sois los que día a día habéis hecho posible esta tesis. Necesitaría un libro entero para agradeceros todo lo que tengo que agradecer. En resumen, gracias por las mañanas, por los cafés, por las comidas, por más cafés, por las cenas, por las noches, por las copas,… Gracias por formar parte de mi vida y haberme hecho reir cuando creía que no podía, y llorar cuando pensaba que no debía. Probablemente debería nombraros a todos y cada uno de vosotros aquí, pero sois demasiados. Espero ser capaz de agradeceros todo en persona. Dejadme, eso sí, que nombre al menos al NanoTeam. Ese gran grupo que "triunfaba" en baloncesto, que es mayoría siempre en el café, que gana (¿o debo decir ganaba?) en las cenas y

copas… Y especialmente quiero destacar a tres personas sin las cuales nada hubiera sido igual. Gracias a nuestra “Doc”, Laura Contreras, por hacer que mi llegada a este grupo fuera tan fácil, y por seguir siempre dispuesta a ayudar en todo por mucho que pasen los años y miles de kilómetros nos separen. Lo reconoceré aquí, sin que sirva de precedente: “You are Laura 1st!” Gracias también a Laura Soriano, por ser tan buena persona. Por tener siempre una sonrisa, y por esos detalles que te hacen maravillosa. No cambies nunca. Y por último, pero no menos importante, ¡gracias Antonio! Sin tu ayuda esto no hubiera sido posible.

(*) Quiero dar las gracias muy especialmente a la profesora Darlene Dartt. Muchísimas gracias por tu ayuda todos estos años, por tu cariño, por tu paciencia con mi inglés y con mi desconocimiento del mundo de la señalización celular. Gracias por lograr que a miles de kilómetros de mi ciudad y de mi gente me sintiera como en casa. Y por el equipo increíble que tienes a quien también le agradezco toda su ayuda, especialmente a Robin Hodges.

(*) *Dear Darlene, this is specially translated for you. Thank you so much for your help and support all these years, for your kindness, for your patience with my English and my ignorance about cell signaling, and for making me feel like I were at home, even when I was thousands of miles away! Thank you also for letting me be part of your incredible team. I want to thank all of them for its help, specially Robin Hodges.*

Gracias a toda la gente que de una u otra manera ha ayudado en esta tesis. Al resto de personal del IOBA, a la gente de la Universidad de Santiago de Compostela, a la del Banco de Tejidos de la Fundación Clínica San Francisco de León, al grupo del profesor Friedrich Paulsen, a toda esa gente maravillosa de Boston… A todos vosotros, y a todos los que no he nombrado: gracias.

Y ya entrando en el terreno más personal, también quiero dar las gracias a ese magnífico grupo de “biotecs” que me llevaron en volandas durante la carrera y me trajeron hasta aquí. Fueron tiempos muy duros para mí que superé gracias a vuestra ayuda. En especial gracias a Sofía, a Maru, a Celia, a Raquel,…

Por supuesto gracias a los verdaderos amigos, esos que están en los buenos momentos y sobre todo en los malos. Con los que parece que no pasa el tiempo aunque lleves meses sin verte. Gracias Vir, por tu ayuda en todo. Gracias, Ainhoa,

por seguir estando tan “loca” como el primer día. Gracias, Ana, por estar a mi lado desde aquellos tiempos en los que hablar de “vaqueros” no era hablar precisamente de pantalones.

Quiero dar las gracias también a mi familia. Mi gran familia. También vosotros sois muchos, así que permitidme que sólo nombre aquí a dos. Gracias Bea, por estar siempre dispuesta a ayudarme con mi “spanglish”. Y gracias Jaime, por el diseño de la portada. Algún día esta tesis valdrá mucho solo por eso.

Debo un agradecimiento muy especial, por supuesto, a mis padres. Mamá, papá, sois sin lugar a duda mi modelo a seguir en esta vida, y los mejores padres que nadie puede tener. Gracias por vuestro apoyo constante e incondicional.

Javi, a ti ya no sé ni qué decirte. Gracias por todo. Por TODO. Por ser como eres, por quererme; y por dejarme quererte. Por haber dedicado todas tus fuerzas en hacerme sonreir una y otra vez; y no sólo a mi. Por no permitirme flaquear cuando no debía, y por dejarme hundirme cuando lo necesitaba; y por rescatarme. Por tu apoyo incondicional a mi carrera. Por no enfadarte cuando estando de vacaciones me dedicaba a trabajar. Por animarme siempre a seguir, a marcharme fuera cuando me necesitabas a tu lado, y por continuar dispuesto a apoyarme. Espero poder devolverte todo esto en algún momento.

A mi hermano, Héctor. Por habernos regalado 17 años maravillosos. 17 años que saben a poco, sí, a muy poco, pero son más que suficiente para haberme dado cuenta de que soy la persona más afortunada, pues soy la única que puede presumir de haber tenido al mejor hermano del mundo. Objetivamente. Todo el que te conoció sabe que es así. Sin que te dieras cuenta me enseñaste con tu ejemplo tantas actitudes necesarias para esta profesión, que me hacen sentir que puedo con todo. Esta tesis es tuya, porque desde que te fuiste, sé que de alguna manera estás en mi dándome fuerza. Y solo gracias a eso me levanto cada mañana, y sonrío. Gracias, hermano. Solo puedo terminar estos agradecimientos con algo que solo tú y yo entenderemos: “Yo, rey de los Filipinos, en base a lo que hagamos, te regalo la botella.”

“Uno, recuerda mirar hacia las estrellas y no hacia abajo, a tus pies. Dos, nunca dejes de trabajar. El trabajo le da significado y propósito a la vida y sin él está vacía. Tres, si tienes la suerte de encontrar el amor, recuerda que está ahí y no lo tires a la basura.”

“*One, remember to look up at the stars and not down at your feet. Two, never give up work. Work gives you meaning and purpose and life is empty without it. Three, if you are lucky enough to find love, remember it is there and don't throw it away*.”

— **Stephen Hawking**

A mis padres que tanto me han apoyado,
a Javi, que siempre está a mi lado, y
a mi hermano, por ser mi ángel y mi inspiración.

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PREFACIO/*PREFACE*

“Todo principio no es mas que una continuación,
y el libro de los acontecimientos se encuentra siempre abierto a la mitad.”

“Every beginning is only a sequel, after all,
and the book of events is always open halfway through.”

— Wisława Szymborska

Biografía Breve / *Short Biography*

Laura García Posadas se licenció en Biotecnología en la Universidad de Salamanca en 2009. Después, finalizó el Máster en Investigación en Ciencias de la Visión de la Universidad de Valladolid en 2010. A continuación empezó a trabajar en el Instituto de Oftalmobiología Aplicada (IOBA) como investigadora predoctoral, donde ha estado realizando su tesis doctoral el el programa de doctorado en Ciencias de la Visión. Además, actualmente está finalizando los estudios de Grado en Psicología en la Universidad Nacional de Educación a Distancia (UNED).

Desde su incorporación al IOBA ha publicado varios artículos sobre inflamación de la superficie ocular y cultivos primarios, y ha presentado su trabajo en varios congresos internacionales. Ha realizado varias estancias de investigación en el *Schepens Eye Research Institute* (EE. UU.) y en *Friedrich-Alexander Erlangen Nüremberg University* (Alemania).

Laura García Posadas graduated with a Biotechnology Bachelor's degree from the University of Salamanca in 2009. Then, she obtained a Master's degree in Visual Sciences from the University of Valladolid. Afterwards, she has been working as a predoctoral researcher in the Institute of Applied Ophthalmobiology (Spanish IOBA), where she is doing a PhD in Visual Sciences. In addition, she is finishing a Bachelor degree in Psychology, in the National University of Distance Education (UNED).

Since her arrival to IOBA, she has published several papers related to ocular surface inflammation and primary cultures, presenting her work in International conferences. She has conducted research stays in Schepens Eye Research Institute (USA), and in Friedrich-Alexander Erlangen Nüremberg University (Germany).

Financiación / *Funding*

Este trabajo de investigación se ha realizado gracias a la financiación de los siguientes proyectos:

- **Barrera corneal, inflamación y terapia potencial.** Acción Integrada con Alemania, DE2009-0085, Ministerio de Ciencia e Innovación. Investigador responsable: Dra. Yolanda Diebold Luque (España) y Dr. Friedrich Paulsen (Alemania). Duración: 01/01/2010 - 31-12-2012

- **Combinación de hidrogeles biomiméticos y nanopartículas como plataformas terapéuticas avanzadas para enfermedades oculares.** Ministerio de Ciencia e Innovación, CICYT MAT-2010-20452-C03-01. Investigador responsable: Dra. Yolanda Diebold Luque**.** Duración: 01/01/2011 – 31/12/2013

- **Identificación de dianas terapéuticas para la reparación de la barrera corneal: diseño de una nueva terapia antiinflamatoria.** Junta de Castilla y León, Convocatoria de la Consejería de Educación de Subvenciones del Programa de Apoyo a Proyectos de Investigación, VA132A11-2. Investigador responsable: Dra. Yolanda Diebold Luque**.** Duración: 30/03/2011 – 30/12/2013

- **Drug Discovery and Development of Novel Eye Therapeutics (3D-NET).** FP7-PEOPLE-2013-IAPP, Marie Curie Industry-Academia Pathways and Partnerships (IAPP) action, Grant Agreement nº. 612218**.** Investigador responsable: Coordinador: Dr. Breandan Kennedy (UCD); IP de la UVA: Dra. Yolanda Diebold Luque. Duración: 1/09/2013 – 31/08/2017

- **Desarrollo de aproximaciones terapéuticas basadas en plataformas nanotecnológicas avanzadas diseñadas específicamente para combatir la ceguera corneal (NanoCorneal).** Ministerio de Economía y Competitividad,

MAT2013-47501-C2-1-R. Investigador responsable: Dra. Yolanda Diebold Luque, Coordinadora. Duración: 01/01/2014 – 31/12/2017

Además, durante el desarrollo de esta tesis doctoral he recibido la siguiente financiación personal de distintos organismos:

- **Beca de iniciación a la investigación del CIBER-BBN**
  (01/06/2009 – 31/05/2010)
- **Beca de colaboración con cargo a proyecto de la Universidad de Valladolid**
  (01/06/2010 – 21/09/2010)
- **Beca de formación de personal investigador de la Universidad de Valladolid**
  (22/09/2010 – 31/07/2011)
- **Ayuda para la formación de personal investigador (FPI) del Ministerio de Economía y Competitividad** (antiguamente Ministerio de Ciencia e Innovación)
  (01/08/2011 – 31/07/2015)
- **Ayuda por la asistencia a cursos, congresos y jornadas relevantes para el desarrollo de tesis doctorales de la Universidad de Valladolid (2012)**
  Asistencia a 2012 *Annual Meeting of the Association for Research in Vision and Ophthalmology* (ARVO), Fort Lauderdale, Florida, EE. UU.
- **Ayuda para la realización de estancias breves en centros de I+D, del subprograma FPI-MICINN (2012)**
  Estancia realizada en *Schepens Eye Research Institute*, Boston, Massachusetts, EE. UU.
- **Ayuda para la realización de estancias breves en centros de I+D, del Ministerio de Economía y Competitividad (2014)**
  Estancia realizada en *Schepens Eye Research Institute*, Boston, Massachusetts, EE. UU.

Contribuciones Científicas / *Scientific Contributions*

El trabajo realizado en esta tesis ha dado lugar a las siguientes publicaciones científicas en revistas indexadas / *Publications out of this thesis*:

1. **Laura García-Posadas**, Laura Contreras-Ruíz, Antonio López-García, Sonia Villarón Álvarez, Miguel Maldonado, Yolanda Diebold. Hyaluronan receptors in the human ocular surface: a descriptive and comparative study of RHAMM and CD44 in tissues, cell lines and freshly collected samples. *Histochemistry and Cell Biology* **2012**; 137:165-176. Índice de Impacto (JCR 2012) = 2.613; Posición de la revista y área: 1/9 Microscopy (**Q1**); 113/185 Cell Biology (**Q3**)

2. **Laura García-Posadas**, Isabel Arranz-Valsero, Antonio López-García, Laura Soriano-Romaní, Yolanda Diebold. A new human primary epithelial cell culture model to study conjunctival inflammation. *Investigative Ophthalmology & Visual Science* **2013**; 54:7143-7152. Índice de Impacto (JCR 2013) = 3.661; Posición de la revista y área: 6/58 Ophthalmology (**Q1**)

3. **Laura García-Posadas**, Laura Contreras-Ruiz, Isabel Arranz-Valsero, Antonio López-García, Margarita Calonge, Yolanda Diebold. CD44 and RHAMM hyaluronan receptors in human ocular surface inflammation. *Graefe's Archive for Clinical and Experimental Ophthalmology* **2014**; 252:1289-1295. Índice de Impacto (JCR 2013) = 2.333; Posición de la revista y área: 20/58 Ophthalmology (**Q2**)

4. **Laura García-Posadas**, Laura Contreras-Ruiz, Laura Soriano-Romaní, Darlene Dartt, Yolanda Diebold. Conjunctival goblet cell function: effect of contact lens wear and cytokines. *Eye & Contact Lens* **2015**. **Revisión invitada**. *In press.* Índice de Impacto (JCR 2013) = 1.679; Posición de la revista y área: 30/58 Ophthalmology (**Q3**)

5. **Laura García-Posadas**, Robin R. Hodges, Dayu Li, Marie A. Shatos, Thomas Storr-Paulsen, Yolanda Diebold, Darlene A. Dartt. Interaction of IFN-γ with cholinergic agonists to modulate rat and human goblet cell function. *Mucosal Immunology* **2015**. *In press.* Índice de Impacto (JCR 2013) = 7.537; Posición de la revista y área: 12/144 Immunology (**Q1**)

Además, el trabajo realizado se ha presentado parcialmente en los siguientes congresos nacionales o internacionales / *Congress contributions:*

1. Antonio López García; **Laura García Posadas**; Sonia Villarón Álvarez; Laura Contreras Ruiz; Isabel Arranz Valsero; Yolanda Diebold. "Citología por cepillado conjuntival (CCC): método incruento de recogida de células de la superficie ocular para su caracterización fenotípica". Congreso de la Asociación Española de Técnicos de Laboratorio (AETEL) 2010, Oviedo, España.

2. **Laura García Posadas**; Laura Contreras Ruiz; Isabel Arranz Valsero; Antonio López García; Margarita Calonge; Yolanda Diebold. "Implication of CD44 and RHAMM hyaluronan receptors in human ocular surface inflammation". The *Association for Research in Vision and Ophthalmology* (ARVO) 2012 Annual Meeting, Fort Lauderdale, EE. UU.

3. **Laura García Posadas**; Dayu Li; Robin R. Hodges; Marie A. Shatos; Yolanda Diebold; Darlene A. Dartt. "Differential effect of Th1 and Th2-type cytokines on rat conjunctival goblet cell function". ARVO 2013 Annual Meeting, Seattle, EE. UU.

4. Antonio López García; **Laura García Posadas**; Margarita Calonge Cano; Yolanda Diebold Luque. "Receptor de ácido hialurónico RHAMM como posible biomarcador de enfermedades inflamatorias de la superficie ocular". AETEL 2013, Alicante, España.

5. **Laura García-Posadas**; Isabel Arranz-Valsero; Ana Fernández; Antonio López-García; Javier Iglesias; Yolanda Diebold. "Development of a bioengineered 3D-model of human conjunctiva". *Tissue Engineering &*

Regenerative Medicine International Society TERMIS-EU 2013, Estambul, Turquía.

6. **Laura García-Posadas**; Isabel Arranz-Valsero; Ana Fernández; Antonio López-García; Javier Iglesias; Yolanda Diebold. "A fibrin-based 3D-model of human conjunctiva".*Functional Analysis & Screening Technologies* (FAST congress) organizado por Cambridge Healthtech Institute FAST Congress 2013, Boston, EE. UU.

7. **Laura García-Posadas**; Dayu Li; Robin R. Hodges; Marie A. Shatos; Yolanda Diebold; Darlene A. Dartt. "Human goblet cell function in an in vitro allergic microenvironment". ARVO 2014 Annual Meeting, Orlando, EE. UU.

8. Yolanda Diebold; **Laura García-Posadas**; Laura Soriano-Romaní; Isabel Arranz-Valsero; Antonio López-García. "Characterization of human conjunctival cells grown in a 3D-model" ARVO 2014 Annual Meeting, Orlando, EE. UU.

9. Antonio López García; **Laura García Posadas**; Laura Soriano Romaní; Yolanda Diebold. "Construcción de tejidos tridimensionales de la superficie ocular usando matrices de fibrina". AETEL 2014, Córdoba, España.

10. **Laura García-Posadas**; Laura Soriano-Romaní; Isabel Arranz-Valsero; Antonio López-García; Yolanda Diebold. "Three dimensional model of human conjunctiva to study oclular surface inflammation". 112th Congress of the German Ophthalmology DOG Congress 2014, Leipzig, Alemania.

11. **Laura García-Posadas**; Laura Soriano-Romaní; Antonio López-García; Margarita Calonge; Yolanda Diebold. "Soluble hyaluronan receptor CD44 secreted by conjunctival cells change in inflammatory conditions". ARVO 2015 Annual Meeting, Denver, EE. UU.

Abreviaturas / *Abbreviations*

$[Ca^{2+}]_i$	Concentración de calcio intracelular	*Intracellular calcium concentration*
3D	Tridimensional	*Three-dimensional*
Ab	Anticuerpo	*Antibody*
ADNc/cDNA	Ácido desoxiribonucleico complementario	*Complementary desoxiribonucleic acid*
AH/HA	Ácido hialurónico	*Hyaluronan acid*
AKC	Queratoconjuntivitis atópica	*Atopic keratoconjunctivitis*
ARNm/mRNA	Ácido ribonucleico mensajero	*Messenger ribonucleic acid*
BAC/BAK	Cloruro de benzalconio	*Benzalkonium chloride*
BC	Citología por cepillado	*Brush cytology*
BCA	Ácido bicinconínico	*Bicinchoninic acid*
bp	Pares de bases	*Base pairs*
BSA	Albúmina sérica bovina	*Bovine serum albumin*
CALT	Tejido linfoide asociado a la conjuntiva	*Conjunctival-associated lymphoid tissue*
Cch	Carbacol	*Carbachol*
CERS	Células epiteliales corneales obtenidas tras cirugía refractiva	*Corneal epithelial cells obtained after refractive surgery*
CFE	Eficiencia de formación de colonias	*Colony forming efficiency*
CK	Citoqueratina	*Cytokeratin*
DMEM	Medio de cultivo Eagle modificado de Dulbecco	*Dulbecco's modified Eagle's medium*
EALT	Tejido linfoide asociado al ojo	*Eye associated lymphoid tissue*
ECM	Matriz extracelular	*Extracellular matrix*
EDTA	Ácido etildiaminotetraacético	*Ethylenediaminetetraacetic acid*
EGF	Factor de crecimiento epidérmico	*Epidermic growth factor*

ELISA	Ensayo de inmunoabsorción enzimática	*Enzyme-linked immunosorbent assay*
ELLA	Ensayo con lectina ligado a enzima	*Enzyme-linked lectin assay*
EMT	Transición epitelio mesénquima	*Epithelial mesenchymal transition*
ERK	Quinasa regulada por señal extracelular	*Extracellular signal-regulated-kinases*
FBS	Suero fetal bovino	*Fetal bovin serum*
GAPDH	Gliceraldehído 3 fosfato deshidrogenasa	*Glyceraldehyde-3-phosphate dehydrogenase*
GPC	Conjuntivitis papilar gigante	*Giant papillar conjunctivitis*
GVHD	Enfermedad injerto contra huesped	*Graft versus host disease*
H/E	Hematoxilina/eosina	*Hematoxylin/eosin*
HARC	Complejo receptor de ácido hialurónico	Hyaluronic acid receptor complex
HBD9	Defensina humana beta 9	*Human beta defensin 9*
HCE	Línea celular de epitelio corneal humano	*Human corneal epithelial cell line*
HPA	*Helix pomatia agglutinin*	*Helix pomatia agglutinin*
IF	Inmunofluorescencia	*Immunofluorescence*
IFN-γ	Interferón gamma	*Interferon gamma*
Ig	Inmunoglobulina	*Immunoglobulin*
IL	Interleuquina	*Interleukin*
IOBA-NHC	Línea celular de epitelio conjuntival humano	*Normal human conjunctival epithelium cell line*
IP3	Inositol trifosfato	*Inositol trisphosphate*
KRB	Krebs-Ringer bicarbonato	*Krebs-Ringer bicarbonate*
LDALT	Tejido linfoide asociado al sistema de drenaje lagrimal	*Lacrimal drenaige associated lymphoid tissue*
MALT	Tejido linfoide asociado a mucosas	*Mucosal associated lymphoid tissue*

MAPK	Proteín quinasa activada por mitógenos	*Mitogen activated protein kinase*
MUC	Mucina	*Mucin*
OCT	*Optimal Cutting Temperature*	*Optimal Cutting Temperature*
PAS	Ácido periódico de Schiff	*Periodic acid Schiff*
PBS	Tampón fosfato salino	*Phosphate buffered saline*
PCL	Policaprolactona	*Polycaprolactone*
PI-3K	Fosfatidil inositol 3 quinasa	*Phosphoinositide 3 kinase*
PIP2	Fosfatidilinositol bifosfato	*Phosphatidylinositol biphosphate*
PLA	Ácido poli-láctico	*Poly-lactic acid*
PLGA	Ácido poliláctico co-glicólico	*Poly-lactic-co-glycolic acid*
RHAMM	Receptor de la motilidad mediada por ácido hialurónico	*Receptor for hyaluronan mediated motility*
RIPA	Ensayo de radioinmunoprecipitación	*Radioinmmunoprecipitation asssay*
rRE	Retículo endoplasmático rugoso	*Rough endoplasmic reticulum*
RT	Temperatura ambiente	*Room temperature*
RT-PCR	Reacción en cadena de la polimerasa con retrotranscripción	*Reverse transcription polymerase chain reaction*
sCD44	CD44 soluble	*Soluble CD44*
SEM	Microscopía electrónica de barrido	*Scanning electron microscopy*
SJS	Síndrome de Stevens Johnson	*Steven's Johnson syndrome*
SS	Síndrome de Sjögren	*Sjögren's syndrome*
TGF-β	Factor de crecimiento transformante beta	*Transforming growth factor beta*
Th	T colaboradora	*T helper*
TNF-α	Factor de necrosis tumoral alfa	*Tumor necrosis factor alpha*
Treg	T reguladora	*T regulator*
UEA-1	*Ulex europaeus* aglutinina I	*Ulex europaeus agglutinin I*
UFL/LFU	Unidad funcional lagrimal	*Lacrimal functional unit*
VKC	Queratoconjuntivitis vernal	*Vernal keratoconjunctivitis*

WB	Western *blotting*	*Western blotting*
ZO	*Zonula occludens*	*Zonula occludens*

RESUMEN EN ESPAÑOL

“Nada en la vida deber ser temido, solamente comprendido.
Ahora es el momento de comprender más, para temer menos”

“*Nothing in life is to be feared, it is only to be understood.*
Now is the time to understand more, so that we may fear less.”

— Marie Skłodowska-Curie

SINOPSIS DEL TRABAJO

En esta tesis doctoral se presenta un nuevo modelo tridimensional de conjuntiva humana. El desarrollo de un nuevo modelo es necesario para superar las limitaciones de los modelos *in vitro* disponibles y, permitir con ello, reducir el número de animales de experimentación que son usados en los ensayos *in vivo* sobre enfermedades inflamatorias de la superficie ocular.

Para desarrollar este nuevo modelo, primero se elaboró un protocolo optimizado para cultivar células epiteliales y fibroblastos a partir de muestras de tejido conjuntival obtenido de donante cadáver. Además, se llevaron a cabo algunos experimentos para seleccionar un biomaterial que resultara adecuado para mantener viables a las células y que sirviera como andamio para el modelo tridimensional de conjuntiva. Las matrices de fibrina resultaron adecuadas para este propósito y fue el biomaterial escogido.

Los fibroblastos se cultivaron en el interior de las matrices de fibrina y las células epiteliales encima. Dichas células epiteliales fueron capaces de estratificar, alcanzando 5-6 capas a los 14 días de cultivo sobre las matrices. Además, se observaron algunas células de tipo caliciforme. Una vez que el modelo tridimensional estuvo puesto a punto, se llevó a cabo una prueba de concepto para demostrar su utilidad para estudiar enfermedades inflamatorias de la superficie ocular.

El modelo tridimensional fue expuesto a distintas condiciones, como el cultivo aireado, la desecación o el tratamiento con citoquinas. Después de la exposición a estas condiciones adversas, se analizaron los cambios producidos en el modelo, tanto los estructurales como en los marcadores celulares. Tanto las condiciones experimentales como los marcadores a analizar fueron seleccionados gracias al conocimiento adquirido en estudios de inflamación en los distintos tipos de células conjuntivales. En dichos estudios, se comprobó la implicación de los receptores de ácido hialurónico CD44 y RHAMM en enfermedades inflamatorias de la superficie ocular, y se analizó también el papel de varias citoquinas sobre las células caliciformes. Los resultados obtenidos en el modelo tridimensional fueron acordes a lo esperado teniendo en cuenta los datos obtenidos en pacientes con patologías

oculares. Por ejemplo, cuando el modelo fue expuesto a IL-13, los niveles de secreción de MUC5AC aumentaron, tal y como ocurre en pacientes con conjuntivitis alérgica donde esta citoquina está elevada. Cuando el modelo se sometió a condiciones de desecación, se encontró un descenso en la expresión de RHAMM, al igual que ocurría en pacientes con síndrome de Sjögren o rosácea.

Los resultados obtenidos en los distintos estudios que componen esta tesis indican que el nuevo modelo tridimensional desarrollado puede ser una herramienta útil para llevar a cabo estudios relacionados con la inflamación conjuntival.

ESTRUCTURA DE LA MEMORIA DE TESIS

Esta tesis se presenta en la modalidad **compendio de publicaciones** y opta a la mención de "**doctorado internacional**". La normativa de la Universidad de Valladolid para esta modalidad exige que: 1) "incluya una introducción, de al menos veinte páginas, que justifique la relación temática de las publicaciones, contenga los objetivos perseguidos, la metodología empleada, los resultados obtenidos y las conclusiones más relevantes"; 2) "debe incluir al menos tres artículos aceptados para su publicación en revistas científicas con factor de impacto"; y 3) "se debe haber realizado una estancia de investigación en un centro extranjero durante al menos 3 meses". El primer requisito se cumple en los apartados "Resumen en español" (páginas 15-70), y "*Summary in English*" (páginas 71-110).

En esta tesis se han incluido un total de ocho estudios (Figura 1), de los cuáles cinco han dado lugar a artículos que están publicados o en prensa (estudios 1-5) y otros tres que están en preparación (estudios 6-8), con lo que se cumple el segundo requisito.

Por último, el cuarto estudio de esta tesis se realizó en el instituto de investigación *Schepens Eye Research Institute*, en Boston (Estados Unidos), cumpliendo con ello el tercer requisito. Los resultados obtenidos del trabajo realizado allí han dado lugar a una publicación científica que se recoge en el estudio 4. Debido a la inclusión de artículos de investigación publicados, y al carácter internacional de la tesis, la mayor parte de esta memoria se ha escrito en inglés.

Después del resumen general, incluido tanto en español como en inglés, esta memoria de tesis se organiza en tres capítulos temáticos en los que se incluyen los ocho estudios (Figura 1).

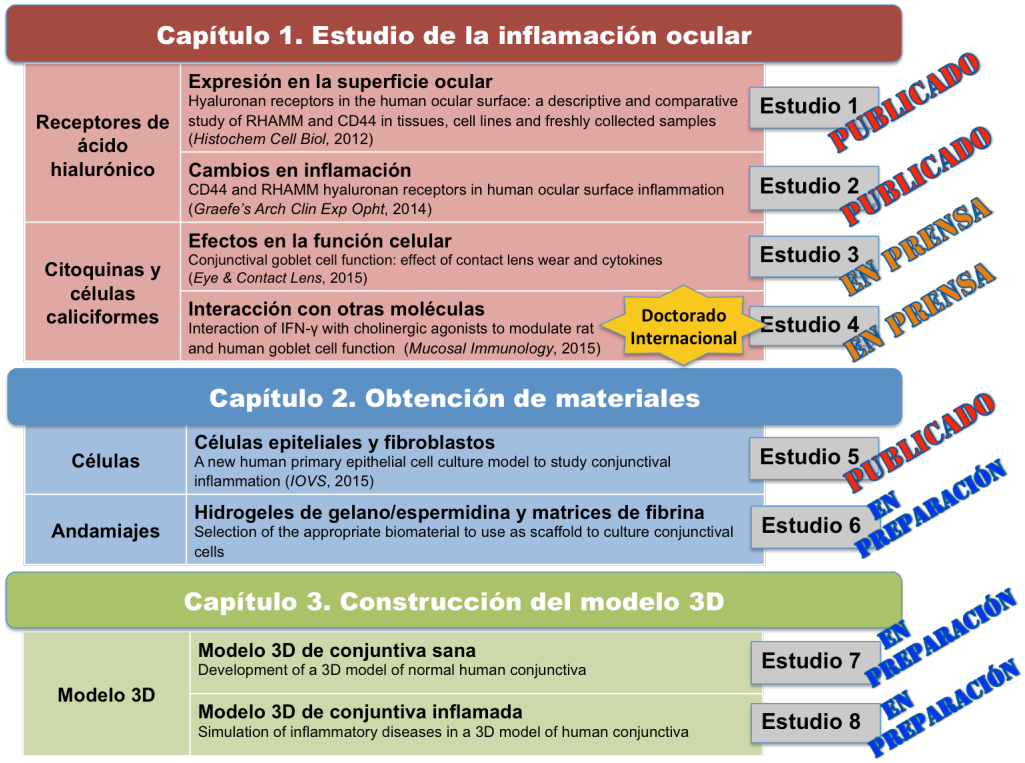

Figura 1. Esquema en el que se incluyen los distintos estudios de esta tesis doctoral agrupados en 3 capítulos.

En el **Primer Capítulo**, "**Estudio de moléculas relevantes en la inflamación ocular**", se han incluido cuatro estudios (que han dado lugar a cuatro artículos) en los que se analiza el papel de moléculas inflamatorias en distintas células conjuntivales. El **estudio 1** es un estudio descriptivo de la expresión de CD44 y RHAMM en los tejidos de la superficie ocular. Se consideró necesario realizar este análisis puesto que el ácido hialurónico se usa en muchos tratamientos oculares, pero sus receptores apenas estaban estudiados a pesar de la demostrada implicación de los mismos en enfermedades extraoculares. Se comprobó que tanto CD44 como RHAMM se expresaban en las células del epitelio conjuntival, y que los niveles de expresión en líneas celulares diferían notablemente de los obtenidos en células procedentes de citologías. Una vez realizado este análisis, se desarrolló el trabajo recogido en el **estudio 2**. En él, se analizó la expresión diferencial de estos receptores, fundamentalmente RHAMM, en células epiteliales recogidas mediante citología de pacientes con enfermedades inflamatorias. Los dos siguientes estudios

se centraron en el papel de las células caliciformes en inflamación. El **estudio 3** es una revisión bibliográfica del efecto de las lentes de contacto y distintas citoquinas en las células caliciformes de la conjuntiva. A continuación, teniendo en cuenta lo recogido en la revisión bibliográfica, en el **estudio 4** se llevó a cabo un estudio funcional en células caliciformes humanas y de rata. En él, se estudió el papel modulador del IFN-γ y el carbacol en la función de dichas células.

Los trabajos del primer capítulo arrojaron datos interesantes sobre el papel de las células conjuntivales en la inflamación. Sin embargo, para avanzar más en su estudio era necesario contar con un buen modelo *in vitro*. Con este objetivo, el **Segundo Capítulo** de esta tesis, "**Obtención de materiales para construir un nuevo modelo tridimensional de conjuntiva humana**", se centró en la búsqueda de los distintos componentes necesarios para desarrollar un modelo *in vitro* adecuado con el que continuar el estudio de la inflamación. Dentro de este bloque, en el **estudio 5** se presenta un protocolo para obtener y expandir fibroblastos y células epiteliales de conjuntiva humana a partir de muestras de donante cadáver. De esta forma se logró evitar el uso de líneas celulares. En el **estudio 6** se realizaron pruebas para determinar qué biomaterial resultaba más adecuado para usar como andamiaje de las células conjuntivales. Se concluyó que las matrices de fibrina eran una buena opción.

Finalmente, el **Tercer Capítulo** de esta tesis, "**Desarrollo de un modelo tridimensional de conjuntiva humana**" se compone de los dos últimos estudios. En el **estudio 7** se desarrolló el modelo tridimensional empleando matrices de fibrina y células epiteliales y fibroblastos conjuntivales. Se llevó a cabo una caracterización de dicho modelo. Por último, en el **estudio 8**, se expuso al modelo tridimensional a distintas condiciones para intentar simular algunas de las enfermedades inflamatorias de la conjuntiva y obtener una prueba de concepto de su utilidad para el estudio de sus enfermedades.

INTRODUCCIÓN

1. SUPERFICIE OCULAR Y UNIDAD FUNCIONAL LAGRIMAL

El término de superficie ocular fue introducido por primera vez por Richard A. Thoft en 1977 (Thoft y Friend, 1977), para referirse a la unidad anatómica y funcional formada por los epitelios de la córnea, la conjuntiva y el limbo, así como por la película lagrimal y los tejidos adyacentes. Su función principal es preservar la visión, protegiendo al globo ocular de la entrada de agentes nocivos a la vez que se mantiene la transparencia corneal.

Años más tarde, y siguiendo la misma filosofía de Thoft de aunar todas aquellas estructuras del ojo que funcionaban de manera conjunta, Michael E. Stern estableció el término de unidad funcional lagrimal (Stern *et al*., 1998). En este caso, a la superficie ocular se unieron las glándulas lagrimales y las interconexiones nerviosas, tanto sensoriales como motoras (Figura 2).

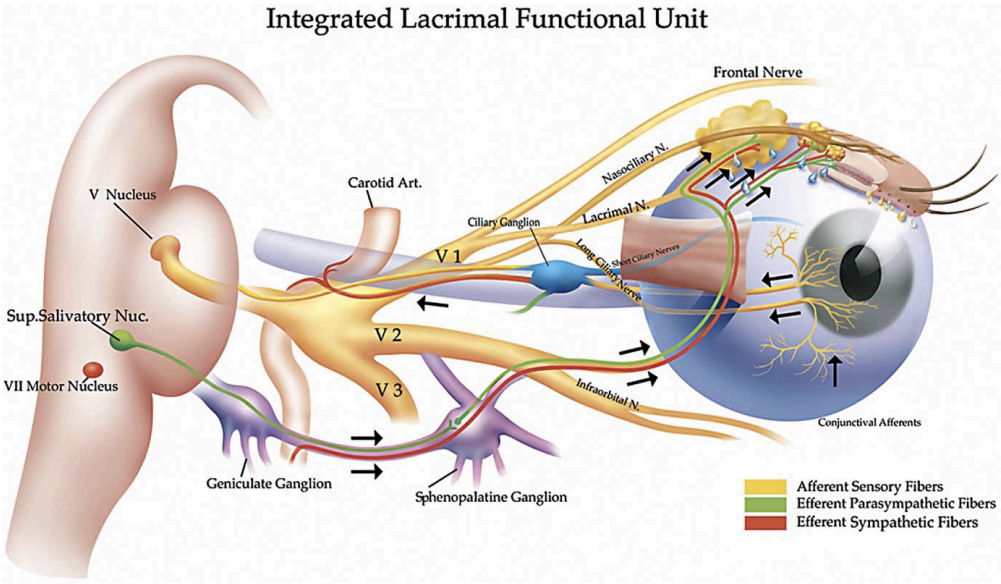

Figura 2. Unidad funcional lagrimal. Imagen tomada de Beuerman et al. The Lacrimal Functional Unit in Dry Eye and Ocular Surface Disorders (Pflugfelder, Beuerman, Stern eds.) Marcel-Dekker Inc., New York, 2004, 1004:11-40.

2. CONJUNTIVA

La conjuntiva es uno de los tejidos que forma parte de la superficie ocular y, por lo tanto, de la unidad funcional lagrimal. Se trata de la membrana semitransparente que cubre la superficie ocular desde el borde externo de los párpados hasta el limbo. Entre sus funciones destaca la producción de moco, que estabiliza la película lagrimal y ayuda a mantener la córnea húmeda y protegida (Dartt, 2006).

Anatómicamente, la conjuntiva se divide en tres zonas:

1) Tarsal o parpebral, que recubre la parte interior de los párpados
2) Bulbar, que cubre la esclera del segmento anterior del globo ocular
3) Fondo de saco o fórnix, que es la unión entre la conjuntiva tarsal y la bulbar

La conjuntiva está formada por un epitelio especializado que reposa sobre un estroma o sustancia propia (Figura 3).

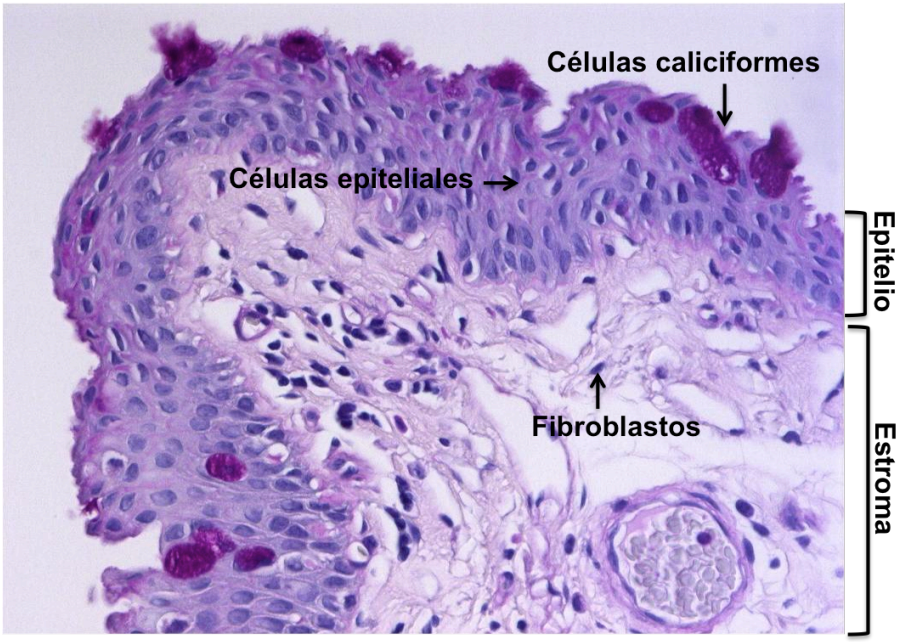

Figura 3. Fotografía de una sección de conjuntiva humana teñida con ácido periódico de Schiff (PAS). Se observa un epitelio estratificado con células caliciformes en las capas más superficiales, teñidas de color fucsia por la acción del colorante PAS. Imagen cedida por el laboratorio de Patología Ocular del IOBA.

2.1. Epitelio

El epitelio conjuntival es un epitelio cuboidal estraficado escamoso no queratinizado. Presenta entre 2 y 10 capas de células epiteliales, dependiendo de la zona de la conjuntiva de la que se trate (Hoang-Xuan et al., 2001). Hay cinco tipos de células epiteliales en la conjuntiva (Figura 4), que fueron descritas en 1989 por K. P. Steuhl en función de sus características ultraestructurales (Steuhl, 1989).

El **tipo I** son las células caliciformes. Son un tipo celular especializado en la secreción de mucinas (Gipson y Argüeso, 2003). La mayor parte de su citoplasma está cargado con gránulos mucosos y normalmente tienen un aparato de Golgi bien diferenciado. Son las únicas células de la conjuntiva que secretan la mucina (MUC) 5AC. Expresan marcadores de epitelio con perfil secretor, como la citoqueratina (CK) 7, y reaccionan a lectinas, que se unen a los glicoconjugados de alto peso molecular que secretan, entre los que se encuentra la MUC5AC (Krenzer y Freddo, 1997). Algunas de las lectinas más empleadas para su detección son las derivadas de *Ulex europeaus* (UEA-1) y *Helix pomatia* (HPA).

El **tipo II** son células epiteliales con un alto contenido de vesículas secretoras. Al observar estas células mediante microscopía electrónica se aprecian en su citoplasma numerosos gránulos secretores electrodensos de gran tamaño, entre 60 y 300 nm. Estas células están distribuidas por toda la conjuntiva, siendo el tipo más común en la conjuntiva humana.

El **tipo III** se reconoce por sus complejos de Golgi bien desarrollados. A menudo se encuentran varios aparatos de Golgi alrededor del núcleo. Se cree que estas células también contribuyen a las secreciones mucosas de la película lagrimal.

El **tipo IV** se caracteriza por la alta presencia de retículo endoplasmático rugoso (rRE) que se observa en su citoplasma. Este tipo es muy frecuente en la conjuntiva humana. También se pueden apreciar pequeñas cantidades de mitocondrias y aparatos de Golgi.

El **tipo V** son células epiteliales que se identifican por su alto contenido en mitocondrias. Debido al elevado número de mitocondrias es raro localizar otros organelos celulares como el rRE o el aparato de Golgi.

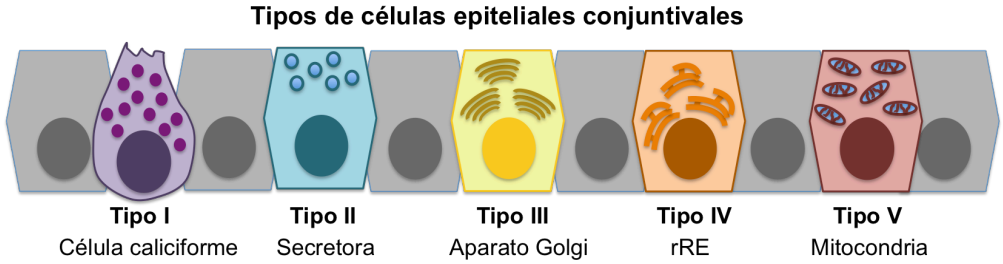

Figura 4. Tipos de células epiteliales que se encuentran en la conjuntiva humana, según lo descrito por K. P. Steuhl en 1989.

Los tipos celulares del II al V a menudo se agrupan y se denominan células epiteliales escamosas estratificadas no caliciformes. En conjunto constituyen aproximadamente el 85-90% de las células del epitelio conjuntival. Expresan marcadores tales como CK4 y CK19. Están unidas entre sí mediante uniones adherentes, entre las que destaca la formada por E-cadherina, y por uniones estrechas, mediante moléculas tales como *zonula occludens* (ZO) 1 y ZO2. Estas células expresan las mucinas MUC1, MUC4 y MUC16, que aportan estabilidad a la lágrima.

Una característica esencial para la diferenciación de las células epiteliales es la polarización (Roignot *et al*., 2013). Las células epiteliales conjuntivales, como las de otros epitelios mucosos, tienen microvellosidades en su superficie apical. Las microvellosidades aumenta la superficie disponible para la adhesion de la lágrima, y están además implicadas en la adhesión celular, la absorción y la secreción. Los cambios o malformaciones en las microvellosidades están presentes en muchas enfermedades. En el caso de la conjuntiva, se han encontrado microvellosidades anormales en varias patologías, como el síndrome de Sjögren y la enfermedad de injerto contra huesped (Mancel *et al*., 1993; Tatematsu *et al*., 2012).

2.2. Membrana basal

La membrana basal es una fina membrana de tejido conectivo que separa el epitelio del estroma subyacente. La membrana basal conjuntival está formada fundamentalmente por colágeno de tipo IV, laminina, nidógeno 1 y 2, y trombospondina-4 (Schlotzer-Schrehardt *et al*., 2007).

2.3. Estroma

El estroma conjuntival está compuesto por moléculas, tales como colágeno, laminina, o ácido hialurónico (AH), entre otras. Además, tiene un importante componente celular. Las células más numerosas del estroma conjuntival son los fibroblastos que, entre otras funciones, se encargan de secretar gran parte de la matriz extracelular que compone el estroma conjuntival.

La conjuntiva es un tejido altamente vascularizado y con abundates vasos linfáticos. También es un tejido con una rica inervación sensorial, lo que hace que se encuentren en él células nerviosas. Finalmente, la conjuntiva tiene un tejido linfoide asociado, conocido como CALT (*Conjunctival-associated lymphoid tissue*) (Knop y Knop, 2000). Junto con el tejido linfoide asociado al sistema de drenaje lacrimal (LDALT) forma el tejido linfoide asociado al ojo (EALT), un componente más del tejido linfoide asociado a mucosas, conocido como MALT. Debido a la presencia del CALT, en el estroma conjuntival están presentes células de la defensa inmune innata, como son los macrófagos y los mastocitos, y células de la respuesta inmune específica, como los linfocitos, las células plasmáticas y las células dendríticas. El CALT no solo ejerce su función protectora sobre la conjuntiva, sino que actúa también sobre la córnea (Knop y Knop, 2005), reforzando así la idea de la conjuntiva como un tejido protector de toda la superficie ocular.

3. IMPLICACIÓN DE LA CONJUNTIVA EN ENFERMEDADES INFLAMATORIAS

3.1. Enfermedades inflamatorias de la superficie ocular

La conjuntiva está implicada en diversas patologías que afectan a la superficie ocular (Figura 5). Y ello se debe, al menos en parte, a su papel protector. Las células de la conjuntiva son participantes activos en la defensa de la superficie ocular. Para ello secretan distintos tipos de moléculas, como citoquinas, quimioquinas, (Enríquez-de-Salamanca *et al*., 2008) o péptidos antimicrobianos como la defensina beta 9 (HBD9), que es secretada por las células epiteliales conjuntivales (Mohammed *et al*., 2010).

Aunque son muchas las enfermedades con afectación conjuntival, tan solo se mencionarán aquí dos de las más comunes, el síndrome de ojo seco y las alergias oculares.

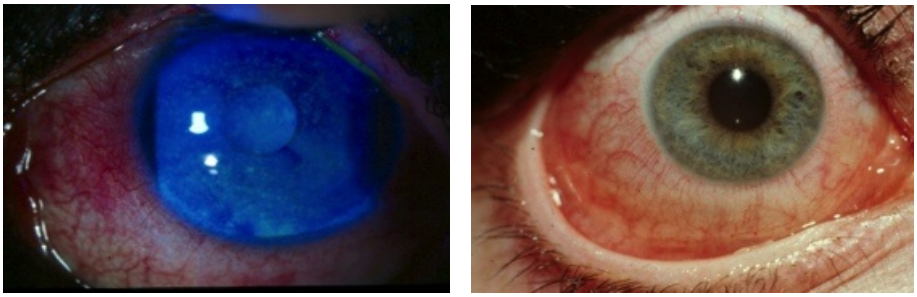

Figura 5. Imágenes de un paciente con síndrome de ojo seco (izquierda) y con conjuntivitis alérgica (derecha), donde se puede observar afectación conjuntival. Imágenes cedidas por la Prof. M. Calonge.

El **síndrome de ojo seco** es una enfermedad inflamatoria multifactorial de la superficie ocular y la película lagrimal que se produce por una disfunción de la unidad funcional lagrimal (Stern *et al.*, 2004; Stern *et al*., 2013). Su alta incidencia (Schaumberg *et al*., 2003; Schaumberg *et al*., 2009) y su gran capacidad para afectar a la calidad de vida de los individuos que la sufren (Schiffman *et al*., 2003), hacen que sea una de las patologías de la superficie ocular que más interés ha despertado en los últimos años. Entre las manifestaciones típicas del ojo seco se encuentran la irritación ocular, el dolor, la visión borrosa y la fotofobia. Las características del epitelio conjuntival de pacientes de ojo seco se estudian a menudo a partir de muestras obtenidas por citología de impresión conjuntival. Se trata de una técnica mínimamente invasiva en la que se coloca un pequeño filtro sobre la superficie conjuntival. Tras presionar el filtro durante unos segundos éste se retira, llevándose con él una o dos capas de células conjuntivales. Después de realizar las tinciones correspondientes se analiza el grado de metaplasia de las células epiteliales y el número de células caliciformes. En las citologías de pacientes con ojo seco puede observarse con frecuencia un menor número de células caliciformes que en las citologías de individuos sanos (Mantelli *et al.*, 2013; Marko *et al*., 2013; Kumar *et al*., 2014).

La **alergia** es una reacción anormal y exagerada del sistema inmune hacia antígenos inofensivos que están presentes normalmente en el ambiente. En las personas que no muestran esta hipersensibilidad, dichos antígenos no producen ninguna respuesta. En las alergias oculares la conjuntiva tiene un papel fundamental. Hay varios tipos de alergia ocular que van desde las formas más leves

(conjuntivitis alérgica estacional y conjuntivitis alérgica perenne) hasta las más severas (queratoconjuntivitis vernal y queratoconjuntivitis atópica) que pueden comprometer la visión debido a la afectación corneal (Leonardi, 2013). En todas estas formas hay participación directa del tejido conjuntival y, en concreto, de las células epiteliales y las células caliciformes (Saban *et al*., 2013). Las alergias oculares se asocian con hipersecreción mucosa y, a menudo, se encuentran mayores densidades de células caliciformes o hiperplasia de las mismas en la conjuntiva de pacientes con estas patologías.

3.2. Moléculas implicadas en enfermedades conjuntivales

El número de moléculas descritas implicadas en las enfermedades inflamatorias que afectan a la conjuntiva aumenta a medida que avanza el conocimiento que tenemos del propio proceso inflamatorio. Debido a la imposibilidad de realizar una revisión exhaustiva sobre todas ellas, se mencionarán tan solo dos tipos: las citoquinas y los receptores de AH.

3.2.1. Citoquinas

Las citoquinas son pequeñas proteínas no estructurales. Su peso molecular varía entre 8 y 40.000 daltons (Dinarello, 2000). Son agentes inmunomoduladores producidos por varios tipos celulares, incluyendo las células epiteliales de la superfice ocular. De hecho, a excepción de los glóbulos rojos, todas las células del organismo son capaces de producir citoquinas y de responder a ellas (Dinarello, 2007). Como reguladores de la respuesta inmune algunas citoquinas ejercen acciones proinflamatorias (Dinarello, 2000), otras tienen efectos antiinflamatorios (Opal y DePalo, 2000) y muchas tienen efectos duales.

Las citoquinas se clasifican en distintos tipos, dependiendo del tipo de célula T colaboradora (*T helper*, Th) que las produce (Steinman, 2007). Los tipos principales son Th1, Th2 y Th17 (Figura 6).

Entre las citoquinas de tipo Th1, dos de las más estudiadas son el factor de necrosis tumoral alfa (TNF-α) y el interferón gamma (IFN-γ). Estas citoquinas suelen estar elevadas en el síndrome de ojo seco. Entre las citoquinas del tipo Th2, la interleuquina (IL) 4, la IL-5 y la IL-13 han sido ampliamente estudiadas en alergias, donde normalmente se encuentran niveles más altos.

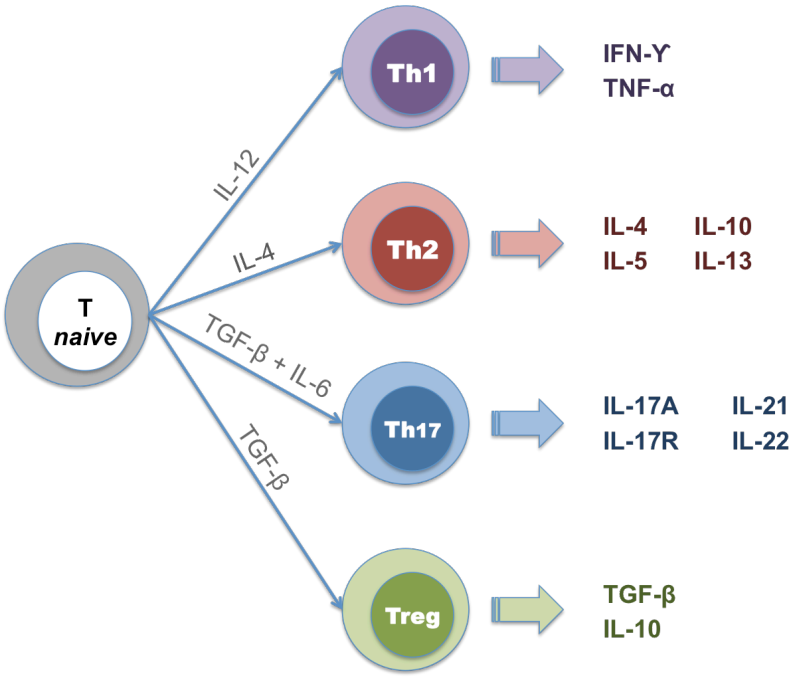

Figura 6. Esquema de las principales citoquinas clasificadas según el tipo de célula Th que las secreta mayoritariamente.

3.2.2. El ácido hialurónico (AH) y sus receptores

El AH es un polisacárido de tipo glicosaminoglicano cuyo peso molecular puede variar notablemente. Forma parte de la matriz extracelular de muchos tejidos, incluidos los oculares, ya que ayuda a absorber agua y a aumentar el espacio intercelular. Además, participa en la remodelación tisular. Un efecto importante del AH es estimular la producción de colágeno por parte de los fibroblastos. Este es el efecto que ha hecho que el AH sea ampliamente utilizado en cosmética.

El AH participa también en el proceso inflamatorio, donde puede presentar un papel dual ligado al tamaño de los fragmentos de AH. El AH de bajo peso molecular tiene efectos proinflamatorios, mientras que hay evidencias de que el AH de alto peso molecular puede proteger la integridad del tejido epitelial, ayuda en el cierre de heridas y puede ejercer acciones antiinflamatorias (Stern *et al.*, 2006; Campo *et al.*, 2009).

Para unirse a las células y ejercer algunas de sus funciones, el AH se une principalmente a dos receptores: el CD44 y el receptor de la movilidad mediada por hialurónico (RHAMM) (Turley, 1982; Entwistle *et al*., 1996). El papel de estos receptores en varios procesos patológicos se ha estudiado desde hace tiempo. Así, hay claras evidencias de su papel directo en enfermedades oncológicas (Giannopoulos *et al*., 2006; Balla *et al*., 2009) y también en enfermedades inflamatorias como la osteoartritis (Dunn *et al*., 2009). El receptor CD44, además de su forma transmembrana, presenta una forma soluble (sCD44), que ha sido ampliamente estudiada en el glaucoma, donde se han encontrado niveles elevados que podrían ser una de las causas del aumento en la presión intraocular que produce la enfermedad (Nolan *et al*., 2007; Giovingo *et al*., 2013).

Sin embargo, a pesar de la importancia de estos receptores en varias patologías y al uso continuo del AH en distintas formulaciones que se aplican sobre la superficie ocular, la presencia de estos receptores y su función específica apenas se han estudiado en el tejido conjuntival.

4. MODELOS DE ESTUDIO DE LA CONJUNTIVA

Debido a la importancia patofisiológica de la conjuntiva y a su implicación en distintas patologías de la unidad funcional lagrimal, su estudio es esencial tanto para comprender el funcionamiento normal de la superficie ocular, como para buscar nuevas estrategias terapéuticas que permitan tratar mejor las enfermedades que la afectan. Previo a los ensayos clínicos que permitirán introducir en el mercado sanitario nuevos fármacos, la investigación debe desarrollarse en el laboratorio, en modelos animales (*in vivo*) y, previamente, en modelos *in vitro*.

4.1. Modelos *in vivo*

Los modelos de estudio *in vivo* implican el uso de animales de experimentación, tales como ratones, ratas, conejos, etc. Al emplear un organismo vivo, la complejidad de las interacciones entre los distintos órganos y sistemas, hace que se obtenga mucha información que se espera pueda ser extrapolada al ser humano. Sin embargo, a pesar de ser necesarios por legislación, estos modelos *in vivo* tiene varias limitaciones relacionadas, fundamentalmente, con aspectos éticos, los elevados costes, la reducida similitud con las estructuras humanas en

estudio y la necesidad de personal con un alto grado de especialización y experiencia, y de instalaciones adecuadas.

4.2. Modelos *in vitro*

La sociedad demanda cada vez más a la comunidad científica que haga esfuerzos mayores por erradicar, o al menos, reducir, la experimentación *in vivo*. En esta línea, los modelos *in vitro* permiten avanzar en el conocimiento celular evitando el uso de animales de experimentación. Esto, junto con el menor coste y la posibilidad de controlar mejor los parámetros de estudio, hace que sea posible probar muchas más condiciones experimentales en los modelos *in vitro* que en los modelos *in vivo*, lo que supone otro valor añadido. Por lo tanto, el desarrollo y uso de modelos *in vitro* más sofisticados debería ser una prioridad en investigación, puesto que no sólo permitiría reducir el uso de animales de laboratorio, sino que también abarataría notablemente los costes de los ensayos preclínicos.

4.2.1. Líneas celulares y cultivos primarios

Las líneas celulares están constituidas por células que se han transformado, de manera espontánea o inducida, y que son capaces de replicarse de manera ilimitada en condiciones controladas en el laboratorio. Además, estas células suelen perder su capacidad de inhibición por contacto, lo que hace que normalmente continúen proliferando una vez que han cubierto por completo toda la superficie de cultivo (lo que se denomina alcanzar la confluencia). Las principales ventajas de las líneas celulares son su disponibilidad y la facilidad de mantenerlas en cultivo, así como la homogeneidad de los resultados que se obtienen, ya que todas las células tienen un mismo origen. Como principal inconveniente destaca el hecho de que al sufrir la transformación que las convierte en líneas celulares, estas células ya no son un fiel reflejo de las células de las que proceden.

Los cultivos primarios son aquellos que se obtienen de células directamente recogidas del organismo. Esto hace que sean mucho más parecidas al tejido original y, por lo tanto, los resultados que se obtienen con cultivos primarios son más fiables y valiosos. Sin embargo, presentan varios inconvenientes o dificultades, que hacen que su uso no esté más extendido. En primer lugar, su disponibilidad es limitada, ya que estas muestras suelen proceder de biopsias o de tejidos obtenidos de donantes cadáver. Además, la investigación con estas muestras está sujeta a

estrictas regulaciones para garantizar el cumplimiento de normas éticas. Y, por último, su cultivo es más difícil y su crecimiento *in vitro* es limitado.

4.2.2. Cultivos en monocapa y cultivos tridimensionales

La manera más habitual de hacer crecer las células en el laboratorio es lo que se denomina un cultivo en monocapa. En ellos, las células, ya sean líneas celulares o cultivos primarios, crecen sobre la superficie especializada de cultivo creando una única capa celular que va recubriendo toda la superficie hasta alcanzar la confluencia.

Por el contrario, los cultivos tridimensionales pretenden recrear la estructura tridimensional que tienen los tejidos. Esto permite estudiar la interacción entre distintos tipos celulares, o ente las células y una matriz extracelular (ECM). La organización de las células en un espacio tridimensional y su relación con ese ambiente extracelular son los factores que determinan su fenotipo, por lo que este tipo de cultivos permite obtener datos más válidos.

Los cultivos tridimensionales pueden emplearse para el estudio de los órganos en condiciones normales o patológicas. El uso de estos cultivos tridimensionales ha ido aumentando con el creciente conocimiento sobre las interacciones célula-célula y célula-ECM que ha aportado el avance de la ingeniería de tejidos.

5. INGENIERÍA DE TEJIDOS

La ingeniería de tejidos es, según Robert S. Langer y Joseph P. Vacanti, “un campo interdisciplinario que aplica los principios de ingeniería y ciencias de la vida para el desarrollo de sustitutos biológicos que restauren, mantengan o mejoren la función de un tejido o de un órgano completo” (Langer y Vacanti, 1993). Por lo tanto, el fin último de la ingeniería de tejidos es el uso clínico. Sin embargo, también pueden emplearse estos órganos o tejidos artificiales como complejos modelos *in vitro*.

Hay dos componentes fundamentales que se combinan en la ingeniería de tejidos: las células y los andamios o andamiajes que las alojan.

5.1. Células

Las células que se emplean en la ingeniería de tejidos pueden ser líneas celulares o células primarias. Además, dependiendo de su origen, las células pueden ser autólogas (si proceden del mismo paciente que recibirá el tejido) o heterólogas (si proceden de otro donante).

Los tipos celulares empleados en la ingeniería de tejidos dependerán, obviamente, del tejido que se quiera reconstruir. En el caso de la conjuntiva, los principales tipos celulares son células epiteliales (incluyendo células caliciformes) y células estromales, principalmente fibroblastos.

5.2. Andamiajes

Los andamiajes son las estructuras acelulares capaces de soportar la formación del tejido en las tres dimensiones. Dan un apoyo estructural temporal a las células y guían su crecimiento. Es por ello que se consideran una parte fundamental de la ingeniería de tejidos.

Un buen andamiaje tiene que ser biocompatible, biodegradable y debe favorecer la adhesión celular. Además, pueden ayudar en el transporte de nutrientes y facilitar la formación de tejidos funcionales (Lee *et al.*, 2011). Para que el tejido artificial sea lo más parecido al real, el biomaterial utilizado para el andamiaje, además de dar soporte a las células, deberá ser lo más parecido posible a la ECM.

Para elaborar los andamiajes se pueden usar biomateriales naturales o sintéticos. Las principales ventajas de los biomateriales naturales frente a los sintéticos son su biocompatibilidad y su mayor parecido a la ECM. Algunos de los biomateriales naturales más empleados como andamiajes son la membrana amniótica, el colágeno, el quitosano, la fibrina y el AH (Rosso *et al.*, 2005), mientras que entre los biomateriales sintéticos destacan el ácido poli-láctico (PLA), el ácido poli-láctico-co-glicólico (PLGA) y la policaprolactona (PCL) (Saltzman, 1999; Putman, 2001).

5.3. Aproximaciones en la superficie ocular

Se han publicado estudios en los que se aplica la ingeniería de tejidos a la superficie ocular. La mayoría de ellos están encaminados a diseñar una córnea artificial que permita sustitutir los trasplantes de córnea convencionales (Griffith *et*

al., 2009) con el fin de tratar a aquellos pacientes con síndrome de insuficiencia límbica (Xu *et al*, 2012). Con este fin, también se ha propuesto llevar a cabo un trasplante de células limbares (Pellegrini *et al*., 2014), células del epitelio oral (Utheim, 2015), y equivalentes conjuntivales (Tanioka *et al*., 2006; Di Girolamo *et al*., 2009). Varios estudios han demostrado el enorme potencial de los cultivos conjuntivales para tratar la enfermedades que conducen a la ceguera y, en concreto, el síndrome de insuficiencia límbica (Di Giralomo *et al*., 2009; Jeon *et al*., 2013).

M. Alaminos y colaboradores (Alaminos *et al*., 2006) desarrollaron un sustituto corneal completo de conejo con el fin de emplearlo como modelo de estudio *in vitro*. Para el desarrollo de este modelo utilizaron los tres principales tipos celulares de la córnea: células endoteliales, fibroblastos y células epiteliales. Como andamiajes emplearon matrices de fibrina y agarosa. Construyeron un cultivo organotípico de córnea que representaba la ultraestructura del tejido nativo. Este trabajo es un buen ejemplo de como la ingeniería de tejidos puede emplearse con fines de investigación *in vitro*, además del uso habitual en medicina regenerativa.

En el año 2007, S. H. Chung y colaboradores (Chung *et al*., 2007) publicaron un modelo tridimensional de conjuntiva humana que producía MUC5AC. La ausencia de producción de este mucina *in vitro* es una de las principales limitaciones de muchos modelos de conjuntiva. Esto se debe a la dificultad de mantener en cultivo células caliciformes. Sin embargo, en este modelo esa limitación está superada. A partir de pequeñas biopsias de conjuntiva lograron cultivar células epiteliales que, posteriormente, fueron sembradas sobre insertos. Mediante cultivo aireado consiguieron que las células epiteliales estratificaran, llegando a obtener 6-8 capas a las dos semanas. En estas condiciones observaron secreción de MUC5AC. Este trabajo aporta un buen modelo de estudio del epitelio conjuntival, pero no de la conjuntiva completa, ya que el estroma no está representado.

A pesar de estos avances, hasta el momento, no se ha publicado ningún modelo tridimensional completo de conjuntiva humana que permita avanzar en el conocimiento de la fisiología del tejido o emplearlo para probar nuevos fármacos o tratamientos.

JUSTIFICACIÓN

A pesar de que la conjuntiva se haya estudiado mucho menos que la córnea, es el principal tejido implicado en algunas de las enfermedades más prevalentes de la superficie ocular y que pueden llegar a afectar a la visión, como son el síndrome de ojo seco o las alergias oculares.

Para avanzar en el conocimiento de los procesos fisiopatológicos de este tejido y encontrar nuevas estrategias terapéuticas que ayuden a tratar las enfermedades mencionadas, es necesario disponer de buenos modelos de estudio.

En el caso de la conjuntiva, consideramos que los modelos disponibles actualmente no resultan suficientes. Se trata de modelos que están excesivamente simplificados al ser monocapas de un único tipo celular. Además, normalmente estas monocapas están formadas por líneas celulares que, tras inmortalizarse, poco se parecen ya a las células que las originaron. Por lo tanto, hay que ser muy cauto a la hora de extraer conclusiones de los experimentos realizados en estos modelos.

Una de las funciones más importantes de la conjuntiva, y que suele estar alterada en los procesos inflamatorios, es la de secretar mucinas que lubriquen, humecten y protejan la superficie ocular. Si bien la mayoría de las células epiteliales conjuntivales son capaces de secretar algún tipo de mucina, hay un tipo de célula que está especializado en esta función. Se trata de las células caliciformes que, además, son las únicas que secretan MUC5AC, una de las mucinas más importantes de la película lagrimal. Por lo tanto, el estudio de las células caliciformes y de su función secretora resulta especialmente relevante.

Teniendo todo esto cuenta, creemos que es necesario desarrollar un nuevo modelo de conjuntiva humana, más complejo e integrador. Para superar las limitaciones de los modelos existentes, éste debería estar elaborado con células primarias y no con líneas celulares. Además, debería representar la estructura tridimensional de la conjuntiva, con un estroma poblado por fibroblastos y un epitelio en el que se intercalen células caliciformes.

Para probar la validez de este modelo para el estudio de enfermedades inflamatorias, es necesario conocer en profundidad el efecto de este proceso en las distintas células de la conjuntiva, de tal forma que después se puedan intentar simular estas condiciones en el modelo tridimensional desarrollado.

Dentro de este contexto se encuadra esta tesis doctoral, cuyo objetivo principal es el desarrollo de un modelo *in vitro* de conjuntiva humana que, por sus características, permita profundizar en el estudio de las enfermedades inflamatorias reduciendo al máximo el uso de modelos *in vivo*.

Para abordar un proyecto de este tipo es necesario contar con el apoyo y la colaboración de un equipo multidisciplinar. El Grupo de Superficie Ocular del IOBA está muy implicado en el estudio de las enfermedades inflamatorias que afectan a la superficie ocular. Para ello, cuenta con especialistas médicos que aportan un punto de vista clínico que nunca debe perderse en la investigación que se centra en aspectos más moleculares. Además, el equipo liderado por la Dra. Yolanda Diebold tiene amplia experiencia en técnicas de cultivos celulares y una carrera establecida en nanomedicina, donde cuenta con colaboraciones en el campo de los biomateriales con investigadores del Departamento de Farmacia y Tecnología Farmacéutica de la Universidad de Santiago de Compostela, y el Banco de Tejidos de la Fundación Clínica San Francisco, de León. Varios proyectos nacionales conjuntos obtenidos en concurrencia competitiva avalan esta colaboración.

Por lo tanto, la necesidad de profundizar en el estudio de la conjuntiva y de probar las nuevas estrategias terapéuticas que se desarrollen dentro del grupo justifica la idea de desarrollar un modelo tridimensional de conjuntiva humana. La trayectoria de todo el Grupo de Superficie Ocular garantiza que se contará con los apoyos y las colaboraciones necesarias para poder llevar a cabo este proyecto de tesis doctoral.

HIPÓTESIS

En esta tesis doctoral se han planteado dos hipótesis relacionadas entre sí:

- Es posible desarrollar un modelo tridimensional de conjuntiva humana que contenga células estromales y epiteliales, incluyendo células de tipo caliciforme, que sean capaces de secretar mucinas.

- Este nuevo modelo tridimensional *in vitro* será capaz de responder a estímulos externos, demostrando así su utilidad para llevar a cabos estudios relacionados con procesos inflamatorios que afecten a la conjuntiva humana.

OBJETIVOS

Objetivo general

- Desarrollar un modelo tridimensional de conjuntiva humana con tres tipos celulares (fibroblastos, células epiteliales y células caliciformes) y una matriz biocompatible que permita el estudio fiable de cambios en la expresión de moléculas asociadas a la inflamación.

Objetivos específicos

1. Estudiar la implicación de los receptores de ácido hialurónico en enfermedades inflamatorias de la superficie ocular (**estudios 1 y 2**).

2. Estudiar el papel de citoquinas relevantes en enfermedades de la superficie ocular sobre la función de las células caliciformes (**estudios 3 y 4**).

3. Establecer cultivos primarios de células epiteliales y estromales de conjuntiva humana (**estudio 5**).

4. Seleccionar un material biocompatible que sirva como andamio para desarrollar un modelo tridimensional (**estudio 6**).

5. Desarrollar un co-cultivo tridimensional de células epiteliales caliciformes y no caliciformes, y de fibroblastos de conjuntiva humana (**estudio 7**).

6. Simular enfermedades inflamatorias de la superficie ocular aplicando distintas condiciones al modelo tridimensional previamente desarrollado (**estudio 8**).

METODOLOGÍA

La metodología empleada para lograr cada uno de los objetivos planteados está descrita de manera detallada en los estudios correspondientes. Por ello, a continuación se presenta tan solo un resumen de las principales muestras biológicas utilizadas y de las principales técnicas usadas.

1. MUESTRAS UTILIZADAS

En el desarrollo de esta tesis doctoral se utilizaron muestras humanas de distinta procedencia. Algunas procedían de tejidos obtenidos a partir de donante cadáver, cedidos por el banco de ojos de la Clínica Barraquer (Barcelona, España) o por los bancos de ojos *Heartland Lions Eye Bank* (Kansas City, MO, EE. UU.) y *Michigan Eye Bank* (Ann Arbor, MI, EE. UU.), siempre bajo acuerdos vigentes y con la aprobación de los Comités correspondientes.

Otras muestras fueron obtenidas directamente de pacientes, mediante citología por cepillado conjuntival (*brush cytology*, BC) o recogidas de pacientes que estaban siendo sometidos a cirugía refractiva. En todos los casos los donantes firmaron un consentimiento informado en el que permitían que se recogieran las muestras correspondientes, así como su uso en investigación. Todos los proyectos en los que se recogieron muestras de pacientes estuvieron aprobados por el Comité Ético de Investigación Clínica.

Todos los experimentos se desarrollaron siguiendo las directrices de la Declaración de Helsinki y siguiendo la normativa del Real Decreto 1716/2011, de 18 de noviembre, por el que se establecen los requisitos básicos de autorización y funcionamiento de los biobancos con fines de investigación biomédica y del tratamiento de las muestras biológicas de origen humano, y se regula el funcionamiento y organización del Registro Nacional de Biobancos para investigación biomédica.

Además de las muestras humanas, en el estudio 4 se usaron también muestras de conjuntiva de ratas Sprague Dawley. Todos los experimentos que

involucraron animales se llevaron a cabo atendiendo a la Declaración de ARVO para el uso de animales en investigación oftalmológica (http://www.arvo.org/About_ARVO/Policies/Statement_for_the_Use_of_Animals_in_Ophthalmic_and_Visual_Research/).

1.1. Líneas celulares

Las líneas celulares de epitelio corneal humano (HCE) de Araki-Sasaki *et al*. (1995) y de epitelio conjuntival humano (IOBA-NHC) de Diebold *et al*. (2003) se emplearon con fines comparativos en experimentos dentro del estudio 1. Además, la línea IOBA-NHC fue utilizada también en experimentos realizados en el estudio 6 de esta tesis doctoral.

1.2. Cultivos primarios

Se desarrollaron cultivos primarios de fibroblastos, células epiteliales y células caliciformes de conjuntiva humana a partir de tejido conjuntival de donante cadáver (Figura 7). Dichos tejidos fueron enviados por la Clínica Barraquer (estudios 5-8) y el *Heartland Lions Eye Bank* y *Michigan Eye Bank* (estudio 4).

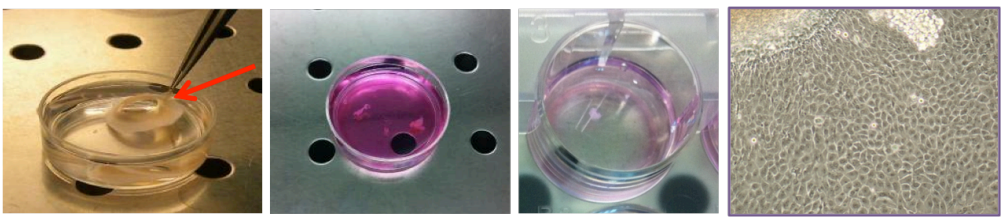

Figura 7. Obtención de cultivos a partir de conjuntiva de donante cadáver (izquierda). Se aisló la conjuntiva a partir del anillo corneoscleral (medio-izquierda). A continuación se realizaron fragmentos de unos 2 mm (explantes) que se colocaron sobre la superficie de placas de cultivo (medio-derecha). A partir de dichos explantes se pudo observar el crecimiento de células epiteliales a partir de 1-2 días (derecha).

También se realizaron cultivos de células caliciformes a partir del tejido conjuntival obtenido de ratas macho Sprague-Dawley, de 4-5 semanas de edad (estudio 4).

1.3. Muestras frescas de pacientes

Se obtuvieron muestras de epitelio corneal de pacientes sometidos a cirugía refractiva (CERS) mediante la técnica de queratectomía fotorrefractiva. Estas muestras fueron empleadas en el estudio 1.

Las muestras de epitelio conjuntival fueron recogidas en donantes sanos y en pacientes con enfermedades de la superficie ocular mediante citología por cepillado conjuntival, para su uso en los estudios 1 y 2 de esta memoria de tesis.

2. DETERMINACIÓN DE PARÁMETROS CELULARES

2.1. Estudio de la proliferación celular mediante el reactivo alamarBlue®

La proliferación celular se determinó en los estudios 5, 6 y 7 de esta tesis mediante el ensayo colorimétrico con alamarBlue® (AbD Serotec, Oxford, Reino Unido). El alamarBlue® es un colorante fluorescente no tóxico que se añade a las células diluido en medio de cultivo. La actividad metabólica de las células reduce este compuesto emitiendo fluorescencia a 590 nm cuando se excita a una longitud de onda de 560 nm. La fluorescencia emitida es proporcional al número de células presentes en el cultivo. Como este compuesto no afecta ni a la viabilidad ni a la proliferación de las células, puede usarse en el mismo cultivo a lo largo del tiempo.

2.2. Estudio de la proliferación de células caliciformes mediante *Cell Counting Kit-8*

La proliferación celular de las células caliciformes de rata del estudio 4 fue determinada con el kit comercial *Cell Counting Kit-8* (Dojindo Molecular Technologies, Gaithersburg, MD, EE. UU.). El producto WST-8 (una sal de tetrazolio) presente en el kit se añadió a las células en cultivo y, tras 45 min de incubación, se retiró el medio. Se leyó la absorbancia del mismo en un espectrofotómetro a 450 nm de longitud de onda. El valor de absorbancia obtenido es proporcional a la proliferación celular.

2.3. Estudio de la viabilidad celular mediante el ensayo de toxicidad *in vitro* XTT

El test XTT es otro ensayo colorimétrico que se utilizó para analizar la potencial citotoxicidad de los hidrogeles de gelano-espermidina analizados en el estudio 6. Las células conjuntivales en contacto con los hidrogeles se incubaron durante 17 h con la sal de tetrazolio XTT (2,3-bis2-metoxi-4-nitro-5-sulfofenil-2Htetrazolio-5-carboxianilida) en medio RPMI sin rojo fenol (Invitrogen-GIBCO, Inchinnan, Reino Unido). Como control positivo de toxicidad se empleó una solución de cloruro de benzalconio (BAC, Sigma-Aldrich, St. Louis, MO, EE. UU.) al 0,001 %. Pasado el tiempo de incubación con XTT se midió la absorbancia de cada pocillo en un espectrofotómetro a 450 nm, siendo el valor obtenido proporcional al número de células presentes en el pocillo.

3. ANÁLISIS DE PROTEÍNAS

3.1. Inmunofluorescencia

La inmunofluorescencia es una técnica de detección de proteínas basada en la reacción antígeno anticuerpo. En ella se utilizaron diversos anticuerpos primarios (ver Tabla 1) que se unieron de manera específica a la proteína de interés. Las condiciones de incubación dependieron del anticuerpo, siendo las más comunes 1 hora a 37º C o toda la noche a 4º C. Después se realizaron tres lavados con PBS para retirar el exceso de anticuerpo primario y se añadió a continuación un anticuerpo secundario marcado con un fluoróforo. El anticuerpo secundario se incubó durante 1 hora a temperatura ambiente. Este anticuerpo secundario se unió al primario, marcando así de manera fluorescente la proteína estudiada. Los núcleos celulares fueron teñidos con Hoechst 33342 (Sigma-Aldrich), dilución 1:1000, durante 10 minutos a temperatura ambiente. Las preparaciones se observaron en el microscopio de fluorescencia Leica CTR6000 (Leica Microsystems, Wetzler, Alemania) donde se realizaron fotografías a distintos aumentos.

Tabla 1. Anticuerpos primarios y lectinas utilizados en los distintos estudios.

Anticuerpo/Lectina	Casa comercial	Dilución IF	Dilución WB	Estudio
CD44	Calbiochem-Merck	1:100	1:500	1
RHAMM	Santa Cruz Biotechnology	1:50	1:500	1
CK4	Abcam	1:100	-	4
CK7	Abcam	1:100	-	4
MUC5AC	Abcam	1:100	-	4
IFN-γ-R1	Novus Biologicals	1:100	-	4
CK19	Dako	1:50	1:1000	5, 7, 8
CK4	Sigma-Aldrich	-	1:250	5
CK7	ThermoScientific	1:100	1:200	5, 7, 8
MUC5AC	EMD Millipore Corp.	1:500	1:500	5, 7, 8
Vimentina	Santa Cruz Biotechnology	1:500	1:200	5
FSP-1	Abcam	1:100	-	5
Ki67	Dako	1:50	-	5, 7, 8
E-caderina	BD Bioscience	1:100	1:1000	5, 7
ZO-1	Invitrogen	1:50	1:250	5
Lectina UEA-1	Sigma Aldrich	1:500	-	4
Lectina HPA	Sigma Aldrich	1:500	-	3, 4, 7, 8

IF: inmunofluorescencia; WB: Western *blotting*

3.2. Electroforesis y Western *blotting*

El Western *blotting* es una técnica de determinación de proteínas que consiste en transferir a una membrana las proteínas previamente separadas en gel de poliacrilamida mediante una electroforesis con dodecil sulfato sódico. Las proteínas se separan en función de su peso molecular y, una vez en la membrana, se lleva a cabo su reconocimiento mediante anticuerpos.

Para llevar a cabo esta técnica es necesario obtener lisados celulares. Esto se consiguió mediante un tratamiento enzimático de las células con el tampón de lisis celular del ensayo de radioinmunoprecipitación (RIPA). Una vez extraidas las proteínas totales se cuantificaron con el kit Pierce BCA Protein assay kit (Thermo

Scientific, Rockford, IL, EE. UU.), basado en ácido bicinconínico (BCA). A continuación, las muestras se cargaron en un gel de poliacrilamida al 10% que se sometió a una corriente eléctrica (15 minutos a 70 voltios y 90 minutos a 110 voltios). De esta forma, mediante electroforesis, se separaron las proteínas en función de su peso molecular. A continuación se llevó a cabo el proceso de Western *blotting* o transferencia a la membrana, en el que las proteínas separadas en el gel se transfieren a una membrana de nitrocelulosa aplicando durante 90 minutos una corriente de 350 miliamperios de intensidad. Finalmente, sobre dicha membrana, se llevó a cabo un proceso de inmunodetección, incubando la membrana con anticuerpos primarios frente a las proteínas de interés (ver Tabla 1). Para concluir con el proceso se añadieron anticuerpos secundarios marcados con luminol y se reveló la membrana, observándose bandas en el lugar en el que se encontraban las proteínas inmunodetectadas.

3.3. Ensayo por inmunoabsorción ligado a enzimas (ELISA)

La determinación de proteínas secretadas se llevó a cabo mediante la técnica de ELISA. Para ello se utlizaron kits comerciales frente a IL-6 (IL-6 ELISA, Diaclone, Besançon, Francia) en los estudios 5 y 8, sCD44 (Human sCD44std Instant ELISA, eBioscience, Viena, Austria) en los estudios 1 y 8, y MUC5AC (Shanghai Yehua Biological Technology Co., Ltd., Shanghai, China) en el estudio 8. En todos los casos se siguieron las indicaciones de los fabricantes. Los límites de detección oscilaron entre 6,25 - 200 pg/ml para la IL-6, 62,5 - 4.000 pg/ml para sCD44 y 5 - 1.000 ng/ml para MUC5AC.

4. ANÁLISIS GENÉTICO

4.1. RT-PCR convencional y electroforesis en gel de agarosa

Para analizar los niveles de expresión genética en los estudios 1 y 4, se realizó una reacción en cadena de la polimerasa (PCR) con transcripción reversa (RT), RT-PCR. Para ello, primero se extrajo el ARN mensajero (ARNm) de las células de interés mediante el kit comercial Rneasy Mini Kit (Qiagen, Valencia, CA, EE. UU.). Después se llevó a cabo la transcripción reversa para obtener así ADN complementario (ADNc). Los genes de interés se amplificaron a partir de ese ADNc

con una PCR convencional, mezclando para ello el ADNc con cebadores para los genes correspondientes (ver Tabla 2), nucleótidos, tampón para PCR comercial (Biotools B&M Labs, S.A., Madrid, España) y Taq polimerasa. Para visualizar el resultado de dicha amplificación se llevó a cabo una electroforesis en gel de agarosa.

4.2. RT-PCR a tiempo real

En otros experimentos (estudios 1, 2 y 4) se llevó a cabo una RT-PCR a tiempo real. Se trata de una variante de la PCR semicuantitativa. Es un avance tecnológico con respecto a la RT-PCR convencional que permite obtener resultados más homogéneos y precisos, de una manera más rápida y con menor riesgo de contaminación. Además, a menudo se alcanza una mayor sensibilidad, por lo que es una técnica más útil cuando se trata de comparar niveles de expresión entre varias muestras. Para realizarla se mezclan 10 ng de ADNc con 1 µl de cebadores (Tabla 2) y 10 µl de SYBR Green PCR Master Mix. La reacción se llevó a cabo en las siguientes condiciones: desnaturalización durante 10 minutos a 95º C, seguida de 40 ciclos de 15 segundos a 95º C (desnaturalización) y 60 segundos a 60º C (elongación). Finalmente la reacción acabó con un ciclo de 90 segundos a 95º C. Después de cada ciclo de elongación se leyó la fluoresencia. El análisis de los datos se llevó a cabo mediante el método semicuantitativo $2^{-\Delta\Delta Ct}$ (Livak y Schmittgen, 2001).

Tabla 2. Cebadores utilizados en los distintos estudios.

Cebador	Casa comercial	Pares de bases	Estudio
CD44	SABioscience-Qiagen	106	1, 2
RHAMM	SABioscience-Qiagen	108	1, 2
GAPDH	Sigma	250	1, 2, 4
P53	SABioscience-Qiagen	188	2
MUC5AC	Sigma-Aldrich	85	4
Human IFN-γ-R1	OriGene Technologies Inc.	169	4
Rat IFN-γ-R1	Qiagen	169	4

5. TÉCNICAS ESPECIALES

5.1. Medida del calcio intracelular

En el trabajo descrito en el estudio 4 se llevó a cabo la medida de los niveles intracelulares de calcio $[Ca^{2+}]_i$ de las células caliciformes de rata y humano. También se evaluaron los cambios que se producían en respuesta a varios estímulos, como fueron el IFN-γ y el carbacol.

Para ello, las células cultivadas en placas de cultivo con fondo de cristal fueron incubadas en tampón KRB (120 mM NaCl, 25 mM $NaHCO_3$, 10 mM HEPES, 4,8 mM KCl, 1,2 mM $MgCl_2$, 1,2 mM NaH_2PO_4, 1 mM $CaCl_2$) con 0,5 % de albúmina de suero bovino (BSA), 8 μM de ácido plurónico F127, 250 μM sulfinpirazona y 0,5 μM Fura-2/AM. La incubación se llevó a cabo durante 1 hora a 37º C. Fura-2/AM es una molécula fluorescente que indica los niveles de $[Ca^{2+}]_i$. Después de la incubación las células se lavaron con el tampón KRB con sulfinpirazona y las placas de cultivo se observaron bajo el microscopio con el sistema de visualización de calcio InCyt Im2 (Intracellular Imaging, Cincinnati, OH, EE. UU.). Este programa permite medir los niveles emitidos de Fura-2 a 505 nm de longitud de onda tras la estimulación a 340 nm y 380 nm.

Para llevar a cabo los experimentos se seleccionaron al menos 10 células individuales y se realizó una lectura basal de 15 segundos antes de añadir ningún compuesto. Se valoró el cambio en el pico máximo de $[Ca^{2+}]_i$, obtenido al restar del valor máximo el valor basal.

5.2. Preparación de matrices de fibrina

Las matrices de fibrina elaboradas en los estudios 6, 7 y 8, se preparon siempre en condiciones de esterilidad y empleando plasma fresco congelado o crioprecipitado obtenido del Centro de Hemoterapia y Hemodonación de Castilla y León (CHEMCYL).

Para su elaboración se mezclaron 400 μl/ml de plasma o 333 μl/ml con 40 μl/ml de ácido tranexámico (Rottapharm, Valencia, España) y 40 μl/ml de cloruro cálcico (Braun, Barcelona, España), todo ello diluido en medio de cultivo. Esta mezcla se colocó sobre el soporte correspondiente (pocillos de placa de cultivo o insertos) y se dejó polimerizar durante al menos 2 horas en un incubador a 37º C.

Cuando se cultivaron fibroblastos en el interior de las matrices, éstos se incorporaron durante el proceso de preparación de la propia matriz, a una concentración de 100.000 células/ml. Las células epiteliales cultivadas sobre las matrices se sembraron encima tras 24 horas de polimerización, con una densidad de 100.000 células/cm^2.

RESULTADOS Y DISCUSIÓN

Esta memoria de tesis se ha estructurado en 8 estudios diferentes. A continuación se presenta un resumen de los resultados de cada estudio.

ESTUDIO 1 - Receptores ácido hialurónico en la superficie ocular humana: un estudio descriptivo y comparativo de CD44 y RHAMM en tejidos, líneas celulares, y muestras recién recogidas

En este trabajo se demostró que los dos principales receptores de AH, CD44 y RHAMM, están presentes en los tejidos de la superficie ocular.

Mediante Western *blotting* se pudo observar que tanto en la conjuntiva como en el limbo había mayores niveles proteicos de RHAMM que de CD44, algo que no ocurría en el tejido corneal. Mediante inmunfluorescencia se vio que en la córnea había mayor distribución de CD44 en las capas basales del epitelio, mientras que en el limbo se detectó mayor intensidad en las capas suprabasales. RHAMM se expresaba en todo el epitelio de la superficie ocular y, además, en el estroma conjuntival. El análisis genético llevado a cabo mediante RT-PCR a tiempo real reveló mayores cantidades de ARNm de CD44 que de RHAMM, tanto en la córnea como en la conjuntiva.

También se analizó la expresión de estos receptores en líneas celulares de córnea (HCE) y de conjuntiva (IOBA-NHC). A nivel proteico se encontraron mayores niveles de RHAMM que de CD44 en ambas líneas celulares, mientras que a nivel genético los niveles de CD44 fueron mayores. Es decir, que se encontró la misma discrepancia entre proteína y ARNm que en el caso de los tejidos.

Por último, se estudió la distribución de estos receptores en células epiteliales primarias de córnea y de conjuntiva obtenidas directamente de donantes sanos. En el caso de la córnea, estas células se obtuvieron de pacientes sometidos a cirugía refractiva y en el caso de la conjuntiva mediante citología por cepillado conjuntival. Los niveles proteicos de RHAMM fueron mayores que los de CD44 en ambos casos, mientras que a nivel de ARNm se detectó más CD44. Al comparar los datos obtenidos en estas muestras con los de las líneas celulares se encontraron

diferencias estadísticamente significativas. Las líneas celulares sobreexpresan el receptor RHAMM, por lo que no se recomienda realizar estudios relacionados con AH en estas líneas celulares.

El receptor CD44 tiene una fracción soluble (sCD44) que fue detectada en un 32% de las muestras de lágrima analizadas.

Este estudio demostró que los receptores de AH están ampliamente distribuidos en la superficie ocular, y que su expresión difiere de unos tejidos a otros. A su vez, se observó que la expresión de CD44 y RHAMM en alguna de las líneas celulares oculares más empleadas difiere notablemente de la expresión en células recogidas directamente de pacientes. Por lo tanto, se puso de manifiesto el gran valor que tiene trabajar con cultivos primarios, y la necesaria cautela que debe tenerse al analizar datos obtenidos de líneas celulares.

ESTUDIO 2 – Los receptores de ácido hialurónico CD44 y RHAMM en la inflamación de la superficie ocular humana

En este estudio se analizaron los niveles de expresión de CD44 y RHAMM en pacientes con enfermedades inflamatorias de la superficie ocular con afectación conjuntival y se demostró que éstos cambiaban en los procesos inflamatorios.

Las muestras de 17 pacientes (41,5 ± 4,9 años; 8 hombres y 9 mujeres) y de 14 donantes sanos (30,1 ± 3,0 años; 6 hombres y 8 mujeres) fueron obtenidas mediante citología por cepillado conjuntival. Los pacientes fueron clasificados en dos grupos: enfermedades inmunes de base atópica y enfermedades inmunes no atópicas. A su vez, se dividieron las muestras según los pacientes tuvieran inflamación leve/moderada o severa. Se aisló el ARNm de las muestras y se analizó la expresión genética de CD44 y RHAMM mediante RT-PCR a tiempo real.

Se observó que en el grupo de enfermedades atópicas severas los niveles de CD44 eran significativamente mayores que en el grupo control de donantes sanos. En este mismo grupo de enfermedades atópicas se detectó un aumento progresivo y estadísticamente significativo en los niveles de RHAMM, llegando a triplicarse los niveles en el caso de los pacientes con inflamación severa.

En el grupo de pacientes con enfermedades inmunes no atópicas se vio una tendencia hacia niveles más bajos de CD44 que en el grupo control, aunque

este cambio no fue estadísticamente significativo. Sin embargo, los niveles de RHAMM en los pacientes con inflamación severa fueron significativamente inferiores que en el grupo de donantes sanos.

Además, se analizó la expresión de p53, un represor de RHAMM, para comprobar si un aumento o un descenso en la expresión de este gen podría ser el responsable de los cambios observados en RHAMM. Sin embargo, no se encontraron resultados relevantes.

En resumen, en este estudio se observaron cambios significativos en la expresión de los receptores de AH en enfermedades inflamatorias que afectan a la conjuntiva. En el caso de patologías de base atópica se detectó una expresión aumentada de CD44 y RHAMM, mientras que en las patologías inmunes de base no atópica se encontró un descenso. Estos resultados indican que los receptores de AH, especialmente RHAMM, están implicados en la inflamación ocular y podrían incluso ser utilizados como potenciales biomarcadores.

ESTUDIO 3 – Función de las células caliciformes conjuntivales: efecto del uso de lentes de contacto y citoquinas

En esta revisión invitada se realizó una búsqueda bibliográfica exhaustiva sobre el efecto de las lentes de contacto y de distintas citoquinas en las células caliciformes.

Primero se llevó a cabo un análisis de las distintas técnicas disponibles para identificar las células caliciformes de la conjuntiva: tinción mediante ácido periódico de Schiff (PAS), inmunodetección de CK7 y MUC5AC, y tinción con lectinas para detectar glicoconjugados.

Se revisaron los distintos modelos *in vitro* existentes para estudiar la función de las células caliciformes. A pesar de la dificultad de cultivar este tipo celular, se han descrito protocolos de cultivo para las células caliciformes de humano, de rata y de ratón, lo que ha permitido avanzar en el conocimiento fisiológico de estas células.

Las células caliciformes están implicadas en varias enfermedades oculares. En el síndrome de ojo seco normalmente hay una disminución en el número y la secreción de las células caliciformes, mientras que en las alergias oculares suelen

encontrarse mayores densidades de células caliciformes. Además, éstas pueden sufrir hiperplasia y presentar mayores tasas de secreción de mucinas. Por último, los pacientes con conjuntivitis papilar gigante (GPC) suelen tener también hipersecreción mucosa.

Hay varios estudios que han intentado analizar el papel de las lentes de contacto sobre las células caliciformes. La mayoría de los estudios indican que el uso de lentes de contacto puede llevar a un descenso en el número de células caliciformes, aunque algunos autores señalan justo lo contrario.

A pesar de la relación de las células caliciformes con distintas enfermedades en las que las citoquinas son muy estudiadas, el efecto de estas últimas sobre dichas células no ha sido ampliamente analizado. En este trabajo se llevó a cabo una revisión sobre este tema. Los principales datos encontrados indican que el IFN-γ produce apoptosis de células caliciformes y un descenso en la secreción, y que citoquinas de tipo Th2, especialmente la IL-13, son capaces de aumentar la proliferación y la secreción de las células caliciformes. Estos datos podrían ayudar a explicar algunos de los signos observados en pacientes.

A lo largo de esta revisión se ha podido ver como el estudio de las células caliciformes ha ido aumentando en los últimos años. Esto ha permitido confirmar que el papel de estas células va más allá de la simple secreción de mucinas, e indica que debe continuarse con el estudio de las células caliciformes.

ESTUDIO 4 – **Interacción del INF-γ con agonistas colinérgicos para modular la función de células caliciformes de rata y humanas**

En este trabajó se llevó a cabo un estudio de la interacción del IFN-γ con el agonista colinérgico carbacol (Cch) para modular la función de las células caliciformes.

Las células caliciformes están inervadas por nervios simpáticos. En condiciones normales, la acetilcolina es capaz de estimular su secreción mediante el aumento de los niveles intracelulares de calcio ($[Ca^{2+}]_i$). En el síndrome de ojo seco suele haber una disminución de la secreción mucosa y un descenso en el número de las células caliciformes. El IFN-γ es una molécula que se encuentra

elevada en esta patología y, por ese motivo, se estudió su interacción con las células calciformes.

Las células cultivadas se caracterizaron mediante inmunofluorescencia y RT-PCR a tiempo real. Además de demostrar que se trataba de células caliciformes (puesto que expresaban CK7 y MUC5AC, y se marcaban con lectinas), se demostró que estas células expresaban el receptor de IFN-γ.

Se observó que el IFN-γ era capaz de aumentar los niveles de $[Ca^{2+}]_i$ de manera dosis dependiente. Lo mismo sucedía con el Cch. Al combinar ambas sustancias se observó que el IFN-γ bloqueaba el aumento de $[Ca^{2+}]_i$ provocado por el Cch y viceversa. Se comprobó que la inhibición que producía el IFN-γ del aumento de $[Ca^{2+}]_i$ provocado por el Cch era dependiente del tiempo, ya que esta inhibición se producía a los 15 minutos, pero no a las 24 horas.

Para analizar las causas de estos cambios en el tiempo, se llevaron a cabo estudios funcionales con y sin $[Ca^{2+}]$ extracelular. Además, se realizaron experimentos con tratamientos previos con tapsigargina, una sustancia que aumenta los niveles de $[Ca^{2+}]_i$ al liberar el Ca^{2+} almanacenado en el retículo endoplasmático, a la vez que impide que esos depósitos intracelulares vuelvan a almacenar Ca^{2+}. Los resultados obtenidos parecen indicar que los efectos a corto plazo se deben al uso de los mismos depósitos de calcio por parte de IFN-γ y Cch.

Además, se observó que el tratamiento durante 24 horas con IFN-γ también bloqueaba el incremento en la secreción de mucinas mediado por Cch por parte de las células caliciformes.

Por último, se demostró que el IFN-γ producía un descenso significativo del 20% en la proliferación de las células caliciformes. Este dato concuerda con los aportados por otros autores que encontraron un incremento de apoptosis en presencia de esta citoquina.

En resumen, en este estudio se demostró que el IFN-γ afectaba a múltiples procesos que controlan la cantidad de mucina producida por las células caliciformes. Afecta a la secreción, pero también al número de células caliciformes presentes en la conjuntiva. Todo esto podría explicar la deficiencia mucosa que suelen sufrir los pacientes con ojo seco, en los que normalmente se detectan niveles elevados de IFN-γ.

ESTUDIO 5 – Un nuevo modelo de cultivo de células primarias epiteliales de conjuntiva humana para estudiar inflamación

En este trabajo se desarrolló y optimizó un protocolo de cultivo de células conjuntivales humanas, para poder utilizarlas como modelo de estudio.

Se aislaron fibroblastos a partir del estroma conjuntival de muestras obtenidas de donante cadáver. Los fibroblastos se cultivaron en medio DMEM/F12 suplementado con antibióticos y un 10% de suero fetal bovino (FBS).

Las células epiteliales de conjuntiva humana se obtuvieron a partir del tejido mediante dos procedimientos: disgregación celular y mediante la técnica de explantes. Se observó que mediante los explantes se obtenían más células de una forma más fácil y rápida, por lo que fue el método de elección.

Se consiguió evitar la contaminación de los cultivos epiteliales por fibroblastos mediante dos técnicas: pre-sembrado y tripsinización diferencial. Con un presembrado de 2 horas se conseguía un cultivo puro de células epiteliales, y lo mismo ocurría si se realizaba una tripsinización diferencial durante 5 minutos para eliminar los fibroblastos.

Una vez que se obtuvieron cultivos de células epiteliales sin contaminación estromal, se probaron cinco medios de cultivo para lograr optimizar la expansión de dichas células. El medio control fue DMEM/F12 suplementado con 10% de FBS, 2ng/ml de factor de crecimiento epidérmico (EGF), 1 µg/ml insulina, 0,5 µg/ml de hidrocortisona, 0,1 µg/ml de toxina colérica, y antibióticos. Además se probó un medio enriquecido en EGF (10 ng/ml), un medio con H_2O_2 (había sido descrito que pequeñas cantidades de H_2O_2 favorecían la adhesión de células de epitelio corneal), un medio condicionado por fibroblastos y un medio con 10% de suero humano en vez de FBS. Los resultados mostraron que el medio condicionado por fibroblastos favorecía la adhesión celular más que ningún otro y era el segundo medio con el que se lograron mejores tasas de proliferación celular, siendo superado tan solo por el medio con suero humano. Por su parte, el medio con suero humano era el que proporcionaba una mayor eficiencia en la formación de colonias. No se encontraron cambios en la expresión del marcador de epitelio conjuntival CK19 entre los distintos medios. Sin embargo, sí que se observaron diferencias en el número de células positivas para el marcador de proliferación Ki67, siendo

superiores en el medio condicionado por fibroblastos y en el medio con suero humano. Con todos estos resultados se determinó que el medio con suero humano era el más adecuado para la expansión *in vitro* de células epiteliales de conjuntiva humana.

A continuación se realizaron sucesivos pases celulares con el medio de elección y se analizaron distintos marcadores específicos de fenotipo epitelial conjuntival secretor. Las células epiteliales expresaban los marcadores CK4, CK19 y CK7 hasta el pase 3. Sin embargo, en los pases 4 y 5 se observaba un descenso e incluso desaparición de dichos marcadores epiteliales, así como un aumento en la expresión de vimentina, lo que indica transición epitelio-mesenquimal. Las células hasta el pase 3 mostraban uniones adherentes (E-cadherina) y uniones estrechas (ZO-1), algo que no ocurría en las muestras de pase 5.

Finalmente, se realizó un pequeño estudio de inflamación celular. Tras tratar a células en distintos pases con TNF-α, se observó que éstas secretaban mayores niveles de IL-6 que los controles sin tratar. El incremento en la secreción de IL-6 tras estimulación con TNF-α ha sido descrito en células epiteliales, por lo que este experimento permitió comprobar que las células cultivadas respondían de manera normal a este estímulo.

En este estudio se desarrolló un protocolo completo de aislamiento y cultivo de células conjuntivales a partir de muestras de tejido procedentes de donante cadáver. Las células epiteliales mantuvieron sus características en cultivo y fueron capaces de responder a estímulos inflamatorios. Esto demuestra que los cultivos de epitelio conjuntival humano realizados mediante el protocolo descrito pueden ser empleados para realizar estudios funcionales.

ESTUDIO 6 – Selección del biomaterial adecuado para su uso como andamiaje de células conjuntivales

El objetivo de este trabajo era encontrar un biomaterial adecuado para elaborar el modelo tridimensional de conjuntiva humana. Para ello, debería permitir el crecimiento celular y la adhesión de células epiteliales en su superficie.

Se probaron dos tipos de biomateriales: hidrogeles de gelano/espermidina (dos formulaciones distintas) y matrices de fibrina obtenidas a partir de plasma humano.

Los hidrogeles de gelano/espermidina presentaban un aspecto macroscópico homogéneo y transparente. Por el contrario, las matrices de fibrina eran más opacas. Sin embargo, el manejo de los hidrogeles de gelano fue más difícil que el de las matrices de fibrina, ya que los primeros eran más frágiles y se rompían fácilmente al manipularlos con las pinzas.

Cuando se añadieron células epiteliales de conjuntiva humana de la línea IOBA-NHC sobre los dos biomateriales se observó una mejor adhesión en las matrices de fibrina. En ellas, las células eran capaces de adherirse a la superficie y proliferar sobre ella. Por el contrario, solo un pequeño porcentaje de células se adhirieron a la superficie de los hidrogeles de gelano/espermidina, y al cabo de unos días esas células morían. La proliferación celular en ambos biomateriales se evaluó mediante el reactivo alamarBlue®**.** Las células en contacto con los hidrogeles de gelano/espermidina presentaban tasas de proliferación muy inferiores a las de las células cultivadas sobre plástico (control). Por el contrario, las mayores tasas de proliferación se hallaron cuando las células se cultivaron sobre la superfice de las matrices de fibrina.

Para comprobar si el aparente problema de biocompatibilidad de los hidrogeles se debía a un problema de citotoxicidad, se evaluó la viabilidad de las células en contacto con los hidrogeles de gelano/espermidina mediante el ensayo de toxicidad *in vitro* basado en el XTT. Los niveles encontrados con los hidrogeles fueron similares a los del control positivo de toxicidad empleado (cloruro de benzalconio), con porcentajes de viabilidad por debajo del 25%.

En resumen, en este trabajo se analizaron dos tipos distintos de andamiajes. Mientras que las matrices de fibrina mostraron buenos resultados, los datos obtenidos con los hidrogeles de gelano/espermidina desaconsejaron su uso con células conjuntivales. En conclusión, las matrices de fibrina fueron el andamiaje elegido para continuar con el proyecto de desarrollo de un modelo tridimensional de conjuntiva humana.

ESTUDIO 7 - Desarrollo de un modelo tridimensional de conjuntiva normal humana

Una vez obtenidas las células y seleccionado el andamio adecuado (las matrices de fibrina) se procedió a elaborar el modelo tridimensional de conjuntiva. Se usaron matrices de fibrina obtenidas a partir de plasma y a partir de crioprecipitado de sangre humana.

Las células epiteliales crecidas sobre las matrices de fibrina estratificaban en distintas capas a lo largo del tiempo, alcanzando hasta 6 capas a los 14 días de cultivo. Además, dichas células mantenían el fenotipo epitelial, corroborado por la expresión de CK19. Mediante inmunofluorescencia se comprobó que algunas de esas células, normalemente las más superficiales, expresaban también CK7. La tinción con la lectina HPA fue también positiva en algunas células. Ambas características son indicativas de la presencia de células caliciformes.

Al analizar la proliferación de las células en las matrices se encontraron diferencias entre los distintos tipos celulares. Los fibroblastos incluidos en el interior de las matrices de plasma mostraron mejores tasas de proliferación que los que estaban en las matrices de crioprecipitado. Por el contrario, las células epiteliales crecían más sobre las matrices de crioprecipitado que sobre las de plasma. El marcador de proliferación Ki67 también se analizó mediante inmunofluorescencia en las células epiteliales, y el porcentaje de células positivas en las distintas condiciones corroboró los datos obtenidos mediante alamarBlue®. Las células que estaban sobre matrices de crioprecipitado tenían un mayor porcentaje de expresión de Ki67 (38,97% a los 3 días y 35,28% a los 7 días) que las que estaban sobre plasma (23,13% a los 3 días y 16,13% a los 7 días).

Al analizar la estructura de las matrices y de las células mediante microscopía electrónica de barrido (SEM) se observó como los fibroblastos mantenían su morfología fusiforme y se integraban bien en la red de fibras en ambos tipos de matrices. Las células epiteliales, por su parte, tapizaban toda la superficie de las matrices. Se observaron microvellosidades en la cara superficial de las células epiteliales, lo que demuestra en parte que están polarizadas en este modelo. Esto es algo que resulta esencial para el desarrollo de sus funciones.

En resumen, en este estudio se desarrolló un modelo tridimensional de conjuntiva humana normal a partir de cultivos primarios y matrices biocompatibles

de fibrina. Al ser un modelo de estudio más completo que los descritos hasta el momento, se cree que pueda ayudar a avanzar en el estudio de las enfermedades inflamatorias de la superficie ocular de una forma más fiable y fidedigna, evitando o disminuyendo con ello el uso de animales de experimentación.

ESTUDIO 8 – Simulación de enfermedades inflamatorias en el modelo tridimensional de conjuntiva humana

En este trabajo se llevó a cabo una prueba de concepto para comprobar la utilidad del modelo tridimensional de conjuntiva humana en estudios *in vitro* de inflamación ocular.

Para ello, la construcción elaborada con matrices de fibrina derivadas de crioprecipitado y células conjuntivales humanas se expuso a distintas condiciones experimentales: control (cultivo en condiciones estándar), cultivo *air-lifted* (en los que las células crecen en la interfase aire-medio de cultivo), desecación (para simular algunos de los hallazgos típicos de ojo seco) y tratamiento con IL-13 (para simular la condición de alergica ocular). En todos los casos las construcciones se mantuvieron en cultivo durante 7 días. En la condición *air-lifted*, las construcciones estuvieron los tres últimos días en cultivo aireado. En la condición de desecación las construcciones estuvieron durante 2 horas en la campana de flujo laminar sin medio cultivo, de tal forma que el flujo de aire de la propia campana simulaba las condiciones adversas. Por último, las construcciones con IL-13 fueron tratadas con 20 ng/ml de esta citoquina durante 24 horas.

Tras 7 días de cultivo las construcciones se fijaron, procesaron y tiñeron con hematoxilina/eosina para su evaluación a microscopía óptica. Se observaron cambios en las distintas condiciones experimentales comparadas con el control. En las construcciones *air-lifted* se observó un aumento en la estratificiación de las capas epiteliales, y en las construcciones tratadas con IL-13 un aumento en el grosor de la matriz. Muchos trabajos previos han demostrado que la condición de cultivo *air-lifted* favorece la estratificación, por lo que los resultados obtenidos en nuestro modelo de conjuntiva humana corroboran también este hecho.

Además, en la condición *air-lifted* se observó un incremento en el número de células de tipo caliciforme teñidas por la lectina HPA, así como en la secreción

de MUC5AC. Datos similares han sido descritos por varios autores en cultivos tridimensionales de epitelio de mucosas.

Para comprobar el estado inflamatorio de las células en las distintas condiciones experimentales se midieron los niveles secretados de IL-6. Estos niveles estaban muy aumentados en las condiciones *air-lifted* y desecación en comparación con el control.

Por último, se utilizó este nuevo modelo tridimensional con las cuatro condiciones experimentales para estudiar los receptores de ácido hialurónico. Los niveles de CD44 y RHAMM se analizaron mediante inmunofluorescencia, y pudo observarse una mayor expresión de ambos en la condición de estimulación con IL-13. Esto concuerda con los datos obtenidos en el estudio 2, donde se vio que los pacientes con enfermedades inmunes de base atópica, en los que la IL-13 suele estar elevada, tenían mayores niveles de expresión de CD44 y RHAMM. Por el contrario, en la condición de desecación se vio que la expresión de RHAMM desaparecía. Este resultado también es compatible con lo observado en el estudio 2 donde se encontró un descenso en los niveles de RHAMM en pacientes con enfermedades inmunes de base no-atópica. Finalmente, se pudo comprobar que las células del modelo tridimensional producían sCD44 y que sus niveles eran mayores en la condición *air-lifted*.

En resumen, en este estudio se ha demostrado que el modelo tridimensional de conjuntiva humana puede ser utilizado para estudiar la inflamación de la superficie ocular y que, con las condiciones experimentales propuestas, se pueden simular algunos de los efectos más habituales de enfermedades inflamatorias tales como el ojo seco y la alergia.

CONCLUSIONES

En esta tesis doctoral se ha desarrollado un nuevo modelo tridimensional de conjuntiva humana. Además, se presenta una prueba de concepto de su uso para el estudio de enfermedades inflamatorias de la superficie ocular. El conocimiento adquirido previamente sobre dichas enfermedades, sobre las células conjuntivales implicadas y sobre algunas de las moléculas más relevantes permitió orientar el desarrollo del nuevo modelo.

Teniendo en cuenta todos los resultados obtenidos en los distintos estudios que conforman esta tesis se concluye lo siguiente:

1. Se ha demostrado que los receptores CD44 y RHAMM están implicados en la inflamación de la superficie ocular y su expresión varía dependiendo de la patología. Esto debería ser tenido en cuenta a la hora de administrar fármacos o lágrimas artificiales que contengan ácido hialurónico. Además, el estudio comparativo de los receptores de ácido hialurónico en muestras obtenidas de pacientes y en líneas celulares ha corroborado una vez más que hay que ser cautos con los resultados obtenidos en líneas celulares, puesto que estos difieren muy a menudo de los que se obtienen con muestras más directas.

2. Las distintas citoquinas que se encuentran elevadas en determinadas patologías inflamatorias que afectan a la conjuntiva tienen efectos diferenciales sobre la función de las células caliciformes. Se ha demostrado que el IFN-γ es capaz de interaccionar con agonistas colinérgicos para modular esta función, lo que indica que las células caliciformes tienen un papel en la protección de la superficie ocular que va más allá de la simple secreción de mucinas.

3. El tejido conjuntival obtenido de donante cadáver es una fuente adecuada de células para desarrollar cultivos primarios de células epiteliales, células caliciformes y fibroblastos conjuntivales, algo que no estaba optimizado hasta el momento. Esta fuente de material humano puede ayudar a reducir el uso de

las líneas celulares que tan a menudo muestran resultados discrepantes con los obtenidos directamente de individuos sanos.

4. Las matrices de fibrina son un buen biomaterial para emplear como andamios en cultivos tridimensionales de conjuntiva humana, puesto que permiten la adhesión y la proliferación de células epiteliales, caliciformes y no caliciformes, y de fibroblastos.

5. Se han establecido unas condiciones estándar con un único medio de cultivo en las que es posible realizar un co-cultivo de fibroblastos, células epiteliales y células caliciformes en una matriz tridimensional de fibrina. En estas condiciones el crecimiento de cada tipo celular alcanza un equilibrio que permite desarrollar un modelo de conjuntiva humana adecuado para llevar a cabo estudios de hasta dos semanas.

6. Se ha podido elaborar y validar un modelo tridimensional de conjuntiva con una estructura similar a la conjuntiva humana, con un epitelio estratificado y polarizado en el que se encuentran células caliciformes que, además, son capaces de secretar MUC5AC de manera diferencial en respuesta a estímulos clínicamente relevantes, tales como la desecación o la presencia de citoquinas inflamatorias.

Estas mismas conclusiones están recogidas en el Epílogo de esta memoria de tesis (página 323).

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SUMMARY IN ENGLISH

“Si tu intención es describir la verdad,
hazlo con sencillez y la elegancia déjasela al sastre”

“*If you are out to describe the truth,*
*leave elegance to the tailo*r”

— Albert Einstein

ABSTRACT

In this Doctoral Thesis report, we have developed a new three-dimensional model of human conjunctiva. The development of a new integrative model was necessary to overcome the limitations of the available *in vitro* models and to allow a reduction in the number of laboratory animals that are used to study ocular surface inflammatory diseases.

For this new model, we developed an optimized protocol to expand epithelial cells and fibroblasts from conjunctival tissue obtained from human cadaveric donors. In addition, some experiments were performed to find an appropriate scaffold within which and upon which the conjunctival cells could develop *in vitro*. Fibrin-based matrices showed good results, and that biomaterial was selected as a scaffold for this model.

Fibroblasts were grown inside the fibrin matrices, and epithelial cells were grown on the surface of the scaffold. Epithelial cells became stratified, reaching 5-6 cell layers at 14 days. In addition, some goblet-like cells were present in the constructs. Once the normal conjunctival three-dimensional model was developed, a proof-of concept for its use to study ocular surface inflammatory diseases was performed. The model was exposed to different conditions such as air-lifted culture, desiccation, or cytokine treatments. After that, changes in different cell markers were evaluated. The markers and the conditions used were selected based on prior knowledge acquired in previous inflammation studies with the different cell types. The results obtained in the three-dimensional model were in accordance with the data obtained from studies with patients. For example, when the model was exposed to interleukin (IL) 13, there was an increase in mucin (MUC) 5AC secretion, as occurs in patients with allergic conjunctivitis. When the model was exposed to desiccation, there was a reduction in the expression of the hyaluronan receptor RHAMM, as seen in patients with Sjögren's syndrome or rosacea.

The results obtained in the different studies that form this Thesis report indicated that the newly developed three-dimensional model can be a useful tool to perform further studies on conjunctival inflammation.

THESIS REPORT STRUCTURE

This Doctoral Thesis report, which is equivalent to a PhD thesis, is elaborated as a "**compendium of publications**" and applies for the **International-awarded Doctoral Thesis Degree.** It has been organized at the University of Valladolid under the regulations of the International Doctorate Committee. The joint requirements are as follows: 1) a general summary, in which the thematic unit of the work is justified, and the objectives, methodology, results, discussion, and conclusions are presented, 2) three articles published in scientific journals within the Journal of Citations Report, and 3) a doctoral stay of at least three months in duration at a research center abroad. The first requirement is fulfilled by the "Summary in Spanish" and the "Summary in English" sections. In this thesis eight different studies are included. Five of them have resulted in published or accepted papers (Studies 1-5), thus fulfilling the second requirement.

The fourth study of this thesis report was performed during my stay at the *Schepens Eye Research Institute/Massachusetts Eye and Ear Infirmary* (Boston, MA, USA). Results obtained in Study 4 have been published and are part of this doctoral thesis. In this way, this thesis report satisfies the third requirement needed to apply for the International-awarded Doctorate Degree.

After the general synthesis, this report is organized into three thematic chapters that present the eight studies (Figure 1).

Chapter 1: **Study of ocular surface inflammation-related relevant molecules** is composed of four studies. In **Study 1**, we analyzed the expression of CD44 and RHAMM hyaluronan receptors in ocular tissues. We considered this study necessary because hyaluronan was, and still is, being used in ocular treatments and artificial tears. However, hyaluronan receptors have not been studied in the ocular surface, despite their implication in cancer and in inflammatory diseases. We demonstrated the presence of CD44 and RHAMM in conjunctival epithelial cells, and we showed that the protein and mRNA expression levels in cell lines differed from those of cytology samples. In **Study 2** we measured hyaluronan receptor expression in conjunctival cells obtained by brush cytology from patients with inflammatory diseases. We found differential mRNA expression of both CD44 and

RHAMM receptors in cells exhibiting different pathologies. Among these, the changes in RHAMM receptor were greater than for CD44 receptor. In **Study 3** we performed a bibliographic review to show the effect of contact lenses and cytokines on conjunctival goblet cell function. This review allowed us to select the most relevant parameters to be studied in **Study 4**, in which we showed that the interaction between IFN-γ and carbachol modulates rat and human goblet cell function.

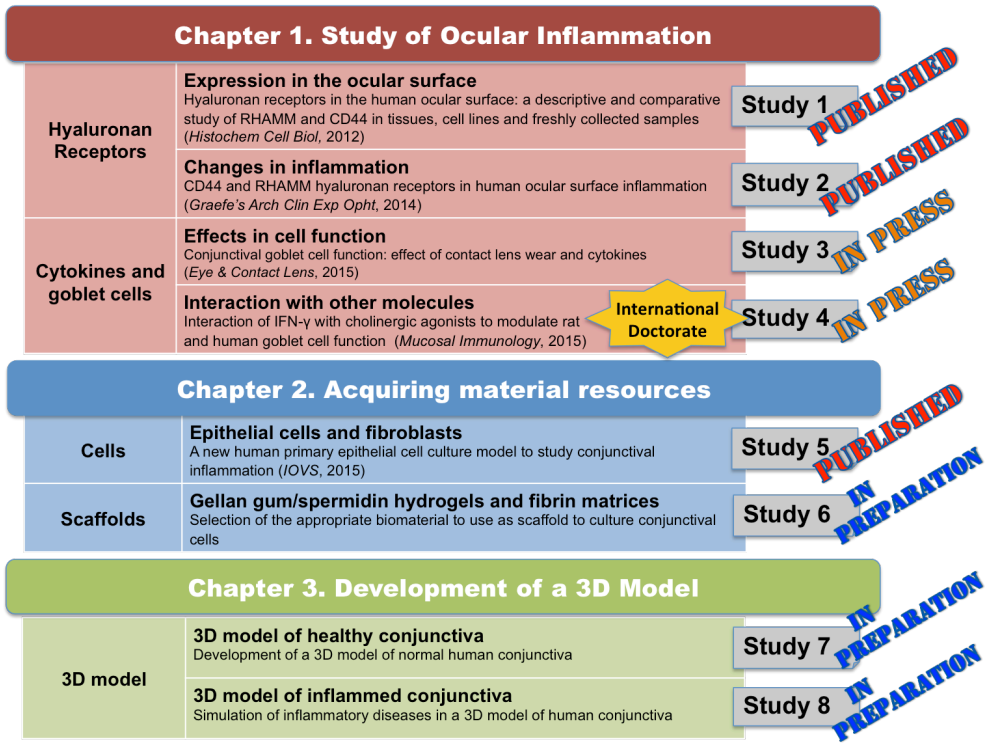

Figure 1. Diagram showing the thesis organization with the three chapters that included the eight studies.

Interesting results regarding the role of conjunctival cells in inflammation were obtained in the Chapter 1. However, to deepen the knowledge of the conjunctiva in inflammatory diseases, such as dry eye or allergy, a good *in vitro* model is necessary. Available models are not complete, and for that reason we considered it necessary to develop a new *in vitro* model of the human conjunctiva that should be three-dimensional and made with primary cells. In the next chapter, **Chapter 2:**

Acquiring material resources to build a new 3D model of human conjunctiva, focused on the obtention of cells and biomaterials. This chapter includes two different studies (Studies 5 and 6 of the thesis). In **Study 5,** an optimized protocol to expand epithelial cells from cadaveric conjunctivas is described. With this protocol, the use of cell lines was avoided. **Study 6** includes the experiments that were carried out with different biomaterials. It concluded with the selection of fibrin matrices as the best scaffold for conjunctival tissue engineering.

Finally, in **Chapter 3: Development of a 3D model of human conjunctiva**, all of the materials previously isolated were put together to engineer a conjunctiva that could serve as an *in vitro* model. In **Study 7**, complete constructs made of fibrin with fibroblasts inside and epithelial cells above were characterized by histological processing, immunofluorescent staining, and scanning electron microscopy (SEM). In **Study 8**, the last one of this thesis, the 3D model developed in the previous study was put under different conditions to simulate conjunctival inflammatory diseases.

INTRODUCTION

1. OCULAR SURFACE AND LACRIMAL FUNCTIONAL UNIT (LFU)

The term “ocular surface” was first introduced by R. A. Thoft (Thoft and Friend, 1977) to refer to the anatomical and functional unit formed by cornea, conjunctiva, and limbal epithelia, along with the tear film and tissue adnexa. The main function of the ocular surface is to preserve sight by protecting the eyeball from external agents and maintaining corneal transparency.

Some years later, M. E. Stern established the term “lacrimal functional unit” (LFU) (Stern et al., 1998). It is composed of the corneal, conjunctival, and limbal epithelia, and the lacrimal glands and neural interconnections (Figure 2).

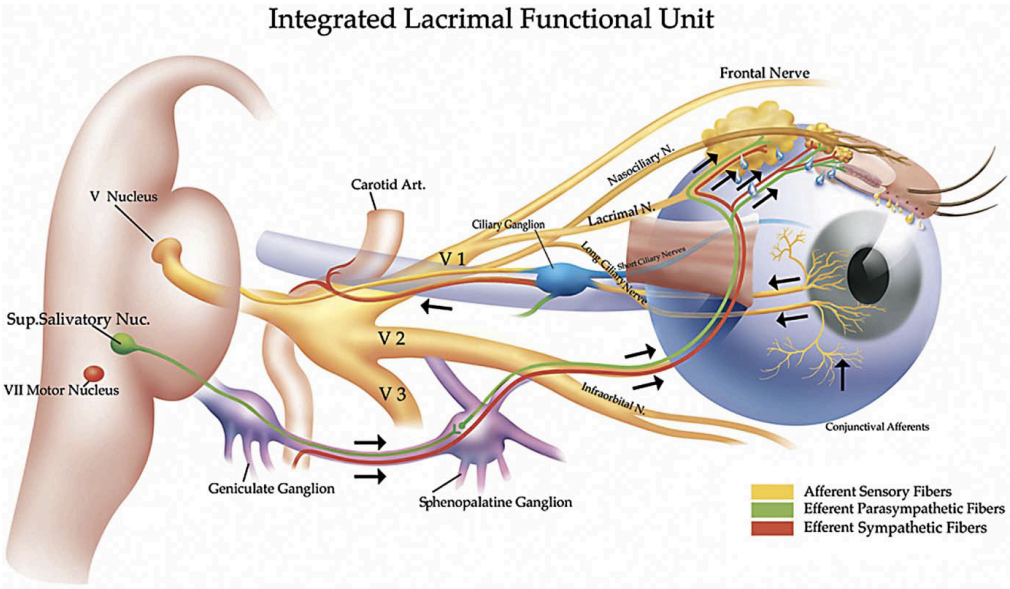

Figure 2. Lacrimal Functional Unit. Picture taken from Beuerman et al. The Lacrimal Functional Unit in Dry Eye and Ocular Surface Disorders (Pflugfelder, Beuerman, Stern eds.). Marcel-Dekker Inc., New York, NY, USA, 2004, 1004:11-40.

2. CONJUNCTIVA

The conjunctiva is part of the ocular surface and the LFU. It is a semitransparent mucous membrane that covers the ocular surface from the eyelids to the edge of the cornea. One of its main functions is to produce mucus that stabilizes the tear film and keep the cornea wet and protected (Dartt, 2006).

Anatomically, the conjunctiva is divided in three parts:

1) The tarsal or palpebral conjunctiva lines the posterior part of the eyelids
2) The bulbar conjunctiva covers the sclera in the anterior segment of the eyeball
3) The fornix forms the junction between palpebral and bulbar conjunctiva

The conjunctiva is composed of a stratified epithelium and an underlying stroma (Figure 3).

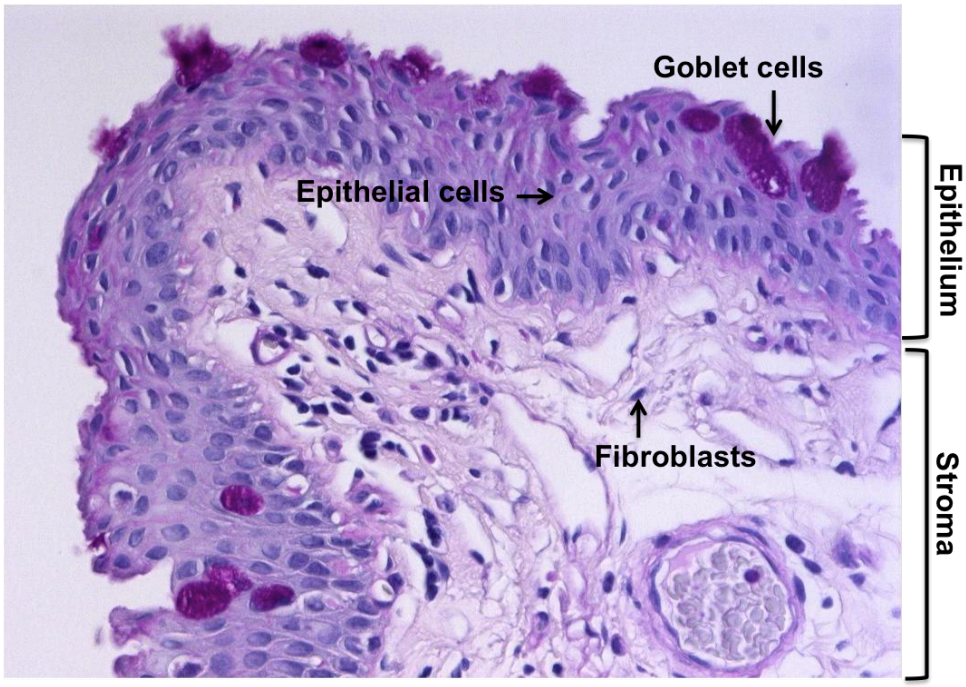

Figure 3. Microphotograph of a conjunctival section. The stratified squamous epithelium contains embedded goblet cells. Image courtesy of Ocular Pathology Laboratory, IOBA.

2.1. Epithelium

The conjunctival epithelium is a stratified squamous non-keratinized epithelium. It has between 2 and 10 epithelial layers, depending on the part of the conjunctiva that is being analyzed (Hoang-Xuan T et al., 2001). Five different types of epithelial cells are present in the conjunctiva (Figure 4). The ultrastructural characteristics were described by K. P. Steuhl (1989).

Type I cells are goblet cells. They are a specialized type of epithelial cell that secretes mucins (Gipson and Argüeso, 2003). They have mucous granules in their cytoplasm, and they usually have a well-differentiated Golgi complex. Goblet cells are the only cell type secreting the mucin MUC5AC in the conjunctiva. This mucin is one of the most important in tears. Goblet cells express secretory epithelium markers (CK7), and their secretory products are stained by lectins that bind high molecular weight glycoconjugates (Krenzer and Freddo, 1997).

Type II cells are epithelial cells with abundant secretory vesicles. They also have numerous 60- to 300-nm electron-dense granules. These cells are distributed throughout the conjunctiva and are the most common cell type.

Type III cells are epithelial cells with a well-developed Golgi apparatus. It is believed that these cells also contribute to the mucous secretions of the tear film.

Type IV cells have large amounts of rough endoplasmic reticulum (rER). This type of cell frequently occurs in human conjunctiva. A small amount of mitochondria and Golgi apparatus is also present in these cells.

Type V cells are mitochondria-rich epithelial cells.

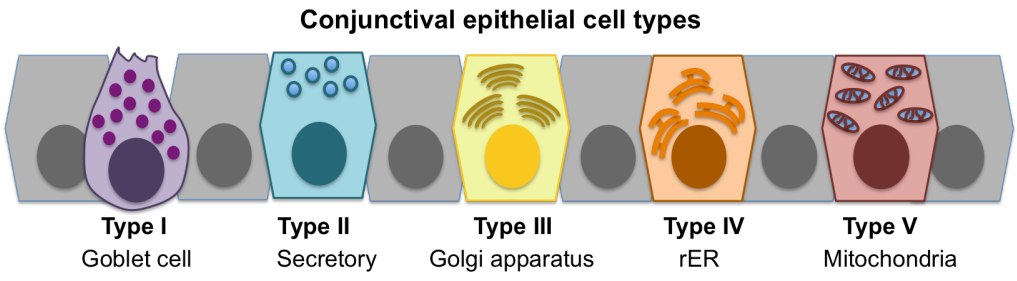

Figure 4. Schematic of the different conjunctival epithelial cell types as described by K. P. Steuhl in 1989.

Cell types II-V are often grouped as squamous stratified non-goblet cells. They constitute approximately 85-90% of all the epithelial cells in the conjunctiva. They are in close contact with each other, showing adherens junctions containing E-cadherin. These cells also have tight junctions, such as the ones formed by zonula occludens (ZO) 1 and ZO2. They express cell markers such as cytokeratin (CK) 4 and CK19 and secrete some mucins, such as MUC1, MUC4, and MUC16. These mucins give stability to the tears.

An essential feature of epithelial differentiation is polarization (Roignot et al., 2013). Conjunctival epithelial cells, as other mucosal epithelia, have microvilli at the apical surface. Microvilli increase the surface area for tear adherence, and are involved in cell adhesion, absorption, and secretion. Abnormal conjunctival microvilli are present in Sjögren's syndrome and graft versus host disease, among other pathologies (Mancel et al., 1993; Tatematsu et al., 2012).

2.2. Basal Membrane

The conjunctival basal membrane is a thin layer of connective tissue that separates the epithelium from the underlying stroma. It is mainly composed of type IV collagen, laminin, nidogen 1 and 2, and thrombospondin-4 (Schlotzer-Schrehardt et al., 2007).

2.3. Stroma

The conjunctival stroma is composed of several molecules, such as collagen, laminin, and hyaluronic acid (HA), among others. It also has an important cellular component. The most abundant cell type in the stroma is the fibroblast. These cells secrete an important amount of the extracellular matrix (ECM) that forms the conjunctival stroma.

The conjunctival stroma has a rich vascular network and abundant lymphatics. In addition, the conjunctiva possesses a rich nerve supply. Finally, there is a significant presence of conjunctival-associated lymphoid tissue (CALT) (Knop and Knop, 2000). Along with the lacrimal drainage-associated lymphoid tissue (LDALT), it forms the eye-associated lymphoid tissue (EALT), a component of the mucosal-associated lymphoid tissue (MALT). Due to the presence of CALT, innate immune defense cells, such as macrophages and mast cells, and specific immune response cells, such as lymphocytes, plasma cells, and dendritic cells, populate the

conjunctival stroma. The CALT protects the conjunctiva and also the cornea (Knop and Knop, 2005). This fact reinforces the idea of the conjunctiva being a protective tissue for the entire ocular surface.

3. CONJUNCTIVAL ROLE IN INFLAMMATORY DISEASES

3.1. Ocular Surface Diseases

The conjunctiva, which protects the exposed anterior segment of the eye, is involved in several inflammatory diseases that affect the ocular surface (Figure 5). Conjunctival epithelial cells are active participants in ocular surface defense, secreting different types of molecules, such as cytokines and chemokines (Enríquez-de-Salamanca et al., 2008) or antimicrobial peptides like human beta-defensin 9 (HBD9) (Mohammed et al., 2010).

Among the large variety of conjunctival diseases, only two of them are included in this summary: dry eye disease and ocular allergy.

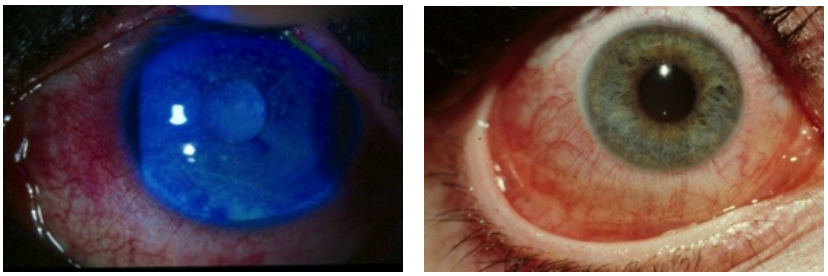

Figure 5. Slit-lamp photographs from a patient with dry eye disease (left) and from a patient with allergic conjunctivitis (right). The conjunctival damage can be seen in both eyes. Images by courtesy of Prof. M. Calonge.

Dry eye syndrome is a multifactorial inflammatory disease of the ocular surface and the tear film. It results from disfunction of the LFU (Stern et al., 2004; 2013). The high prevalence of this disease (Schaumberg et al., 2003; 2009) and its high impact in the quality of life of patients (Schiffman et al., 2003) makes dry eye disease one of the most studied pathologies in the ocular surface. The typical symptoms are itching, pain, blurred vision, and photophobia. The conjunctival

epithelium of these patients can be analyzed by impression cytology. This is a minimally invasive technique that consists of applying a small filter in the conjunctiva. After some seconds, the filter is recovered and the attached cells (one or two layers of conjunctival epithelium) are fixed and stained. Morphological features such as cell shape or metaplasia, and the number of goblet cells are determined. Lower numbers of goblet cells are usually found in impression cytologies from dry eye patients (Mantelli et al., 2013; Marko et al., 2013; Kumar et al., 2014).

Allergy is an abnormal and exaggerated immune response to normally harmless antigens that do not trigger any reaction in non-allergic patients. Conjunctival tissue plays a key role in ocular allergies. There are several types of allergies, ranging from mild forms (seasonal of perennial allergic conjunctivitis) to the most severe ones (vernal keratoconjunctivitis and atopic keratoconjunctivitis) where sight can be seriously compromised because of the potential impairment of the cornea (Leonardi, 2013). In all of these allergic disorders, a direct participation of conjunctival tissue exists. Specifically, squamous epithelial cells and goblet cells are reported to take an active role in the allergic response (Saban et al., 2013). Ocular allergies are frequently associated with mucus hyperproduction, and higher densities and/or hyperplasia of goblet cells are found in allergic patients.

3.2. Molecules Involved in Conjunctival Diseases

The number of molecules that are linked to ocular surface inflammation is growing continuously and simultaneously with our knowledge of inflammatory disease processes. The impossibility of reviewing all of these molecules led us to choose only two types to describe here: cytokines and HA receptors.

3.2.1. Cytokines

Cytokines are small non-structural proteins with a molecular weight ranging from 8 to 40,000 Daltons (Dinarello, 2000). They are immunomodulatory agents produced by different cell types, including epithelial cells in the ocular surface. In fact, every cell except the erythrocyte can produce and respond to cytokines (Dinarello, 2007). As regulators of the immune response, some cytokines are proinflammatory (Dinarello, 2000), others exert anti-inflammatory actions (Opal y DePalo, 2000), and others have both effects.

Cytokines can be classified into different subtypes, depending on the type of T helper (Th) cell that produces them (Steinman, 2007). The main subtypes are Th1, Th2, and Th17 (Figure 6).

Among Th1 type cytokines, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) are the most studied. These two cytokines are usually present in higher amounts in dry eye patients. Among Th2-type cytokines, interleukin (IL) 4, IL-5, and IL-13 have been widely studied in allergy research, where elevated levels have been described.

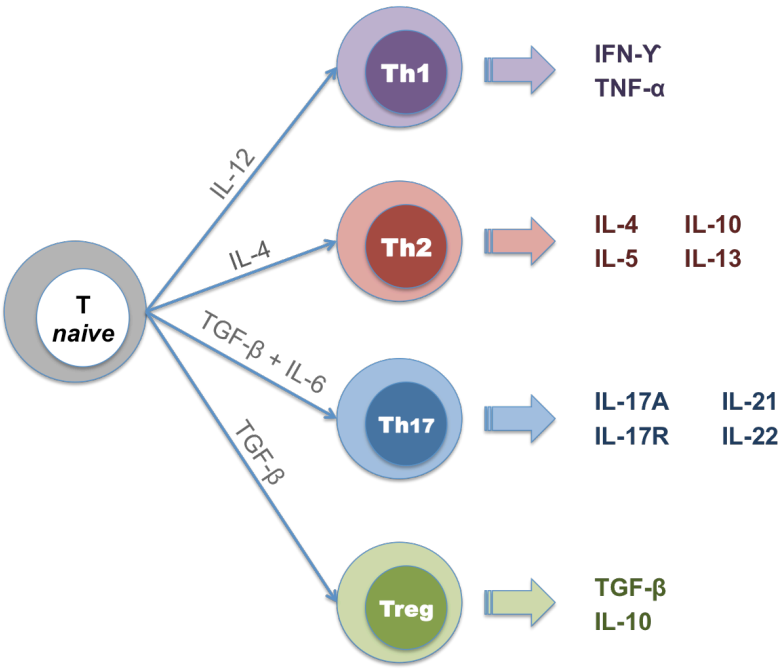

Figure 6. Diagram showing the main cytokine subtypes, classified according to the Th cell type that produces them.

3.2.2. Hyaluronic Acid (HA) and its Receptors

HA or hyaluronan consists of glycosaminoglycans with different molecular weights. They form the ECM of ocular and other tissues. The main functions are to retain water and increase the intercellular space, as well as to participate in tissue remodeling. One important effect of HA is to stimulate collagen production by fibroblasts. For this reason, HAs are broadly used in cosmetics.

HA also participates in the inflammatory process, exerting dual actions depending on the size of the HA fragments. Low molecular weight HAs have proinflammatory effects, whereas high molecular weight HAs help to maintain tissue integrity, participate in wound healing, and provide anti-inflammatory actions (Stern et al., 2006; Campo et al., 2009).

To bind cells and exert some of its functions, HAs have two main receptors: CD44 and Receptor for Hyaluronic Acid Mediated Motility (RHAMM) (Turley, 1982; Entwistle et al., 1996). The role of these receptors in pathological processes has been studied for a long time. There is clear evidence regarding their direct involvement in oncologic diseases (Giannopoulos et al., 2006; Balla et al., 2009) and in inflammatory pathologies such as osteoarthritis (Dunn et al., 2009). CD44 has a transmembrane form and a soluble part (sCD44) that is produced by shedding or alternative splicing. Higher levels of sCD44 are present in the aqueous humor of eyes with glaucoma and this fact has been proposed as one of the causes of increased intraocular pressure that leads to glaucoma (Nolan et al., 2007; Giovingo et al., 2013).

However, despite the apparent importance of these receptors in several pathologies and the continuous use of HA in ocular pharmaceutical formulations, the presence of these receptors and their specific role has not been extensively studied in conjunctival tissues.

4. MODELS TO STUDY THE ROLE OF THE CONJUNCTIVA IN NORMAL AND INFLAMMED OCULAR SURFACES

The physiological importance of the conjunctiva and its implication in several pathologies of the LFU makes it essential to study and understand the normal ocular surface behavior. Before performing clinical trials to test new drugs, research must be done first using *in vitro* models and then on *in vivo* models with laboratory animals.

4.1. *In vivo* Models

In vivo models utilize live experimental animals, such as mice, rats, rabbits, etc. Animal models are useful tools in research because, due to the complexity of system interactions, the results are more likely to resemble what might be expected

in humans. In addition, they yield large amounts of information. However, they present several limitations, mainly ethics-related issues, and more practical issues such as elevated costs, inexact similarity with human equivalent tissues, and the need of specialized and experienced staff and facilities.

4.2. *In vitro* Models

Because of the important concerns cited above regarding living animal research models, *in vitro* models allow researchers study and learn from *in vitro* systems, thus reducing the reliance on the use of animal models. Another advantage of *in vitro* models is that several biological parameters can be easily controlled at the same time. In addition, the use of these models results in a more cost-effective approach to develop pre-clinical studies, as it is possible to test many more experimental conditions than with *in vivo* models. Therefore, the development and use of more advanced, sophisticated *in vitro* models is highly desirable.

4.2.1. Cell Lines and Primary Cultures

Cell lines are constituted of transformed cells that can undergo an infinite number of cell divisions under controlled laboratory conditions. The transformation can be spontaneous or artificially induced. As a consequence of the transformation process, cell lines lose the property of contact inhibition. Loss of this trait allows them to continue growing after confluence in cell culture has been reached. The main advantages of cells lines are availability, easy culture technique, and homogeneous results. The main disadvantage is that after the transformation that makes them cell lines, these cells are in some ways different from the original ones. Some of the native characteristics are lost, and they are not as reliable as the original cells.

Primary cultures are established from cells obtained directly from the organism under study, usually from biopsy or cadaveric tissue. For this reason, they are more similar to the original tissue than cell lines, and results obtained using primary cultures are more valuable. However, they have some inconveniences. First, human tissue availability is quite limited and subjected to strict regulations to guarantee ethical compliance. In addition, it is difficult to culture these cells and their *in vitro* growth is limited.

4.2.2. Monolayer Cultures and Three-Dimensional Cultures

The most common way to grow either cells lines or primary culture cells in the laboratory is in a monolayer culture. In monolayer cultures, cells grow attached to a specialized culture surface, forming a single layer of cells that covers the entire surface, at which point the culture has reached a state of confluence. In contrast, three-dimensional cultures approximate the original three-dimensional structure of the donor tissues. Such a complex structure would allow better studies of the interaction between different cells types or between cells and the ECM. The organization of cells in a three-dimensional space and the relation with the extracellular environment are the main factors that determine cell phenotype. For that reason, research using three dimensional cultures reveals more valuable results in terms of the actual similarity with the living *in vivo* tissue.

Three-dimensional cultures can be used to study organs or tissues in normal or pathological states. The use of these cultures is being increased with the growing knowledge of cell-cell and cell-ECM interactions that tissue engineering is providing.

5. TISSUE ENGINEERING

Tissue engineering was described by R. Langer and J. P. Vacanti as “an interdisciplinary field that applies the principles of engineering and life sciences to the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ” (Langer and Vacanti, 1993). Thus, the ultimate goal of tissue engineering is the clinical use of the engineered tissues. However, these artificial tissues or organs can also be used as complex *in vitro* models. There are two main components in any tissue engineered tissue: cells and scaffolds that support cell growth.

5.1. Cells

Both cell lines and primary cells can be used to engineer a tissue regardless of the final use planned for it. Depending on the origin of the cells, these can be autologous (from the same patient) or heterologous (from a different donor). The cell types used in tissue engineering depend, obviously, on the tissue that is going to be engineered. In the conjunctiva, the main cell types are epithelial cells (including goblet cells) and stromal cells, mainly fibroblasts.

5.2. Scaffolds

Scaffolds are the acellular component that maintain the formation of the tissue in three dimensions. They provide cells with structural support and guide their growth. For these reasons scaffolds are an essential part of tissue engineering.

A good scaffold should be biocompatible, biodegradable, and should favor cell adhesion. Also, it can help in nutrient transport and facilitate functional tissue formation (Lee et al, 2011). To develop an artificial tissue as similar to the native tissue as possible, the scaffold should also be similar to the native ECM.

Both natural and synthetic biomaterials can be used to elaborate scaffolds. The main advantages of natural biomaterials are their biocompatibility and their greater resemblance to the native ECM. Some of the most frequently used natural biomaterials are amniotic membrane, collagen, chitosan, fibrin, and HA (Rosso et al., 2005), whereas the most frequent synthetic biomaterials are poly-lactic acid (PLA), poly-lactic-co-glycolic acid (PLGA), and polycaprolactone (PCL) (Saltzman, 1999; Putman, 2001).

5.3. Ocular Surface Approaches

Several studies applying tissue engineering resources into the ocular surface field have been published. Some of them are focused on developing an artificial cornea to substitute for conventional corneal grafts (Griffith et al., 2009) in patients suffering from limbal stem cell deficiency (Xu et al., 2012). With this purpose, the transplant of limbal epithelial cells (Pellegrini et al., 2014), oral epithelial cells (Utheim, 2015), and conjunctival equivalents (Tanioka et al., 2006; Di Girolamo et al., 2009) have also been proposed. Several studies report the huge potential of using conjunctival cells for treating blindness, and specifically limbal stem cell deficiency (Di Giralomo *et al*., 2009; Jeon *et al*., 2013).

Alaminos and collaborators (Alaminos et al., 2006) recently developed a complete rabbit corneal substitute to be used as an *in vitro* model. To develop this model they used the three main corneal cell types: endothelial cells, fibroblasts, and epithelial cells. Fibrin and agarose matrices were used as scaffolds for this model. They constructed an organotypic cornea model that resembles the ultrastructure of a normal native cornea. This study is a good example of how tissue engineering can also be used for research purposes.

In 2007, Chung and collaborators (Chung et al., 2007) reported the development of a three-dimensional model of human conjunctiva that secreted MUC5AC. The lack of MUC5AC production *in vitro*, probably due to the difficulty of culturing goblet cells, is the main limitation of several conjunctival models reported so far. However, in the model described by Chung et al., this limitation was overcome. Starting from small conjunctival biopsies, Chung and colleagues cultured human goblet cells that were later seeded in Transwell-clear 3450 culture inserts with a polyester membrane. Using an air-lifted culture technique, these cells became stratified in different layers, reaching 6-8 layers after two weeks. In these conditions they observed MUC5AC secretion. This research provides a good model to study conjunctival epithelium, but not the whole conjunctiva because the stroma was not present.

Despite the progress made with conjunctival tissue equivalents, a complete three-dimensional conjunctival model has not been described until now. Thus, there remains a need for a good human model to increase our understanding of normal and pathological conjunctival physiology and to test new treatments.

MOTIVATION

Although the conjunctiva is less often studied than the cornea, it is the main tissue implicated in some prevalent and potentially sight-threatening ocular surface diseases, such as dry eye or ocular allergy. To make progress in the study of the pathophysiological processes affecting the conjunctiva and to find new therapies to treat those diseases, it is mandatory to have good *in vitro* models.

We understand that currently available models for conjunctiva are not adequate because they are oversimplified. Usually they consist only of cell monolayers formed by transformed cell lines that are no longer similar to the actual progenitor tissue. Therefore, caution should be taken when conclusions are drawn from results obtained with these models.

One of the main functions of the conjunctiva is to secrete mucins that lubricate and protect the ocular surface. This function is usually altered in inflammation. Most conjunctival epithelial cells are able to secret mucins, but the main secretory cell is the goblet cell, which is the only cell type in the conjunctiva that secretes MUC5AC, one of the most important mucins in the tear film. Hence, the study of goblet cell function becomes especially relevant.

Bearing all this in mind, we feel a new, more complex and integrative conjunctival model is necessary. To overcome the limitations of existing models, the new model should be derived from primary cells and not from cell lines. In addition, the model should recapitulate the three-dimensional structure of the conjunctiva, with at least a stroma populated with fibroblasts and an epithelium containing mucin-secreting goblet cells.

To test the efficacy of this model in the study of inflammatory diseases, the different types of cells that participate in the inflammatory process should have already been identified. In this way, the most relevant findings can be simulated in the three-dimensional model.

This doctoral thesis was developed in the context of this framework. The main aim was to develop a three-dimensional *in vitro* model of human conjunctiva that allows researchers to deepen the knowledge of conjunctival inflammatory diseases while minimizing the use of animals.

To face this challenge, a multidisciplinary team was needed. The Ocular Surface Group from IOBA has provided leadership in the study of inflammatory diseases for a long time. The team relies on medical experts that provide the clinical point of view to understand the pathological processes. Additionally, the team is led by Dr. Y. Diebold who has expertise in cell culture techniques and an established career in the field of nanomedicine. Effectiveness of the team relies in part on collaborations in the biomaterials field, such as researchers from the Department of Pharmacy and Pharmaceutical Technology from the University of Santiago de Compostela (Spain) and the Tissue Establishment San Francisco Clinic Foundation from León (Spain). Several competitive national joint grants support these collaborations.

Therefore, the need for further pathophysiological study of the conjunctiva and the need to test new therapeutic strategies that are being developed by the nanomedicine team support the idea of developing a three-dimensional model of human conjunctiva. The career path within the Ocular Surface Group from IOBA is a guarantee of achieving these goals and making progress in this challenging thesis project.

HYPOTHESES

It is possible to develop a three-dimensional model of human conjunctival tissue containing stromal and epithelial cells, and this model can incorporate goblet-like cells able to secrete mucins.

This *in vitro* model would be capable of responding to external stimuli, and would be suitable to study inflammatory processes affecting the conjunctiva.

OBJECTIVES

General Objective

To develop a three-dimensional model of human conjunctiva composed of three cell types: fibroblasts, epithelial cells, and goblet cells. The model should be developed in a biocompatible matrix and allow reliable studies related to inflammation.

Specific Objectives

1. To determine the role of the HA receptor in ocular surface inflammatory diseases (**Studies 1 and 2**).
2. To determine the role of cytokines expressed in ocular surface diseases on goblet cell function (**Studies 3 and 4**).
3. To establish primary cultures from human conjunctival epithelial and stromal cells (**Study 5**).
4. To select an appropriate biomaterial to be used as a scaffold in the development of a three-dimensional model (**Study 6**).
5. To develop a three-dimensional co-culture of human conjunctival goblet, non-goblet epithelial cells, and fibroblasts (**Study 7**).
6. To simulate ocular surface inflammatory diseases in the three-dimensional model by the application of different stimuli (**Study 8**).

METHODOLOGY

Detailed descriptions for the methodologies used in the experiments are included in each study. For that reason only general techniques are summarized in this section.

1. SAMPLES

Human conjunctival tissues to prepare primary cultures were obtained post-mortem from cadaveric donors. These samples were from Barraquer Eye Bank (Barcelona, Spain), Heartland Lions Eye Bank (Kansas City, MO, USA), and Michigan Eye Bank (Ann Arbor, MI, USA), in all cases with valid agreements and approval from Institutional Boards.

Conjunctival and corneal samples for the analyses performed in Studies 1 and 2 were obtained from living individuals. Prior approval from the Clinical Research Ethics Committee was obtained, and each patient signed an informed consent. All the experiments were in accordance to the Tenets of the Declaration of Helsinki and to the Spanish regulations in biomedicine and biological samples.

Rat goblet cells were obtained from Sprague-Dawley rat conjunctivas. Experiments were performed in accordance with the ARVO Declaration for Use of Animals in Research (http://www.arvo.org/About_ARVO/Policies/Statement_for_the_Use_of_Animals_in_Ophthalmic_and_Visual_Research/).

1.1. Cell Lines

The human corneal epithelial (HCE) cell line (Araki-Sasaki et al., 1995) and the normal human conjunctival (IOBA-NHC) epithelial cell line (Diebold et al., 2003) were used in Study 1 for comparison purposes.

1.2. Primary Cultures

Primary cultures from fibroblasts, epithelial, and goblet cells were stablished in Studies 4, 5, 7, and 8 from the human conjunctival tissue (Figure 7) following the explant technique. In addition, goblet cells were also cultured from Sprague-Dawley rats in Study 4.

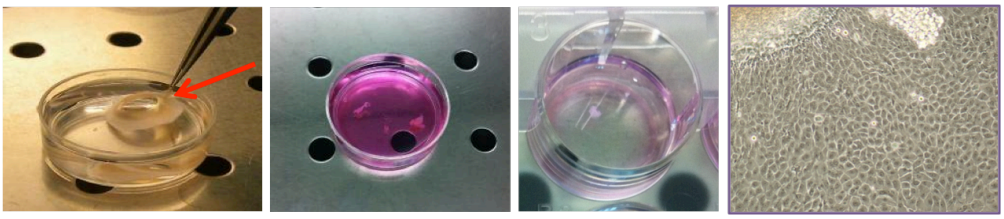

Figure 7. Cell expansion from cadaveric conjunctiva. The conjunctival tissue was isolated from corneoscleral rims (left). Small pieces (explants) were placed in the surface of 12-well plates (middle left, middle right). Cell growth from the explants was observed after 1-2 days (right).

1.3. Freshly Collected Samples

Conjunctival epithelial cells used in Studies 1 and 2 were collected by brush cytology from patients with active ocular surface inflammatory diseases and from healthy volunteers. Corneal epithelial cells analyzed in Study 1 were obtained from patients undergoing refractive surgery.

2. CELL PARAMETER MEASUREMENTS

2.1. Cell Proliferation Measurement with the Cell Proliferation Assay Reagent alamarBlue®

Cell proliferation was determined in Studies 5, 6, and 7 with the colorimetric assay alamarBlue® (AbD Serotec, Oxford, UK). alamarBlue® dye was applied to cultured cells and after 4-6 h incubation, and the fluorescence was read at 590 nm. Fluorescence levels were related to cell proliferation.

2.2. Goblet Cell Proliferation Measurement with Cell Counting Kit-8

Cell proliferation of conjunctival rat goblet cells was determined in Study 4 with Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA). Tetrazolium salt WST-8 included in the kit was diluted in culture medium and added to cultured cells. Absorbance was read at 450 nm after 45 min incubation. The absorbance values obtained were proportional to cell proliferation.

2.3. Cell Viability Measurement with *In Vitro* Toxicology Assay kit, XTT based

Cell viability was determined in Study 6 with the *In Vitro* Toxicology Assay kit, XTT based (Sigma-Aldrich, St. Louis, MO, USA) to evaluate potential cytotoxicity of gellan gum hydrogels. As in the other proliferation assays, the XTT assay was based on a tetrazolium salt that becomes reduced by mitochondrial dehydrogenases in viable cells. XTT was added to the cells and incubated for 17 hours. The absorbance, which is proportional to cell viability, was read at 450 nm.

3. PROTEIN ANALYSIS

3.1. Immunofluorescence Detection

Immunofluorescence staining is based on antigen-antibody reactions. A primary antibody binds the protein and, later, a secondary antibody with a fluorescent label binds the primary antibody. A list of all the different primary antibodies used in this thesis is found in Table 1. This technique was routinely used in Studies 1, 4, 5, 7, and 8.

3.2. Electrophoresis and Western Blotting

Western blotting is a method for identification of proteins that have been previously separated according to their molecular weight by electrophoresis in a polyacrylamide gel. Proteins are then transferred from the gel to a membrane, where they are subsequently detected using specific antibodies. This technique was used to detect different proteins in Studies 1 and 5. A list of the primary antibodies used and their working dilutions is presented in Table 1.

3.3. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a biochemical assay used to detect an antigen that is present in a liquid phase and is based on the antigen-antibody reaction. Secreted proteins, such as IL-6 (Studies 5 and 8), sCD44 (Studies 1 and 8), and MUC5AC (Study 8) were measured by ELISA following the assay manufacturer's instructions (Diaclone, Besançon, France, for IL-6 ELISA; eBioscience, Vienna, Austria, for sCD44 Instant

ELISA; Shanghai Yehua Biological Technology Co., Ltd., Shanghai, China, for MUC5AC ELISA).

Table 1. Primary antibodies and lectins used in the different studies

Antibody/Lectin	Reference	IF dilution	WB dilution	Study
CD44	Calbiochem-Merck	1:100	1:500	1
RHAMM	Santa Cruz Biotechnology	1:50	1:500	1
CK4	Abcam	1:100	-	4
CK7	Abcam	1:100	-	4
MUC5AC	Abcam	1:100	-	4
IFN-γ-R1	Novus Biologicals	1:100	-	4
CK19	Dako	1:50	1:1000	5, 7, 8
CK4	Sigma-Aldrich	-	1:250	5
CK7	ThermoScientific	1:100	1:200	5, 7, 8
MUC5AC	EMD Millipore Corp.	1:500	1:500	5, 7, 8
Vimentin	Santa Cruz Biotechnology	1:500	1:200	5
FSP-1	Abcam	1:100	-	5
Ki67	Dako	1:50	-	5, 7, 8
E-cadherin	BD Bioscience	1:100	1:1000	5, 7
ZO-1	Invitrogen	1:50	1:250	5
UEA-1 lectin	Sigma Aldrich	1:500	-	4
HPA lectin	Sigma Aldrich	1:500	-	3, 4, 7, 8

IF: immunofluorescence; WB: Western blotting

4. GENETIC ANALYSIS

4.1. Conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Electrophoresis in Agarose Gel

To determine genetic expression levels, RT-PCR followed by electrophoresis in agarose gel was performed in Studies 1 and 4. Previously, mRNA was isolated with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The primers used are listed in Table 2. Specific conditions for each primer are detailed in the corresponding studies.

4.2. Real Time RT-PCR

This technique is a technological advance of conventional RT-PCR. It provides some advantages such as speed, reproducibility, and low contamination risk. In this thesis, a semiquantitative variant was performed in Studies 1, 2, and 4. The isolated mRNA was reversed transcribed to cDNA that was then mixed with primers (Table 2) and SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Data was analyzed with the $2^{-\Delta\Delta Ct}$ semiquantitative method (Livak y Schmittgen, 2001).

Table 2. Primers used in different studies

Primer	Reference	Base pairs	Study
CD44	SABioscience-Qiagen	106	1, 2
RHAMM	SABioscience-Qiagen	108	1, 2
GAPDH	Sigma	250	1, 2, 4
P53	SABioscience-Qiagen	188	2
MUC5AC	Sigma-Aldrich	85	4
Human IFN-γ-R1	OriGene Technologies Inc.	169	4
Rat IFN-γ-R1	Qiagen	169	4

5. SPECIAL TECHNIQUES

5.1. Intracellular Calcium Measurement

In Study 4, intracellular calcium levels $[Ca^{2+}]_i$ were measured in cultured rat and human goblet cells stimulated with IFN-γ and carbachol. Cells were incubated with Krebs-Ringer bicarbonate (KRB) buffer (120 mM NaCl, 25 mM $NaHCO_3$, 10 mM HEPES, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1 mM $CaCl_2$) with 0.5% bovine serum albumin (BSA), 8 μM F127 pluronic acid, 250 μM sulfynpirazone, and 0.5 μM Fura-2/AM for 1 hour at 37ºC. After incubation, the cells were rinsed in KRB buffer with sulfinpyrazone and under the microscope with the calcium visualizing system InCyt Im2 (Intracellular Imaging, Cincinnati, OH, USA). At least 10 individual cells in each assay were selected for analysis. The peak change in $[Ca^{2+}]_i$ was recorded.

5.2. Fibrin Matrices Preparation

Fibrin-based matrices were prepared from either human fresh frozen plasma (Studies 6 and 7) or cryoprecipitates (Studies 7 and 8), obtained from CHEMCYL (Valladolid, Spain). Matrices were produced by mixing plasma (400 µl/ml) or cryoprecipitate (333 µl/ml) with 40 µl/ml tranexamic acid (Rottapharm, Valencia, Spain) and 40 µl/ml calcium chloride (Braun, Barcelona, Spain), all diluted in DMEM/F12 culture medium (Invitrogen-GIBCO, Inchinnan, UK). In matrices where fibroblasts were incorporated, 100,000 cells/ml were added to the matrix mix before polymerization. When epithelial cells were cultured on the surface of the matrices, 100,000 cells/cm^2 were added 24 hours after matrix formation.

SUMMARY OF RESULTS

Chapter 1: Study of Ocular Surface Inflammation-Related Relevant Molecules

- Hyaluronan receptors are expressed in the ocular surface. CD44 expression was constant for the entire ocular surface epithelium, whereas higher levels of RHAMM were found in the conjunctival tissue compared to the corneal tissue. This was the first study reporting the presence of RHAMM in the conjunctiva. In addition, we demonstrated that sCD44 was present in normal human tears.
- CD44 and RHAMM were implicated in conjunctival inflammation. Higher levels of CD44 and even higher levels of RHAMM were present in patients with immune-atopic diseases such as vernal and atopic keratoconjuntivitis. In contrast, lower levels of RHAMM were detected in patients with immune non-atopic diseases such as rosacea or Sjögren's syndrome.
- Several proinflammatory and anti-inflammatory cytokines interact with goblet cells in different situations. It has been described that IFN-γ and TNF-α that are usually found in higher levels in dry eye patients with decreased goblet cell proliferation and secretion. In contrast, IL-4, IL-5, and IL-13 were increased in ocular allergy and induced goblet cell differentiation, proliferation, and increased secretion.
- IFN-γ affected rat and human cultured goblet cell proliferation and secretion. In addition, IFN-γ interacted with carbachol to modulate goblet cell function, each blocking the effect of the other.

Chapter 2: Acquiring Material Resources to Build a New Three-Dimensional Model of Human Conjunctiva

- We developed a protocol to expand epithelial cells from cadaveric conjunctival tissue. Standard culture medium supplemented with human serum provided the best proliferation rates. Cells expressed epithelial and goblet cell markers such as CK4, CK7, CK19, E-cadherin, and MUC5AC.

- Gellan gum/spermidin hydrogels and fibrin matrices were tested as scaffolds for human conjunctival cells. Gellan gum hydrogels were not compatible with the studied cells. In contrast, fibrin-based matrices made from fresh frozen plasma allowed conjunctival epithelial cell growth on the surface of the matrix and inside the matrix.

Chapter 3: Development of a Three-Dimensional Model of Human Conjunctiva

- We developed a three-dimensional conjunctival model with human primary cells derived from conjunctival tissues and fibrin matrices made from plasma and cryoprecipitates. Fibroblasts were grown inside the matrices and epithelial cells on the surface of them. Epithelial cells became stratified on the surface of the matrices, reaching 5-6 layers at 14 days, and they maintained the conjunctival epithelial cell phenotype. Cell proliferation rates varied depending on the specific matrix composition. Epithelial cells grew better in cryoprecipitate matrices, whereas fibroblasts showed higher proliferation rates inside plasma matrices. The presence of mucin-secreting cells was observed 3 days after establishing the cultures and were no longer present after the seventh day of culture.
- The three-dimensional model was exposed to different conditions to mimic inflammatory diseases, such as dry eye or allergic conjunctivitis. When the model was exposed to desiccation, RHAMM expression was decreased, similarly to what happened in patients with immune non-atopic disease. In contrast, when IL-13 was added, there was an increase in MUC5AC secretion. This is one of the main clinical findings in allergic diseases. Thus, this model reacts differentially to the analyzed stimuli, and the responses were in accordance to what occurs in the inflammation-related studies included in Chapter 1 of this Thesis.

CONCLUSIONS

In this Doctoral Thesis report the development of a new three-dimensional model of human conjunctiva is presented. Also, proof-of-concept of its use to study ocular surface inflammatory diseases is provided. The existing knowledge of the diseases, including the cells and molecules implicated in the pathophysiology, allowed us to focus the development of this model upon the most relevant findings.

Bearing in mind all of the results obtained in the different studies accomplished, we conclude the following:

1. We demonstrated that CD44 and RHAMM hyaluronan receptors are implicated in ocular surface inflammation. Expression of the hyaluronan receptors changed depending on the etiology of the disease. This should be taken into account when artificial tears or drugs containing HA are prescribed to patients. In addition, comparison of the results based on cell lines and on fresh samples obtained from patients highlighted once again the caution that researchers should exert when extrapolating results obtained from cell lines.

2. Cytokines that are altered in several ocular surface inflammatory diseases, such as dry eye and ocular allergy, had differential effects on goblet cells. We showed that IFN-γ interacts with cholinergic agonists to modulate goblet cell function in terms of proliferation and secretion, indicating that goblet cells have a protective role beyond mucin secretion.

3. Conjunctival tissue obtained from cadaveric donors, a resource that has been neglected for a long time, provided suitable conjunctival epithelial cells and fibroblasts to construct the conjunctival models. This source of human tissue can help to reduce the use of human cell lines, which can drift far from the originating healthy human tissues in terms of marker expression.

4. Fibrin-based matrices were a good biomaterial to develop a three-dimensional scaffold for human conjunctival cells. The properties of these matrices likely facilitated cell adhesion and allowed the proliferation of different cell types.

5. We established standard conditions with a specific culture medium to co-culture conjunctival fibroblasts, epithelial cells, and goblet cells. In these conditions, the performance of each cell type reached an equilibrium that allowed development of an appropriate three-dimensional model suitable to perform different laboratory studies up to two weeks.

6. We designed, constructed, and validated an *in vitro* three-dimensional conjunctival model with a structure similar to that of the native human conjunctival tissue. The model included a stratified and polarized epithelium containing goblet cells that secreted mucins in response to relevant stimuli, such as desiccation or cytokine exposure.

These conclusions are also included in the Epilogue of this Thesis report (page 325).

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Chapter 1

STUDY OF OCULAR SURFACE INFLAMMATION-RELATED RELEVANT MOLECULES

"A veces no es suficiente saber lo que significan las cosas,
a veces hay que saber lo que no significan"

"*Sometimes it's not enough to know what things mean,*
sometimes you have to know what things don't mean"

— Bob Dylan

Study 1

Hyaluronan receptors in the human ocular surface: a descriptive and comparative study of RHAMM and CD44 in tissues, cell lines and freshly collected samples

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Histochem Cell Biol (2012) 137:165–176
DOI 10.1007/s00418-011-0878-z

ORIGINAL PAPER

Hyaluronan receptors in the human ocular surface: a descriptive and comparative study of RHAMM and CD44 in tissues, cell lines and freshly collected samples

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Accepted: 18 October 2011 / Published online: 18 November 2011

Histochemistry and Cell Biology. 2012;137:165-176.

ESTUDIO 1

El ácido hialurónico fue descubierto por primera vez en el vítreo del ojo. Desde entonces, y debido a sus propiedades, su uso se ha ido extendiendo hasta estar presente en muchos de los productos que usamos a diario, desde la cosmética a los fármacos.

En el ojo, el ácido hialurónico se ha usado en lágrimas artificiales y también en el desarrollo de nuevas plataformas terapéuticas de liberación controlada. Sin embargo, sus receptores no han sido apenas estudiados en la superficie ocular. Y eso, a pesar de su demostrada importancia en otras patologías sistémicas como el cáncer.

En este estudio se analiza la expresión de los receptores de ácido hialurónico en tejidos de la superficie ocular, en líneas celulares de la córnea y de la conjuntiva, y también en muestras epiteliales de los mismos tejidos recién recogidas de pacientes, así como en lágrima.

STUDY 1

Hyaluronic acid or hyaluronan was first discovered in the vitreous of the eye. Since then, its use has been extended and now it is present in many of the products we use everyday, from cosmetics to drugs.

In the eye, hyaluronic acid is included in different artificial tear formulations. Also, it is an important component of drug delivery systems used to obtain a controlled release of different ocular drugs. Although its receptors are implicated in several systemic diseases, they have not been deeply studied in the ocular surface.

This study assesses the expression of hyaluronan receptors in corneal and conjunctival epitelial cell lines, in ocular surface tissues, as well as in freshly collected epitelial cells and tear samples from patients.

Hyaluronan Receptors in the Human Ocular Surface: a Descriptive and Comparative Study of RHAMM and CD44 in Tissues, Cell Lines and Freshly Collected Samples

ABSTRACT

The purpose of this study was to demonstrate the presence of the receptor for hyaluronan-mediated motility (RHAMM) in human conjunctival epithelium and in two widely used cell lines from human corneal (HCE) and conjunctival (IOBA-NHC) epithelia. We compared the distribution of RHAMM proteins and mRNAs in human ocular surface tissues (corneal, limbal and conjunctival), HCE and IOBA-NHC cell lines, and corneal and conjunctival epithelia primary samples from healthy donors with the previously identified hyaluronan receptor CD44. We also aimed to determine if soluble CD44 (sCD44) was present in human tears, as it could have a role in the interaction of the tear fluid with hyaluronan. Protein expression was evaluated by Western blots and immunofluorescence microscopy. mRNA expression was evaluated by RT-PCR and Q-PCR. sCD44 was analyzed by ELISA in culture supernatants and in human tears. We describe the expression of RHAMM in human healthy conjunctiva and in HCE and IOBA-NHC cells at both protein and mRNA levels, and the presence of sCD44 in human tears. Furthermore, we detected CD44 and sCD44 expression variations in *in vitro* inflammatory conditions. This study also focused on the necessary caution with which the conclusions extracted from cell lines should be made, and in the great value of using primary samples as often as possible.

Keywords: RHAMM; Hyaluronan receptors; CD44; Ocular surface; Conjunctiva ; Epithelial cells; Inflammation; Eye

INTRODUCTION

Interactions between epithelial cells and the extracellular matrix are essential for the maintenance of tissue architecture. An abundant component of the extracellular matrix is hyaluronan (HA), a glycosaminoglycan that is implicated in development, tissue remodeling, creation of cell-free spaces, and disease processes like inflammation and tumorigenesis (Sherman et al. 1994; Turley et al. 1991). For these reasons it has been deeply analyzed in many organs, including the eye. The way in which HA exerts its functions is by interacting with cell-surface receptor proteins. Although others exist, there are two main receptors, CD44 and the receptor for HA-mediated motility (RHAMM) that have been characterized and cloned (Entwistle et al. 1996).

CD44 is reported to be the main HA receptor (Aruffo et al. 1990). It is a transmembrane glycoprotein found in a wide variety of cell types, including leukocytes, fibroblasts, keratinocytes, and epithelial cells (Isacke and Yarwood 2002). It has a variety of functions such as cellular adhesion, HA degradation, lymphocyte activation, lymph node homing, myelopoiesis and lymphopoiesis, angiogenesis, and release of cytokines (Sneath and Mangham 1998). Through alternative splicing, the CD44 gene can give rise to different isoforms, all highly glycosylated (Naor et al. 1997). In the cornea, CD44 plays an important role in wound healing (Yu et al. 1998). A soluble form of CD44 (sCD44) has been detected in circulation and in other body fluids (Cichy and Puré 2003). In the eye, this form has been studied in the aqueous humor, and suggested as a potential protein marker for primary open-angle glaucoma (Budak et al. 2009). However, to our knowledge, it has not been studied in the tear film.

RHAMM (Hardwick et al. 1992; Turley 1982) is part of a multimeric cell surface-bound complex, termed the HA receptor complex (HARC), present in the cell surface or secreted into the media. The capability of HARC to bind HA, however, has been exclusively attributed to the RHAMM protein (Abetamann et al. 1996). The interaction between HA and RHAMM triggers a phosphorylation cascade implicated in cell signaling, cell migration, and proliferation, along with being involved in modulation of acute and chronic inflammation (Turley et al. 2002). RHAMM has neither a signal peptide nor a membrane domain, despite which it is present not only as an intracellular protein in the nucleus, but also in the cell membrane (Turley et al.

2002). It is thought to be linked to the plasma membrane by either a glycosylphosphatidylinositol anchor or by a so far unidentified linker protein (Assmann et al. 1999). As a result of alternative splicing and posttranscriptional modifications (mainly glycosylations), a remarkable heterogeneity of RHAMM proteins exists, with molecular masses ranging from 52 to 125 kDa. RHAMM forms are not highly expressed in most normal mammalian cell types, such as fibroblasts, endothelial cells, macrophages, immature thymocytes, or B-cell lineages, but are commonly overexpressed in many advanced cancers (Gust et al. 2009; Hamilton et al. 2007).

Due to the implications of these receptors in several diseases, they have been carefully studied in many tissues. However, this is not the case of the ocular surface, which comprises the cornea, the conjunctiva and the sclerocorneal limbus. Many severe problems in the ocular surface, mainly in the corneal and conjunctival epithelia, eventually cause blindness. It is important to know which molecules are involved in these diseases, to find good biomarkers or target proteins for new therapies. One of those could be the HA receptors, but they are not very well defined. In fact, there are no processes describing the expression and distribution of these receptors in the ocular surface. A great part of the studies are carried out in cell lines. CD44 is present in human ocular surface tissues, such as cornea (Zhu et al. 1997), limbus (Lerner et al. 1998), and conjunctiva (Hughes et al. 2006). It is also present in the human corneal epithelial (HCE) cell line (Hornof et al. 2008) and in the normal human conjunctival (IOBA-NHC) cell line (De la Fuente et al. 2008). Ahmad et al. (2008) reported the presence of RHAMM in corneal and limbal epithelial cells. As far as we know, there is no evidence of RHAMM presence in human conjunctiva or in the HCE and IOBA-NHC cell lines.

Therefore, this study was aimed, firstly, to determine whether or not epithelial cells from human conjunctiva express RHAMM. Secondly, by comparing the CD44 and RHAMM expressions in corneal and conjunctival tissues with that in HCE and IOBA-NHC cell lines, we could gain insight regarding the suitability of using cell lines as models for *in vitro* processes. We also aimed at determining whether sCD44 is present in tear fluid, and if it is detectable. Finally, we wanted to show some preliminary data indicative of the possible implication of these receptors in ocular surface inflammation.

MATERIALS AND METHODS

Materials

All materials used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated. DMEM/F12, fetal bovine serum (FBS), penicillin and streptomycin were from Invitrogen-GIBCO (Inchinnan, UK). Human epidermal growth factor (EGF) and bovine insulin were from Invitrogen (Eugene, OR, USA). Cholera toxin was purchased from Gentaur (Brussels, Belgium). Eight-well multichamber Permanox® slides were from Nunc (Roskilde, Denmark) and Optimal Cutting Temperature (OCT) compound was from Sakura (Tokyo, Japan). BCA assay was from Pierce (Rockford, IL, USA). HeLa cell extract and mouse brain extract came from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). All the cytokines used for the *in vitro* inflammation model were from PeproTech (London, UK). Rat anti-CD44 monoclonal and goat anti-RHAMM polyclonal antibodies (Abs) were from Calbiochem-Merck (Darmstad, Germany) and Santa Cruz Biotechnology Inc., respectively. The secondary Abs for Western blotting were purchased from Jackson Immuno-Research Laboratories, Inc. (West Grove, PA, USA), and those for immunofluorescence assays were from Invitrogen. Acrylamide and Tris buffered saline (TBS) used in Western blotting experiments were from Bio-Rad Laboratories (Hercules, CA, USA). Vectashield® mounting media was from Vector Laboratories (Burlingame, CA, USA). Human sCD44std Instant ELISA was from eBioscience (Vienna, Austria). The buffer used to recover tears was Assay Buffer (Millipore Corporation, Billerica, MA, USA). Kits for RNA isolation were from Qiagen (Valencia, CA, USA), and Quant-iT^{TM} RNA assay and SuperScript Vilo cDNA kit were from Invitrogen. All reagents for reverse transcription polymerase chain reaction (RT-PCR) were from Biotools B&M Labs S.A. (Madrid, Spain), except Blue JuiceTM Gel Loading Buffer 109 (Invitrogen). CD44 and RHAMM primers were purchased from SABioscience-Qiagen (Frederick, MD, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were from Sigma. SYBR Green PCR Master Mix was from Applied Biosystems (Carlsbad, CA, USA). The ChemiDoc® gel documentation system (Bio-Rad Laboratories) was used to visualize the acrylamide and agarose gels, and the Quantity One software was used to analyze the resulting images. A Leica DMI 6000B fluorescence microscope (Leica Microsystems, Wetzlar,

Germany) was used to visualize immunofluorescence preparations and LAS AF Lite software (Leica Microsystems) was used to analyze them.

Human tissues

Human corneal, limbal and conjunctival tissues were obtained from cadaveric donors and used as controls. Tonsil tissues from cadaveric donors were used as positive controls. Human corneal and conjunctival epithelial cells, and tear samples from healthy donors were collected and used as described below. This study followed the tenets of the Declaration of Helsinki and had the approval of the Institutional Review Board of the University of Valladolid. Informed consent was obtained from every donor.

Corneal epithelial cells were obtained from healthy donors (n = 8; mean age ± standard error of the mean: 34.6 ± 2.8 years) undergoing photorefractive keratectomy. From four of the donors, the corneal epithelial cells obtained after refractive surgery (CERS samples) were recovered in 1 ml of culture medium for immunofluorescence microscopy. These samples were washed with phosphate buffered saline (PBS) and incubated with 0.05%trypsin-ethylenediaminetetraacetic acid for 15 min to dissociate cells. For the other four donors, the CERS samples were recovered in 700 µl of lysis buffer for RNA extraction.

Conjunctival epithelial cells were obtained by brush cytology (BC) from healthy volunteers (n = 8; 25.4 ± 1.9 years). Donors had no ocular or systemic disease or topical treatment within the previous 6 months. BC was performed on the upper tarsal conjunctiva of one eye of each donor. Topical double anesthetic Colircusí (Alcon Cusí S.A., Barcelona, Spain) was applied. The conjunctiva was brushed twice in the same spot with the Cytobrush Plus GT (Medscand AB, Malmö, Sweden). After sampling, the epithelial cells were immediately detached from the brush by gentle rotation for 30 s in an Eppendorf tube containing 1.4 ml of culture medium (DMEM/F12 supplemented with 10% FBS) for immunofluorescence assays. For RNA extraction, the epithelial cells were detached into 700 µl of RLT lysis buffer (from RNA isolation Qiagen kit) plus 14.5 M b-mercaptoethanol.

Tear samples were obtained from healthy donors (n = 28, 20–50 years old, 11 males and 17 females) who were not under ocular or systemic medications. Tear collection was performed as previously described (Enriquezde-Salamanca et al. 2010). Briefly, 8 µl of tears were collected (4 µl from each eye) using glass capillary

micropipettes. Each sample was then diluted 1:2.5 in a sterile collection tube containing Assay Buffer.

Cell lines and culture conditions

HCE cells (Araki-Sasaki et al. 1995) from passages 42 to 52 were cultured in DMEM/F-12 supplemented with 15% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml EGF, 0.5%dimethylsulfoxide, 5 µg/ml insulin, and 0.1 l µg/ml cholera toxin.

IOBA-NHC conjunctival cells (Diebold et al. 2003) from passages 71 to 87 were grown in DMEM/F-12 supplemented with 10% FBS, 5,000 U/ml penicillin, 5 mg/ml streptomycin, 2.5 µg/ml fungizone, 2 ng/ml human EGF, 1 µg/ml bovine insulin, 0.1 µg/ml cholera toxin, and 0.5 µg/ml hydrocortisone.

Both cell lines were cultured at 37º C in a 5% CO_2–95% air atmosphere. The medium was changed every other day, and the cultures were observed daily by phase contrast microscopy.

***In vitro* inflammation model**

HCE and IOBA-NHC cells were seeded in 24-well plates and grown until confluence. Then, cells were maintained for 24 h in serum free medium, before treating them with TGFβ (10 ng/ml), TNFα (25 ng/ml) or IL13 (20 ng/ml) for 48 h. Controls were untreated cells. The cytokines and their concentrations were selected according to the *in vitro* inflammation model previously described (Enriquezde-Salamanca et al. 2008).

Electrophoresis and Western blot analysis

Cadaveric tissues from the cornea, conjunctiva, and limbus, and HCE and IOBA-NHC cells were separately homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer with a cocktail of proteases inhibitors (100 µl/ml phenylmethylsulfonyl fluoride, 6 µl/ml aprotinin, and 100 nM sodium orthovanadate). Cadaveric tonsil, included as control for CD44 and RHAMM, was treated similarly. Also, HeLa cell extract and mouse brain extract were used as controls for CD44 and RHAMM, respectively. The controls were selected as recommended by the manufacturer. After incubation on ice for 30 min, the samples were centrifuged at 14,000 rpm for 30 min at 4º C. To isolate proteins from the membrane fraction of

IOBA-NHC cells, an additional step was performed. After the centrifugation, the supernatant was collected and centrifuged at 35,000 rpm for 1 h at 4º C. Then, the pellet containing the membrane fraction was resuspended in 250 µl of RIPA buffer, mixed with sample buffer, boiled and stored until used. The protein concentration was measured by BCA assay.

Proteins in each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels according to the method of Laemmli (1970). Proteins were then transferred to nitrocellulose membranes, according to Towbin et al. (1979), and blocked for 1 h in TBS containing 0.05% Tween-20, 5% milk, and 4% FBS. Membranes were incubated with primary Abs (Table 1) for 2 h, then washed in TBS, and incubated with horseradish peroxidase-conjugated secondary Abs (Table 1) at room temperature (RT) for 1 h. Immunoreactive bands were visualized by a chemiluminescence method using the ChemiDoc® gel documentation system and images were analyzed with the Quantity One software.Equal loading was proved by GAPDH detection.

Immunofluorescence assays

Corneal, conjunctival, limbal, and tonsil tissues from cadaveric donors were rinsed in 30% sucrose in PBS, placed in 30% sucrose at 4º C overnight, embedded in OCT compound, and frozen. Cryostat sections (7 µm) were collected in poly-L-lysine-treated slides and kept at -80º C until used. HCE and IOBA-NHC cells were seeded onto eight-well multichamber Permanox™ slides and grown until confluence was reached. CERS cells (n = 4) and conjunctival epithelial cells obtained by BC (n = 4) were fixed on slides by cytocentrifugation with a Shandon Cytospin (Southern Products, Cheshire, UK) at 800 rpm for 5 min.

Tissue cryosections, HCE cells, IOBA-NHC cells, CERS samples, and BC samples were fixed in ice-cold methanol, washed in PBS, and blocked in PBS containing 4% goat or donkey serum, 0.3% Triton® X-100, and 1% bovine serum albumin, at RT for 1 h. The slides were then incubated with primary Abs (Table 1) at 4º C overnight. Alexa Fluor®-conjugated secondary Abs (Table 1) were used for 1 h at RT. Cell nuclei were counterstained with Hoechst dye. The preparations were coverslipped, mounted, and viewed under an epifluorescence microscope (Leica DMI 6000B). Negative controls included the omission of primary Abs. The specificity of anti-CD44 and anti-RHAMM was previously tested by blocking them with an

excess of HA. Image fluorescence was quantitatively evaluated by the mean grayscale value determined by the microscope's software. The background of the negative controls was also quantified, and this value was subtracted from that of the experimental samples. This allowed making a relative comparison between different images.

Table 1: Antibody (Ab) concentrations (µg/ml) used in Western blotting and in immunofluorescence assays

		Species	Western blotting	Immunofluorescence assays
CD44	1st Ab	Rat	1.25	10
	2nd Ab	Goat	0.15	6
RHAMM	1st Ab	Goat	0.4	4
	2nd Ab	Donkey	60	6

sCD44 instant enzyme-linked immunosorbent assay (ELISA)

An instant ELISA was developed to determine the levels of sCD44 in cell culture supernatants from HCE and IOBA-NHC cells exposed to proinflammatory cytokines (TGFβ, TNFα and IL13), and in tear samples from healthy donors (n = 28). The assay was performed according to the manufacturer's protocol. Culture medium and Assay Buffer used to collect the tear samples were used as negative controls.

Reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR (Q-PCR)

Total RNA from control tissues, HCE cells, IOBA-NHC cells, CERS samples (n = 4) and BC samples (n = 4), was isolated using the RNeasy Mini Kit. Contaminating DNA was eliminated by digestion with RNase-Free DNase Set.

Total RNA concentration was measured with the QuantiT TM RNA assay. The extracted RNA was reverse-transcribed to cDNA with the SuperScript Vilo cDNA kit following the manufacturer's protocol.

RT-PCR was conducted on aliquots of cDNA samples using the Mastercycler Personal Thermocycler (Eppendorf, Hamburg, Germany). Each reaction contained 1 µl of gene-specific primer pairs (Table 2), 50 ng of cDNA, 5 µl of PCR buffer 109, 1 µl dNTPs, and 1 µl Taq polymerase in a final volume of 50 µl.

Negative controls with omission of cDNA were included for all RT-PCR experiments. The level of GAPDH mRNA for each sample was used as an endogenous control. Thermocycling conditions were 95º C for 2 min, followed by 39 cycles of 95º C for 20 s, 60º C (primer-specific annealing temperature) for 30 s, 72º C for 40 s and a final cycle of 72º C for 10 min. RT-PCR products and the 100 base pair (bp) ladder markers were mixed with 2 µl of Blue JuiceTM Gel Loading Buffer 109 and were resolved on 2% agarose gels. Gel images were captured with the ChemiDoc® gel documentation system and analyzed with the Quantity One software.

Table 2: Primers for reverse transcription PCR

Primer	Primer sequence	Size product
GAPDH	Sense: 5'-GAACGTGAAGGTCGGAGTCAAC-3' Antisense: 5'-CGTGAAGATGGTGATGGGATTTC-3'	250 bp
CD44	Unknown: Ref. PPH00114A	106 bp
RHAMM	Unknown: Ref. PPH09857A	108 bp

bp, base pair

The Q-PCR reaction was performed with 10 ng cDNA, 1 µl primers and 10 µl SYBR Green PCR Master Mix in a final volume of 20 µl. The PCR reaction was run under the following conditions: denaturation at 95º C for 10 min, followed by 40 cycles of 95º C for 15 s and 60º C for 60 s, and a final cycle of 95º C for 90 s. All reactions were performed in duplicate. The levels of GAPDH for each sample were used as endogenous controls and a non-template control was included to detect DNA contamination. To assure the specificity of the PCR products, a melting curve analysis was performed. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis

Statistical analysis was done using Statistical Procedures for the Social Sciences software (SPSS 15.0, SPSS Inc, Chicago, IL, USA). Data were expressed as means ± standard error of the means. A Levene's test was performed to assure the equality of variance, and after that, a one-way ANOVA was done. Then, pairwise comparisons (Tukey test) were performed. When there was no equality of variance, a robust test was performed (Brown–Forsythe test). After that, pairwise comparisons

were done with Games–Howell test. Differences were considered to be significant when $p \leq 0.05$.

RESULTS

HA receptors in human ocular surface tissues

Different isoforms of CD44 and RHAMM were detected by Western blot analysis in protein extracts of the cadaveric corneal, conjunctival, and limbal tissues, as well as tonsil, the positive control (Fig. 1a, b). In all cases, major bands of 58 kDa for CD44 and of 57 kDa for RHAMM were identified. Volumetric analysis of the immunoreactive bands showed similar levels of CD44 expression in all ocular tissues (Fig. 1c; Table 3). RHAMM expression was higher in the conjunctival and limbal extracts compared to the corneal extracts ($p = 0.016$ and 0.017, respectively). For cornea, there was no difference in the expression of CD44 and RHAMM; however, for conjunctiva and limbus, the RHAMM levels were significantly higher than the CD44 levels ($p = 0.014$ and 0.007, respectively, Table 3).

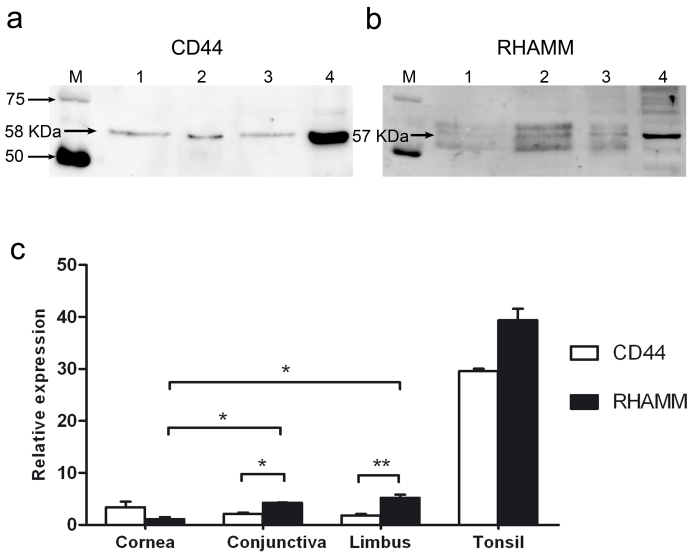

Fig. 1 Western blot analysis of CD44 (**a**) and RHAMM (**b**) expression in human tissues. M molecular weight marker, 1 Cornea, 2 Conjunctiva, 3 Limbus, 4 Tonsil. **c** Volumetric analysis of immunoreactive bands (n = 3). Higher levels of RHAMM than of CD44 were detected in human conjunctiva and limbus. *p B 0.05; **p B 0.01

Table 3: Summary of hyaluronan receptors expression levels in all samples studied

		Protein		mRNA	
		CD44	**RHAMM**	**CD44**	**RHAMM**
TISSUES	**Cornea**	+	+	+++	+
	Limbus	+	++	N/D	N/D
	Conjunctiva	+	++	+++	+
CELL LINES	**HCE**	++	+++	++	++
	IOBA-NHC	+	+++	++	++
PRIMARY SAMPLES[a]	**CERS samples**	++	+++	+++	+
	BC samples	+	++	+++	+

Protein levels were determined by Western blotting or immunofluorescence[a], and mRNA levels were determined by RT-PCR. CERS samples: corneal epithelial cells obtained after refractive surgery; BC samples: conjunctival epithelial cells obtained by brush cytology. Expression intensity was arbitrarily estimated as: (+) weak, if expression levels were in the first third of intensity range; (++) medium, if expression levels were in the second third; (+++) strong, if expression levels were in the upper third of intensity range. N/D: Not determined

We studied the distribution of both HA receptors in cadaveric corneal, limbal, and conjunctival tissues by immunofluorescence microscopy. No fluorescent signal was detected in negative controls, as expected (data not shown). Primary antibodies against CD44 (Fig. 2a–c) and RHAMM (Fig. 2d–f), detected by the immunofluorescent signal, were distributed mainly in the epithelium of all of the ocular surface tissues. In the corneal epithelium, the staining of CD44 was somewhat more pronounced in the deeper layers (Fig. 2a), whereas in the limbus, it had stronger intensity in the suprabasal layers compared to the basal ones (Fig. 2b). RHAMM was not expressed in either corneal or limbal stromas (Fig. 2d, e), but it was detected in conjunctival stroma (Fig. 2f).

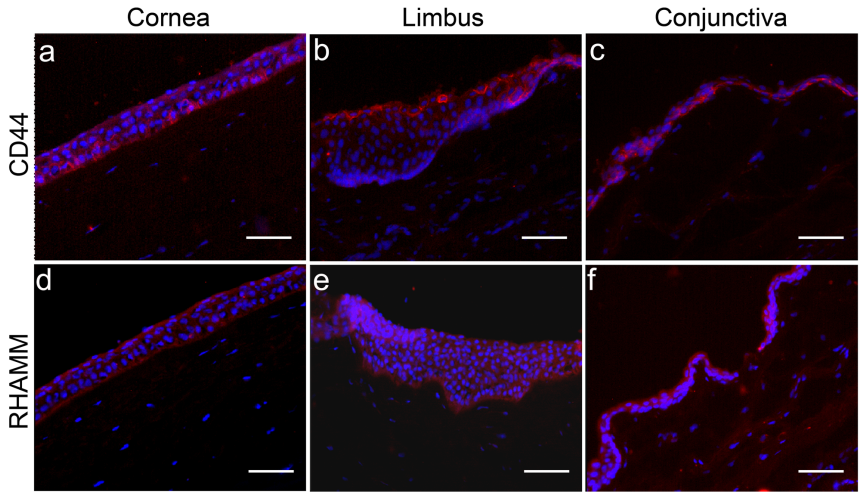

Fig. 2 Expression of HA receptors in cadaveric ocular surface tissues. CD44 (**a–c**) and RHAMM (**d–f**) expression (red) was evident in all tissues. Nuclei were counterstained with Hoechst dye (blue). Representative images of three independent experiments. Scale bars a–f: 50 lm

Both CD44 and RHAMM mRNAs were detected in corneal and conjunctival tissues by RT-PCR (Fig. 3a). For conjunctiva, the levels of CD44 mRNA, measured by Q-PCR, were significantly higher than that of RHAMM (p = 0.017, Fig. 3b; Table 3).

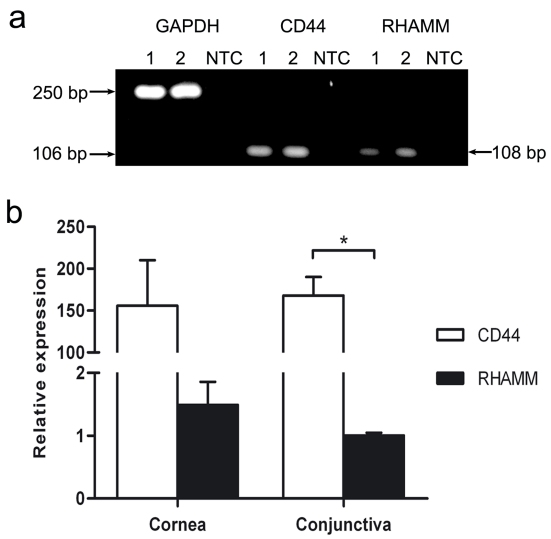

Fig. 3 RT-PCR analysis **a** of CD44 and RHAMM mRNA expression in human ocular surface tissues. 1 Cornea, 2 Conjunctiva, NTC Nontemplate control. **b** Analysis of Q-PCR (n = 3). Expression of the CD44 gene was higher than expression of the RHAMM gene in human conjunctiva. *p ≤ 0.05

HA receptors in corneal and conjunctival cell lines

Major CD44 (Fig. 4a) and RHAMM (Fig. 4b) bands were detected in Western blots of corneal and conjunctival cell lines. Isoforms of both receptors were also in the membrane fraction of the IOBA-NHC cells, where the immunoreactivity was the strongest in the 57 kDa form of CD44. Weaker immunoreactive bands were also detected for RHAMM at higher (100–200 kDa) molecular masses. RHAMM levels were higher than CD44 levels in IOBA-NHC cells ($p = 0.044$), as determined by the volumetric analysis (Fig. 4c; Table 3).

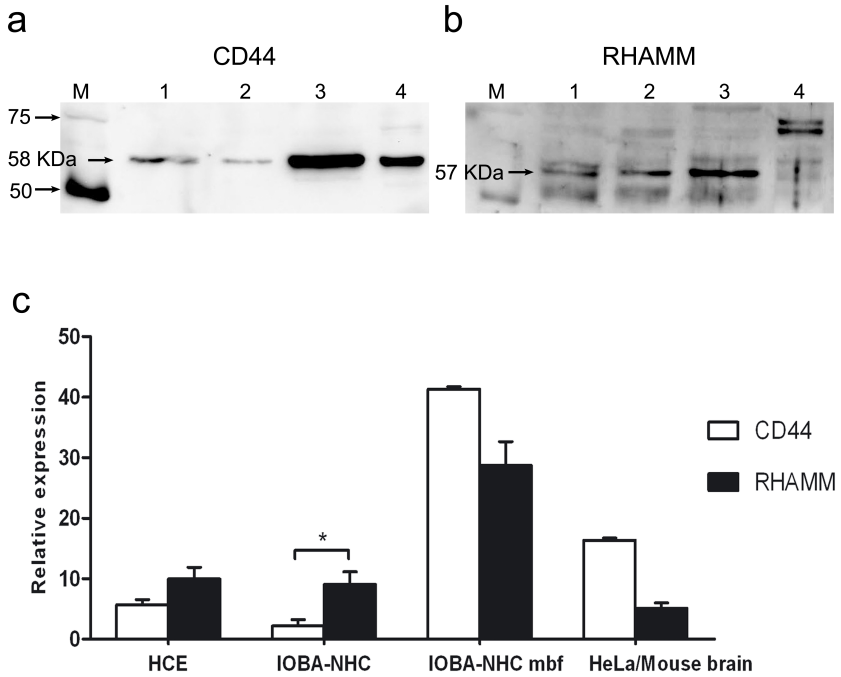

Fig. 4 Western blotting analysis of CD44 (**a**) and RHAMM (b) expression in human cell lines. M molecular weight markers, 1 HCE, 2 IOBA-NHC, 3 IOBA-NHC membrane fraction, 4 Positive controls: HeLa cell extract for CD44 and mouse brain extract for RHAMM. c Volumetric analysis of immunoreactive bands (n = 3). *p B 0.05

Negative controls for immunofluorescence assays did not show any signal (data not shown). Immunofluorescent signals for both CD44 and RHAMM were also detected in HCE and IOBA-NHC cells (Fig. 5). In both cell lines, CD44 was evenly distributed among the cells, and CD44 and RHAMM were localized to the plasma membrane, with less intensity also being present in the cytosol.

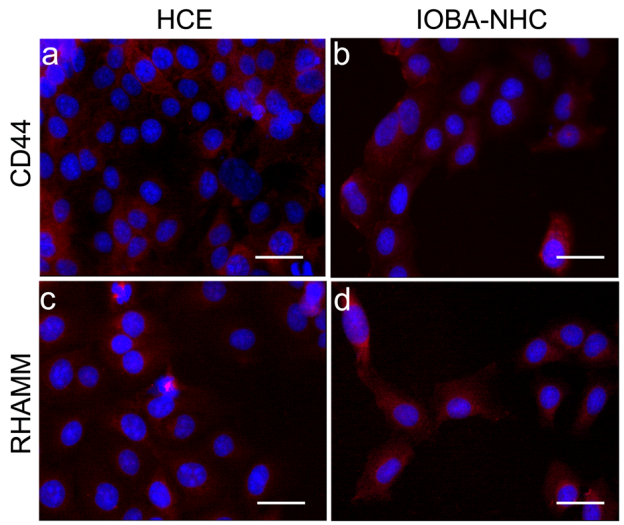

Fig. 5 Expression of HA receptors in ocular surface cell lines. CD44 (a-b) and RHAMM (c-d) were present (red) in HCE (a, c) and IOBA-NHC cell lines (b, d). Nuclei were counterstained with Hoechst dye (blue). Representative images of 3 independent experiments. (Magnification, x40)

By RT-PCR, CD44 and RHAMM mRNAs were detected in HCE and IOBA-NHC cells (Fig. 6a). According to Q-PCR results, both cell lines had higher levels of CD44 that those of RHAMM ($p = 0.015$ for HCE and $p = 0.007$ for IOBA-NHC). HCE cells had higher levels of RHAMM than IOBA-NHC ($p = 0.047$, Fig. 6b). Both cell lines had significantly higher mRNA levels of RHAMM than did any of the other tissues or primary samples taken by CERS or BC.

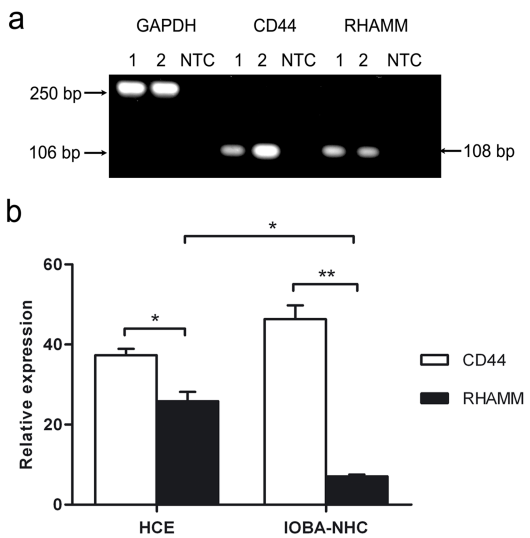

Fig. 6 RT-PCR analysis (a) of CD44 and RHAMM gene expression in human ocular surface cell lines. 1, HCE; 2, IOBA-NHC; NTC, non-template control. (b) Analysis of Q-PCR (n=3), showing higher levels of CD44 than of RHAMM in both cell lines, and higher RHAMM levels in HCE than in IOBA-NHC cells. *p≤0.05; **p≤0.01

HA receptors expression in CERS, BC and tears samples

CD44 and RHAMM were detected by immunofluorescence microscopy in the CERS samples and in the BC samples from healthy donors (Fig. 7). As expected, in negative controls no fluorescent signal was detected (data not shown). The CD44 signal was predominantly membrane-associated in both CERS and BC samples (Fig. 7a, b). RHAMM had a high intensity in the plasma membrane in CERS samples (Fig. 7c), whereas in BC samples it was more diffuse, with some expression in the plasma membrane, but also in cytosol and nucleus (Fig. 7d). Quantitative evaluation of the fluorescence in both CERS and BCsamples showed significantly higher intensity of RHAMM than CD44 ($p = 0.029$ and $p = 0.006$, respectively; Table 3).

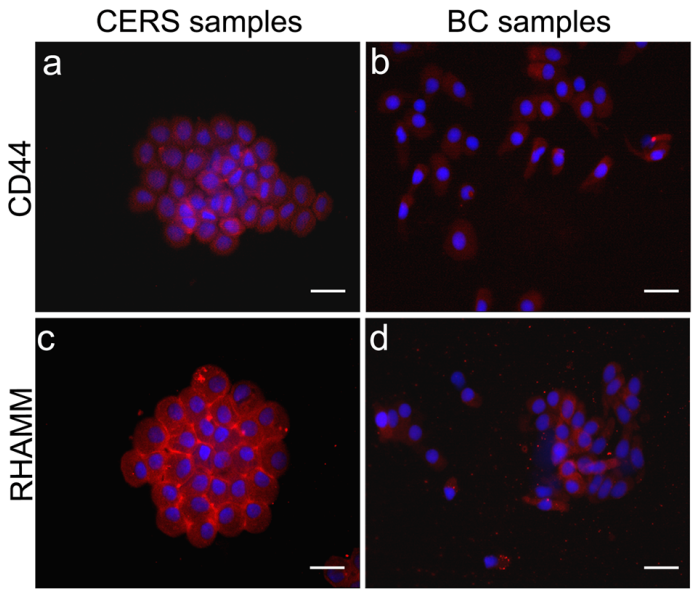

Fig. 7 CD44 (a-b) and RHAMM (c-d) immunofluorescence. In these representative images, both HA receptors (red) were expressed in corneal epithelial cells obtained after refractive surgery (CERS samples) and in the conjunctival epithelial cells obtained by brush cytology (BC samples). The fluorescence intensity for RHAMM receptors was higher than that for CD44 receptors. Representative images of 4 independent experiments (Magnification, x40)

Both CD44 and RHAMM mRNAs were detected in all of the CERS and BC samples (Fig. 8a). For each of the primary samples, the CD44 mRNA level was higher than that for RHAMM ($p = 0.010$ for CERS and $p < 0.001$ for BC). Both HCE and IOBA-NHC cells had higher levels of RHAMM than CERS and BC samples, respectively ($p = 0.030$ and $p = 0.010$, respectively).

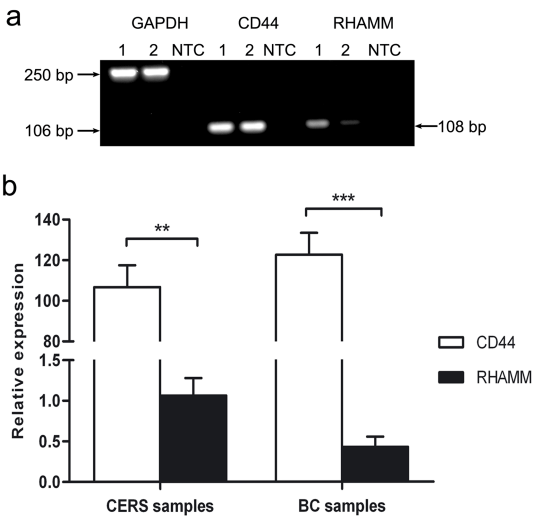

Fig. 8 RT-PCR analysis (a) of CD44 and RHAMM gene expression in corneal epithelial cells obtained after refractive surgery (CERS samples) and in conjunctival epithelial cells obtained by brush cytology (BC samples). 1, CERS samples; 2, BC samples; NTC, non-template control. (b) Analysis of Q-PCR (n=3). **p≤0.01;***p≤0.005

The sCD44 was detected in 32% of all the tear samples (29.4% in male samples and 36.36% in female samples) with a concentration of 1.72 ± 0.38 ng/ml, showing that this molecule is present in tears. No sCD44 was detected in the negative control.

HA receptors expression in inflammatory conditions

The expression of both CD44 (Fig. 9a) and RHAMM (Fig. 9b) was studied by Q-PCR in HCE and IOBA-NHC cells exposed to cytokines to simulate inflammatory conditions. We found significantly higher levels of CD44 mRNA in HCE cells exposed to TGFβ ($p < 0$ 0.001), TNFα ($p = 0.048$) or IL13 ($p = 0.047$).

We also studied the secretion of sCD44 by cytokine exposed cells (Fig. 9c) to culture medium, and found a significant reduction when IOBA-NHC cells were exposed to IL13 ($p < 0.001$), while no significant changes were observed after TFGβ or TNFα exposure. No sCD44 was detected in the negative control, as expected.

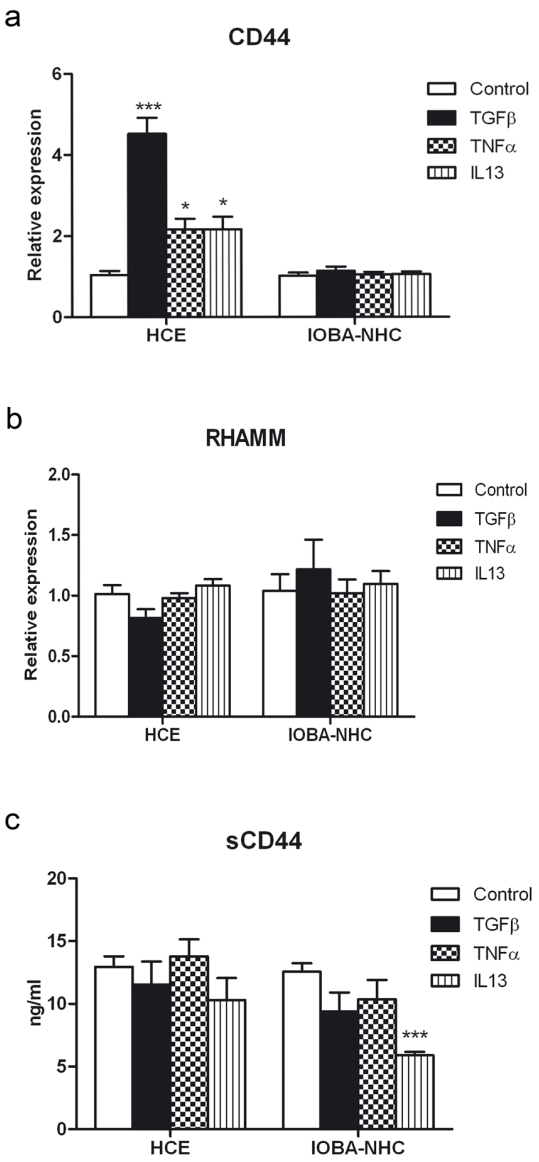

Fig. 9 Analysis of HA receptors in inflammatory conditions. Q-PCR analysis of CD44 (a) and RHAMM (b) gene expression in HCE and IOBA-NHC cells exposed to TGFβ, TNFα and IL13 (n=3). There were higher levels of CD44 in HCE cells exposed to the cytokines compared with the unexposed control. (c) sCD44 levels in cell culture supernatants of HCE and IOBA-NHC cells exposed to TGFβ, TNFα and IL13 (n=3). There were lower levels of sCD44 in IOBA-NHC cells exposed to IL13. *p≤0.05; **p≤0.01; ***p≤0.005

DISCUSSION

We report the presence and cellular distribution of RHAMM in conjunctival epithelial cells of healthy human donors, and the presence of sCD44 in normal human tears. We also compared the presence of RHAMM with another HA receptor, CD44, in freshly collected corneal and conjunctival cells as well as with HCE and IOBA-NHC cell lines.

Our previous work with nanoparticles made of HA and other biopolymers showed slightly higher internalization rates in HCE cells than in IOBA-NHC cells (Contreras-Ruiz et al. 2011). Moreover, when doing preliminary studies in the cell lines in our laboratory, we have noticed variations in the mRNA levels of these receptors, when those cell lines are stimulated with different cytokines. These data point out a possible relevance of these receptors in pathological conditions affecting the ocular surface.

For these reasons we are interested in a complete study and characterization of these important molecules in the normal and healthy ocular surface. To determine if these cell lines are a good *in vitro* model for this kind of assay, we characterized HA receptors in ocular surface epithelia and the only available soluble form in tears (sCD44). We wanted to know if the expression of these receptors in the cell lines was similar to that of human ocular surface tissues and freshly collected samples.

CD44 has been described in human corneal and conjunctival cells (Hughes et al. 2006; Lerner et al. 1998; Zhu et al. 1997). However, to our knowledge, a comparison of its expression levels between different tissues has not been reported. In this study we determined that CD44 expression is rather constant for the entire ocular surface epithelium, with no significant differences in either the protein or mRNA levels. Additionally, CD44 is present in the human corneal and conjunctival cell lines used in this work (de la Fuente et al. 2008; Hornof et al. 2008).

Interestingly, we have been able to detect sCD44 in 9 out of 28 tear samples (32% of all the tear samples). The reason why sCD44 was not detected in the remaining 68% of tear samples may be related with the detection limit of the kit used; however, it is the most sensitive commercially available kit to measure sCD44. Another limitation that may explain the observed differences is the small amount of tear sample that one can collect without stimulating reflex tear production (4 µl/eye).

Baseline sCD44 levels have been detected in several body fluids, such as serum, lymph or saliva, which may support our hypothesis of baseline levels also in human tears, where sCD44 could have a physiological role. Also, detected tear levels are similar to those measured in saliva (Franzmann et al. 2005). More experiments with a larger study group are warranted to extract stronger conclusions.

No reports describe the presence of RHAMM in conjunctiva and just one indicates its presence in corneal and limbal suprabasal cells but not basal cells (Ahmad et al. 2008). Our data showed that RHAMM is present throughout the conjunctival epithelium and confirm its presence in corneal and limbal tissues. However, we observed a different distribution pattern in the limbus from that reported by Ahmad et al. (2008). We found that RHAMM expression was present throughout the entire limbal epithelium, with no differences between basal and suprabasal layers. We also described RHAMM expression in both corneal and conjunctival cell lines studied.

Moreover, RHAMM, as well as CD44, was clearly detected in CERS and BC samples from healthy donors. BC is a minimally invasive technique that enabled us to obtain small amounts of human conjunctival cells, with a minimum of discomfort for the donor. Even with the small amount of tissue collected, it was enough to accomplish the study of HA receptor mRNAs. Our results in this respect contrast with those of Fujihara et al. (1997), who could not detect CD44 expression in their BC samples and concluded that CD44 was not expressed in normal human conjunctival epithelial cells. However, subsequent works from different groups confirmed its presence (De Saint et al. 2004; Hughes et al. 2006), as substantiated by our results.

Western blot assays showed different isoforms of CD44 and RHAMM in ocular surface tissues and cells. The results obtained in whole cell extracts and membrane fractions of IOBA-NHC cells indicated a relatively high expression of the non-membrane-associated RHAMM forms, in contrast to CD44. This is supported in the literature where intracellular RHAMM is described (Maxwell et al. 2008; Slevin et al. 2007).

Interestingly, we found differences among the cell lines, tissues, and BC samples. Both HCE and the IOBA-NHC cell lines had higher RHAMM mRNA levels than did the tissues or the primary samples. Moreover, the lines also had higher protein levels of RHAMM than did the corneal or conjunctival tissues. That

overexpression may be related to the fact that the cell lines are immortalized, and RHAMM is implicated in malignant transformation and proliferation (Maxwell et al. 2008; Pilarski et al. 1994; Sohr and Engeland 2008). Also, both cell lines had lower protein levels of CD44 compared to RHAMM. This can be explained by the concept of molecular redundancy, as suggested for these HA receptors (Nedvetzki et al. 2004). Accordingly, in the absence of CD44, RHAMM supports some of its functions, which could explain the reduced levels of CD44 when RHAMM is overexpressed.

Additionally, some of the differences found between protein and mRNA expression levels could be due to posttranslational modifications. CD44 and RHAMM are strongly glycosylated (Sherman et al. 1994; Underhill 1992), which can mask epitopes and consequently make protein immunodetection difficult. Post-translational modifications can also affect protein stability. Discrepancies in RHAMM expression levels could be also partially attributed to differences in cell cycle phase. Several studies confirm that RHAMM is cell cycle-regulated and that there can be discrepancies between RHAMM mRNA levels and protein levels (Buganim and Rotter 2008; Sohr and Engeland 2008).

RHAMM protein levels were higher in conjunctiva than in cornea. Although CD44 is postulated as the main HA receptor, our results suggest an unexpected role of RHAMM in conjunctiva because its levels are similar to or even higher than those of CD44. The actual physiological relevance of this HA receptor remains to be determined.

Our results in the *in vitro* inflammation model showed some variations in the expression of CD44 in cells stimulated with inflammatory cytokines compared with the unstimulated controls. That points at a possible implication of CD44 in ocular surface inflammation. In contrast, we did not see differences in the expression of RHAMM between different conditions. The fact that RHAMM is overexpressed in these cell lines could explain why its mRNA levels are not increased as CD44 levels are. Additionally, we detected some differences in the levels of sCD44 in the cell culture supernatants of IOBA-NHC cells exposed to IL13. Taking these data all together, it seems that there could be changes in the HA receptors in the ocular surface when an inflammatory process is underway.

It is important to know these receptors in normal and pathological conditions, because several topical formulations with HA are being used in ophthalmology. For this reason, our finding about the presence of sCD44 in human

tears is especially important, as it could be very relevant to the interaction with eye drops. In fact, artificial tears with HA are widely used in some inflammatory processes, like dry eye disease, and they could be exerting some unknown actions, since the interaction with HA receptors has not been studied.

CONCLUSIONS

In light of these results, we conclude that human ocular surface tissues express both CD44 and RHAMM receptors for HA. This is the first work that describes RHAMM presence in conjunctiva and confirms RHAMM expression in the cornea and limbus. We also show that sCD44 is present in human tears. Furthermore, this study reveals that HCE and IOBA-NHC cells express these receptors in a similar, but not identical, way to corneal and conjunctival tissues, respectively. This means that conclusions based on HA receptor assays in those cell lines need to be made with caution. Finally, we show that the expression of these receptors is somehow altered in inflammatory conditions, what could implicate that they could play a role in ocular surface inflammation.

ACKNOWLEDGMENTS

Authors thank Professor Margarita Calonge, M.D., Ph.D., for critical reading of the manuscript, Ester Carreño, M.D. for technical assistance to obtain tear samples, and Ignacio Amat, M.D. for his help in the immunofluorescence study of HA receptors. The authors also acknowledge the assistance of Britt Bromberg Ph. D. ELS of Xenofile Editing (http://www.xenofileediting.com) for assistance in the final editing and preparation of this manuscript.

Financial support was provided by FEDER-CICYT MAT 2010-20452-CO3-01, Spanish Ministry of Science and Education, Regional Grant VA056A08, Networking Research Center on Bioengineering, Biomaterial and Nanomedicine (CIBER-BBN), Ministry of Health, Spain, FPI-Ministry of Science and Innovation and FPU-Ministry of Education Scholarship Programs.

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Study 2

CD44 and RHAMM hyaluronan receptors in human ocular surface inflammation

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Graefes Arch Clin Exp Ophthalmol (2014) 252:1289–1295
DOI 10.1007/s00417-014-2686-3

INFLAMMATORY DISORDERS

CD44 and RHAMM hyaluronan receptors in human ocular surface inflammation

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Received: 14 June 2013 / Revised: 27 February 2014 / Accepted: 28 May 2014 / Published online: 12 June 2014

Graefe's Archive for Clinical and Experimental Ophthalmology. 2014;252:1289-1295.

ESTUDIO 2

En el estudio anterior se demostró la presencia de los receptores de ácido hialurónico en la superficie ocular. También se vieron variaciones en la expresión de uno de esos receptores (RHAMM) en la línea celular de conjuntiva tratada con distintas citoquinas para simular condiciones inflamatorias. Sin embargo, se observó también que las líneas celulares no expresaban estos receptores de la misma manera que las muestras recién recogidas de pacientes, por lo que esos datos deben ser corroborados.

Con esos antecedentes, se decidió llevar a cabo un estudio piloto en un grupo reducido de pacientes con distintas enfermedades inflamatorias con afectación conjuntival. De esta forma, en este estudio se pretende mostrar la implicación de estos receptores, especialmente RHAMM, en las patologías inflamatorias de la superficie ocular.

STUDY 2

The previous study established the presence of hyaluronan receptors in the ocular surface. Variations in the levels of RHAMM were found in the conjunctival cell line treated with inflammatory cytokines. However, changes in the expression of these receptors between cell lines and primary cells were also observed. For that reason, those data should be corroborated.

To address this issue, a pilot study with a reduced number of patients with inflammatory diseases was accomplished. Thus, the aim of this study was to show the involvement of hyaluronan receptors, especially RHAMM, in inflammatory diseases of the ocular surface.

CD44 and RHAMM Hyaluronan Receptors in Human Ocular Surface Inflammation

ABSTRACT

Background: CD44 and RHAMM hyaluronan (HA) receptors have been studied in several systemic diseases such as osteoarthritis and cancer. However, not too much is known about their role in ocular surface disorders. The purpose of this research was to determine if CD44 and RHAMM are implicated in human ocular surface inflammation.

Methods: Upper tarsal conjunctival epithelial samples from patients with active ocular surface inflammation (n = 17) and healthy controls (n = 14) were recovered by brush cytology. Patients were evaluated by an ophthalmologist and classified in different groups according to the etiology (immune atopic diseases or immune non-atopic diseases) and inflammation intensity (mild/moderate or severe). CD44, RHAMM, and p53 mRNAs were measured using Real-Time PCR.

Results: CD44, RHAMM, and p53 mRNAs were detected in all samples. In immune atopic diseases, higher levels of CD44 and RHAMM mRNAs were present, reaching a 300% increase for RHAMM in severe inflammation ($p < 0.001$). In contrast, in immune non-atopic diseases, the HA receptors were downregulated. CD44 tended to decrease up to 30% in severe patients ($p = 0.06$), and RHAMM decreased 40% in severe inflammation ($p = 0.021$).

Conclusions: RHAMM may be implicated in severe ocular surface inflammation affecting the upper tarsal conjunctiva.

Keywords: hyaluronan receptors, RHAMM, CD44, ocular surface, inflammation

INTRODUCTION

Ocular surface inflammation can be due to different factors such as infection, autoimmunity, and external injuries among others. In all of these pathological conditions, epithelial cells and the extracellular matrix (ECM) undergo changes in protein composition and levels. ECM proteins provide not only structural support to cells but also function as signalling molecules for inflammatory cells [1]. In fact, the ECM becomes altered in different pathologies affecting the eye [2, 3].

An abundant component of the ECM is hyaluronan (HA), a crucial glycosaminoglycan in many cellular processes, including tissue remodelling, tumorigenesis, inflammation, and immune regulation [4]. To trigger a response, HA must bind cells through cell-surface receptors. Two of the most studied HA receptors are CD44 and the receptor for HA-mediated motility (RHAMM) [5]. CD44 plays an important role in different events occurring during the inflammatory process, such as leukocyte recruitment, cell-matrix interactions, and HA degradation [6]. RHAMM has both intracellular functions such as control of cell cycle, and extracellular functions such as locomotion [7, 8]. The main RHAMM repressor is p53, a tumour suppressor protein implicated in apoptosis [9, 10].

The presence of CD44 has been described in cornea, limbus, and conjunctiva [11, 12], whereas little is known about RHAMM expression in the ocular surface [13]. Abnormal levels of both CD44 and RHAMM receptors have been described in some diseases, such as leukemia [14], osteoarthritis [15], and retinoblastoma [16], but little has been published regarding the ocular surface. Recently, we have reported the expression of RHAMM in human conjunctiva (cell lines of normal human conjunctival epithelium, fixed tissues, and brush cytology samples), as well as changes in both HA receptors in human ocular cell lines when exposed to several inflammatory cytokines [17]. To further determine a possible involvement of CD44 and RHAMM in inflammation, we analysed their mRNA expression in human ocular surface inflammatory diseases. The purpose of this study was to determine if gene expression for these receptors was altered in pathological inflammatory states.

METHODS

Subjects

This study followed the tenets of the Declaration of Helsinki and had the approval of the Ethics Committee of the University of Valladolid, Valladolid, Spain. Seventeen Caucasian patients and 14 Caucasian healthy control subjects were recruited. Patients were recruited at the Ocular Immunology Unit of the Institute for Applied Ophthalmobiology (IOBA, Valladolid, Spain). An informed consent was signed by each donor after the procedure was fully explained.

Inclusion criteria for the patients were 10-70 years of age, a well-established diagnosis of chronic ocular inflammatory disease, and a clear etiology. Exclusion criteria for both patients and healthy controls were pregnant or nursing women, use of contact lenses, any ocular disease in control subjects, any other ocular surface disease other than the one responsible for the current inflammatory state in patients, use of HA-based artificial tears, or any systemic disease such as leukemia and osteoarthritis known to alter levels of HA receptors. Additional exclusion criteria for healthy control subjects were systemic disease or medications of any kind, ocular topical medications of any kind (including any artificial tear use). Allergies, even mild, were excluded.

The clinical phenotype, i.e., specific diagnosis and degree of inflammation, of the recruited patients was assessed by an ophthalmologist who specialized in ocular surface diseases (author MC). Patients were classified in different groups according to the etiology and the inflammation intensity according to Efron Grading Scale [18].

Sample collection and processing

One brush cytology (BC) sample was taken from the upper tarsal conjunctiva of patients and healthy subjects for RNA extraction as previously reported by our group [19, 20]. In patients with both eyes affected, the most inflamed eye was the one chosen to perform the BC. Briefly, after topical application of the double anesthetic Colircusí® (Alcon Cusí S.A., Barcelona, Spain), the conjunctiva was scraped twice in the same spot with the Cytobrush Plus GT (Medscand AB, Malmö, Sweden). The cells were detached in an Eppendorf tube containing 700 µl

of RLT lysis buffer (Qiagen, Valencia, CA, USA) containing 14.5 M β-mercaptoethanol.

Real Time polymerase chain reaction (RT-PCR)

Messenger RNA (mRNA) from BC samples was isolated using the RNeasy Mini Kit (Qiagen), and contaminating DNA was eliminated by digestion with RNase-Free DNase Set (Qiagen). Total RNA concentration was measured with the Quant-iT RNA assay (Invitrogen, Inchinnan, UK). The extracted RNA was reverse-transcribed to cDNA with the SuperScript Vilo cDNA kit (Invitrogen) following the manufacturer's protocol.

The RT-PCR reaction was performed as previously described [17] using the following primers: CD44 (Ref. PPH00114A), RHAMM (Ref. PPH09857A), and p53 (Ref. PPH00213E), all from SABiosciences-Qiagen (Frederick, MD, USA), and glyceraldehyde phosphate dehydrogenase (GAPDH) from Sigma (St. Louis, MO, USA). All reactions were performed in duplicate. The levels of GAPDH for each sample were used as endogenous controls, and a non-template control was included to detect DNA contamination. To assure the specificity of the PCR products, a melting curve analysis was performed. Relative mRNA expression levels were determined using the $2^{-\Delta\Delta Ct}$ method [21].

Statistical analysis

Statistical analysis was done using the Statistical Package for the Social Sciences software (SPSS 15.0, SPSS Inc., Chicago, IL, USA). Data were calculated as means ± standard error of the mean (SEM). To compare two groups a Student's t-test was done. For more than two groups a one-way analysis of variance (ANOVA) was done after assuring equality of variance (Levene's test). After that, pairwise comparisons (Tukey test) were performed. In the absence of variance equality, a robust test was performed (Welch test), and the Games-Howell test was used for pairwise comparisons. Differences were considered to be significant when $p \leq 0.05$.

RESULTS

Clinical characteristics of patients and controls

Thirty-one subjects were recruited for this pilot study: 17 patients with inflammatory diseases affecting the ocular surface (41.5 ± 4.9 years; 8 males and 9

females), and 14 healthy volunteers (30.1 ± 3.0 years; 6 males and 8 females). There was no statistically significant difference in the age between the patients and healthy subjects. The individual characteristics of each patient are shown in **Table 1**.

Table 1. Ocular surface inflammation characteristics in enrolled patients

Case N°	Gender/Age	Eye	Etiology	Inflam grade	Elapsed time since disease onset	Other ocular diseases	Ocular topical treatment
1	F / 54	OS	Rosacea	1	8 years	-	Artificial tears
2	F / 23	OD	AKC	3	3 years	-	Artificial tears
3	F / 23	OS	AKC	2	3 months	Chemical burn in OD	Antiallergics
4	M / 11	OD	VKC	1	4 years	-	Antihistamines
5	M / 69	OS	DED 2nd to GVHD	2	3 years	YAG laser in OS (5 yr before); Cataract surgery (10 yr before); Ocular hypertension	Lubricants; prostaglandin; artificial tears
6	M / 52	OS	DED 2nd to GVHD	3	4 years	-	Artificial tears; glucocorticoid; autologous serum; antibiotics
7	F / 59	OS	AKC	2	6 months	-	Artificial tears
8	F / 47	OD	Rosacea	1	9 months	-	-
9	F / 59	OD	SS 2nd to SLE	1	7 years	Blepharitis	Lubricants; artificial tears
10	M / 12	OS	VKC	1	NDA	NDA	NDA
11	M / 67	OD	Rosacea	4	9 months	-	Artificial tears; β-adrenergic receptor blocker
12	M / 51	OD	AKC	4	1 year	Phlyctenulosis	- (no treatment)
13	M / 21	OS	VKC	3	15 months	-	Antiallergics
14	F / 39	OS	Stevens-Johnson syndrome	4	9 years	Amniotic membrane graft (3 times before)	Artificial tears; glucocorticoids; lubricants
15	M / 19	OS	VKC	2	11 years	-	-
16	F / 68	OS	Rosacea	2	2 years	Wagner's disease; Cataract surgery (7 yr before); Ocular hypertension (6 m before)	β-adrenergic receptor blocker; glucocorticoid
17	F / 31	OS	Rosacea	1	2 years	-	Artificial tears

OD, right eye; OS, left eye; AKC, atopic keratoconjunctivitis; VKC, vernal keratoconjunctivitis; DED, dry eye disease; GVHD, graft versus host disease; SS 2nd to SLE, Sjögren syndrome secondary to systemic lupus erythematosus; NDA, no data available.

By etiological group, 8 patients had immune atopic diseases and 9 had immune non-atopic diseases (**Table 2**).

Table 2. Pathological groups under study with individual diseases included in each one

Group (n; mean age)	Disease	n	Mean age
Immune atopic	AKC	4	39.0 ± 9.4
(8; 27.4 ± 6.3)	VKC	4	15.7 ± 2.5
	Rosacea	5	59.0 ± 4.6
Immune non-atopic	GVHD	2	60.5 ± 8.5
(9; 54.0 ± 4.4)	SJS	1	39
	SS	1	59

AKC: Atopic keratoconjunctivitis; VKC: Vernal keratoconjunctivitis; GVHD: Graft versus host disease; SJS: Stevens-Johnson's syndrome; SS: Sjögren's syndrome

CD44 and RHAMM gene expression

In the RT-PCR studies, mRNA for both receptors was detected in all samples. There were no significant differences in mean CD44 or RHAMM mRNA levels when all of the patients, regardless of disease etiology or intensity, were collectively compared to the healthy controls; however, the variability of expression was much higher in the patients. No relationship was found between age of healthy controls and expression levels of either CD44 or RHAMM mRNA. However, men (n = 6) expressed 20% less CD44 mRNA than women (n = 8) ($p = 0.019$, Student's t-test, data not shown).

When we studied the expression of CD44 and RHAMM mRNAs by etiology, there were no significant differences in CD44 gene expression between normal and inflamed eyes. However, RHAMM gene expression was significantly lower in eyes with immune non-atopic inflammatory disease than in healthy eyes ($p = 0.026$, Games-Howell test). There was also a tendency toward higher levels of RHAMM in immune atopic patients compared to healthy controls ($p = 0.067$).

To know if the expression of HA receptor mRNAs was influenced by the severity of the current inflammatory flare-up, patients with mild/moderate inflammation (degrees 1 and 2) and severe inflammation (degrees 3 and 4) within each etiology group (atopic diseases and immune-based diseases) were compared, as follows.

Immune atopic diseases

For immune atopic patients with moderate inflammation, there was no significant difference in the expression of CD44 mRNA compared to healthy control subjects (**Figure 1a, left**). However, for immune atopic patients with severe inflammation, the expression of CD44 mRNA was significantly higher ($p = 0.05$, Games-Howell test).

Bigger differences were found in the expression of RHAMM mRNA (**Figure 1a, right**). While the expression of RHAMM mRNA in patients with moderate inflammation was not significantly higher than in healthy controls, patients with severe inflammation had a 3-fold increase compared to the controls ($p < 0.001$, Tukey test). The levels in the severely affected patients were also significantly higher than in those with moderate inflammation ($p < 0.001$, Tukey test). In summary, an increase of HA receptor mRNAs was present in atopic diseases.

Immune non-atopic diseases

Immune non-atopic patients with severe inflammation had a tendency for lower levels of CD44 mRNA than healthy controls ($p = 0.06$, Tukey test, **Figure 1b, left**). For RHAMM, this decreased expression in severe patients was more pronounced (40%) and statistically significant ($p = 0.021$, Tukey test, **Figure 1b, right).** In summary, HA receptor mRNA levels were lower in patients with immune non-atopic diseases compared to healthy control subjects.

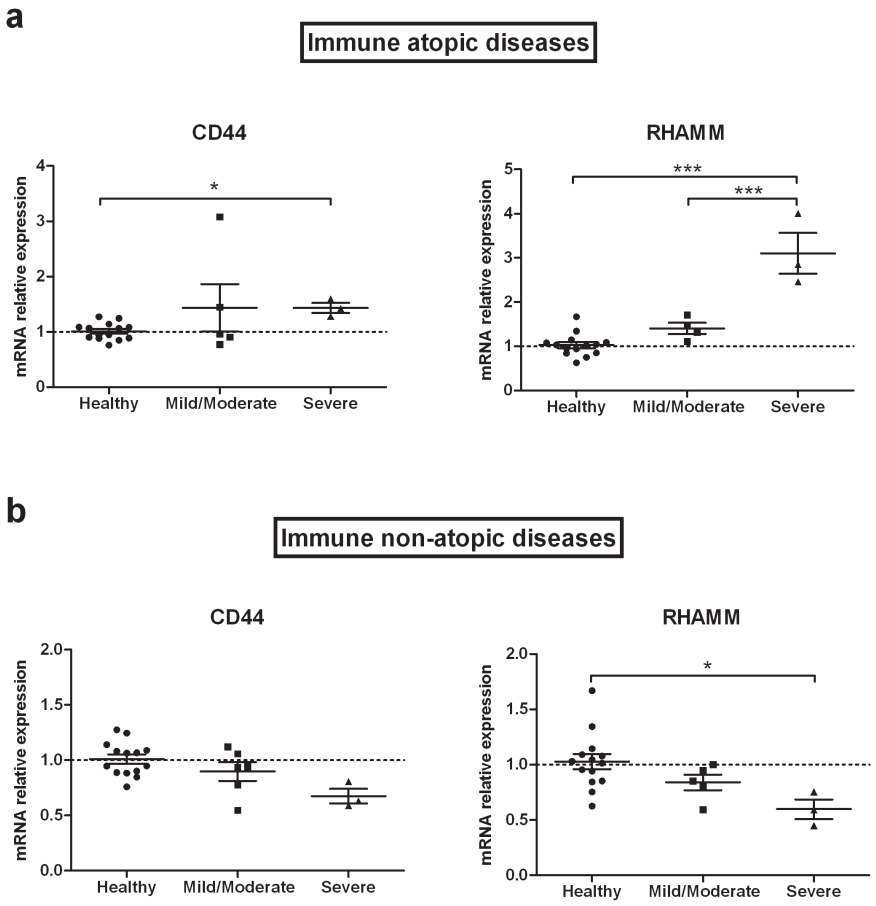

Figure 1. Inflammation intensity-associated hyaluronan receptor expression in immune atopic and immune non-atopic disease groups. **a)** CD44 and RHAMM were significantly overexpressed in atopic patients with severe inflammation. **b)** In immune non-atopic diseases, there was a tendency toward lower levels of CD44. RHAMM expression was significantly decreased in the severely affected group. * $p \leq 0.05$; *** $p \leq 0.005$

p53 gene expression

p53 mRNA levels were measured to determine if it was overexpressed and possibly acting as a repressor for RHAMM. p53 mRNA was detected in all samples. Patients with inflammation expressed lower levels of p53 than healthy controls ($p = 0.012$, Student's t test). In immune atopic patients there were no significant differences in p53 gene expression compared to healthy subjects (**Figure 2a**). However, patients with immune non-atopic diseases expressed lower levels of p53

than healthy controls (p = 0.005, Tukey test). The levels in mild/moderate immune-based inflammation were significantly lower than in healthy controls (p = 0.009, Tukey test, **Figure 2b**). In summary, inflamed eyes expressed lower p53 levels than healthy eyes, especially in immune non-atopic diseases.

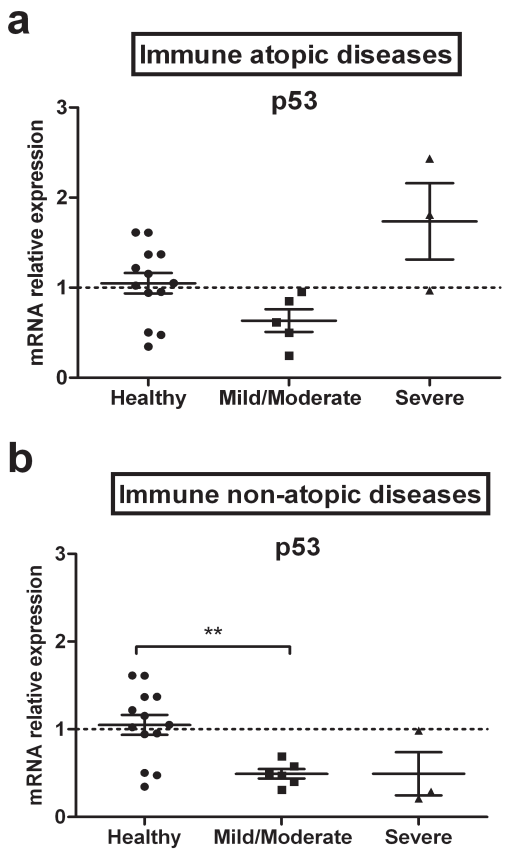

Figure 2. Inflammation intensity-associated p53 mRNA expression. **a)** There were no significant differences in the expression of p53 in either mild/moderate or severely affected patients with atopic diseases. **b)** p53 expression was significantly decreased in mild/moderate patients with immune non-atopic diseases. ** p ≤ 0.01

DISCUSSION

In the present study we showed that CD44 and RHAMM HA receptor mRNAs are expressed in human conjunctival cells harvested by BC from healthy subjects and from patients with ocular surface inflammation. While BC is a minimally invasive technique, patients with conjunctival inflammation may suffer some pain from it. For this reason, it is not ethically acceptable to perform several BCs on the same patient. Additionally, cells recovered from different BC samples may not be

the same because the most differentiated cells are found in the external layers. These will be recovered only with the first brush [19]. Due to this limitation, we were limited to performing only a single type of analysis, the RT-PCR for HA receptor gene expression. The amount of recovered cells was not enough to study protein expression by Western blotting, and it is difficult to quantify differences in expression by immunofluorescence microscopy. Therefore, we studied only mRNA expression, due to the great accuracy of the RT-PCR technique.

The expression levels of RHAMM receptor mRNA varied more among the different groups than did those of CD44. In general, RHAMM receptor mRNA expression levels were higher in immune atopic patients than in healthy control subjects or in those with immune non-atopic diseases. Further, the expression was higher in the more severe cases. This may implicate RHAMM in inflammatory conditions affecting the upper tarsal conjunctiva.

Our results showed that CD44 and RHAMM mRNA levels did not vary among different ages, but higher levels of CD44 were measured in healthy women than in men. This difference could be due to hormonal factors. Dave et al. [22] showed an increase in CD44 levels in rats with higher levels of progesterone receptor-A, which indicates a relationship between progesterone and CD44. Durst et al. [23] reported that estradiol increased CD44 expression in human cell lines. Both progesterone and estradiol levels are higher in females than in males, which supports our results.

The classification of patients according to their etiology revealed some striking differences in the expression of RHAMM mRNA *versus* healthy controls. For instance, there was a 200% increase in immune atopic patients and a 25% decrease in immune non-atopic diseases. This suggests an involvement of RHAMM in ocular surface inflammation.

However, the most relevant results are those obtained when we grouped our samples according to not only the etiology but also the intensity of the inflammation. Both severe groups showed significantly different levels of RHAMM when compared to healthy subjects. It is noteworthy that the variations were completely different depending on the etiology group, ranging from a 300% increase in severe atopic patients to a 40% decrease in immune-non atopic states.

We now propose a hypothetical molecular model to explain these results (**Figure 3**). Some inflammatory cytokines that are overexpressed in atopic

inflammation promote HA production in a variety of cell types [24]. Interestingly, we have reported a significant increase in CD44 mRNA expression in corneal epithelial cells exposed to some of these cytokines [17]. HA has a higher affinity for CD44 than for RHAMM [25]. For that reason one can assume that most of the HA present in the ECM is bound to CD44 under normal conditions. However, when there is an increase in HA level, it probably binds RHAMM as well. The HA/RHAMM complex triggers a signal transduction cascade that leads to increased RHAMM mRNA production and protein overexpression, which then moves to cell surface [26]. The result is higher levels of RHAMM that will bind the abnormally high levels of HA. However, the high levels of HA/RHAMM complex may also increase CD44 levels [27]. Then, CD44 will preferentially bind HA, inducing phagocytosis to eliminate HA and intracellular signals to stop ECM overproduction [28]. Inflammation due to this signalling cascade would end when both receptors reached equilibrium.

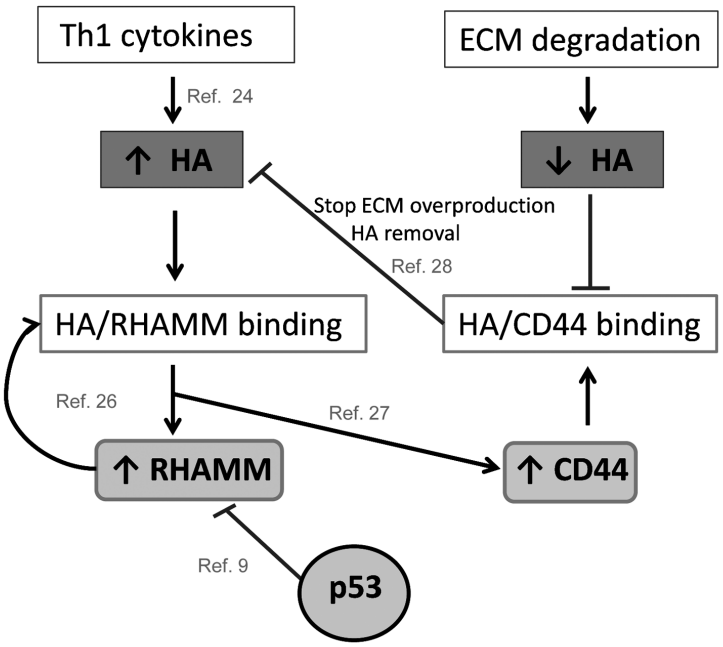

Figure 3. Hypothetical model. Proposed mechanism of RHAMM upregulation in immune atopic inflammation and downregulation in immune non-atopic inflammation.

In contrast to patients with atopic inflammatory disease, we found a decrease of CD44 and RHAMM mRNA levels in immune non-atopic disease

patients with severe inflammation. In several diseases included in this group, e.g., rosacea and Sjögren syndrome, ECM degradation and remodelling has been reported as a cause or as a consequence of the pathology [29, 30]. A degraded ECM could mean lower HA levels than in normal conditions, and this in turn could lead to a downregulation of HA receptors (**Figure 3**).

The main RHAMM repressor is p53 [9]. This protein controls cell cycle, and it is implicated in apoptosis. The reported higher levels of apoptosis in several ocular surface diseases [20] could be due to higher levels of p53. This correlates with the lower RHAMM mRNA levels, which could be due to p53-mediated downregulation (**Figure 3**). Our proposed explanation of HA receptor regulation in ocular inflammatory diseases deserves further study.

Currently, artificial tears containing HA are being used in patients with ocular surface inflammation, particularly in patients with moderate-to-severe dry eye disease. However, the interaction of the instilled HA with the HA receptors present in the ocular surface are not known. Our findings show that there is a need for determining if these compounds are beneficial, harmful, or neither.

In summary, by sampling the upper tarsal conjunctiva, we have shown that RHAMM mRNA expression levels vary with inflammatory conditions affecting the ocular surface. These variations are dependent on the etiology and severity of the inflammation, which suggests that the underlying mechanisms implicated in each group are completely different. We have detected the highest variations in RHAMM mRNA when the inflammation was severe, but similar changes, although lower in intensity, were present in mild/moderate cases. This study provides an overview of HA receptor expression in different inflammatory diseases affecting the human ocular surface and suggests that RHAMM may be implicated in upper tarsal conjunctiva severe inflammation. Since there was no information about the expression of RHAMM in any ocular surface disease, we considered necessary to first accomplish a pilot study like this one. The main limitation of this research is the small sample size. However, even with a low number of patients, several statistically significant differences have been found. Now, we consider that our results should stimulate and guide further research. The potential usefulness of RHAMM as a biomarker of and/or therapeutic target for ocular surface inflammation is worth exploring.

ACKNOWLEDGMENTS

This study was partially presented at 2012 ARVO Annual Meeting (Fort Lauderdale, FL, USA).

Authors thank Dr. José M. Herreras, MD, PhD for helping in patient recruitment, Sonia Villarón, MD, Darío Victoria, MD and Beatriz Ramirez, MD for technical assistance in obtaining brush cytology samples. We also thank Itziar Fernández (IOBA-University of Valladolid) for statistical advice. The authors also acknowledge the assistance of Britt Bromberg, PhD, ELS, of Xenofile Editing (http://www.xenofileediting.com) for the final editing and preparation of this manuscript.

This work was supported by FEDER-CICYT Grant MAT-2010-20452-C03-01 and FPI Scholarship program (Ministry of Science and Innovation, Spain) and Regional JCyL Grant VA132A11-2.

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Study 3

Conjunctival goblet cell function: effect of contact lens wear and cytokines

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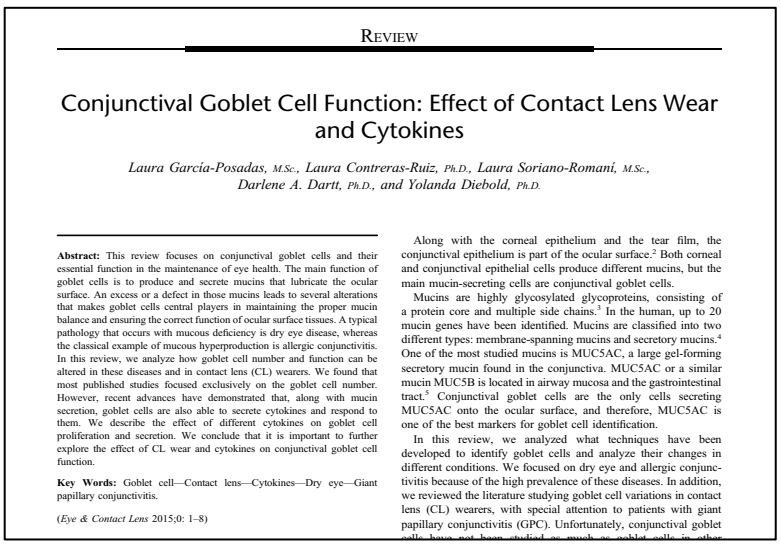

REVIEW

Conjunctival Goblet Cell Function: Effect of Contact Lens Wear and Cytokines

Laura García-Posadas, M.Sc., Laura Contreras-Ruiz, Ph.D., Laura Soriano-Romaní, M.Sc., Darlene A. Dartt, Ph.D., and Yolanda Diebold, Ph.D.

Abstract: This review focuses on conjunctival goblet cells and their essential function in the maintenance of eye health. The main function of goblet cells is to produce and secrete mucins that lubricate the ocular surface. An excess or a defect in those mucins leads to several alterations that makes goblet cells central players in maintaining the proper mucin balance and ensuring the correct function of ocular surface tissues. A typical pathology that occurs with mucous deficiency is dry eye disease, whereas the classical example of mucous hyperproduction is allergic conjunctivitis. In this review, we analyze how goblet cell number and function can be altered in these diseases and in contact lens (CL) wearers. We found that most published studies focused exclusively on the goblet cell number. However, recent advances have demonstrated that, along with mucin secretion, goblet cells are also able to secrete cytokines and respond to them. We describe the effect of different cytokines on goblet cell proliferation and secretion. We conclude that it is important to further explore the effect of CL wear and cytokines on conjunctival goblet cell function.

Key Words: Goblet cell—Contact lens—Cytokines—Dry eye—Giant papillary conjunctivitis.

(*Eye & Contact Lens* 2015;0: 1–8)

Along with the corneal epithelium and the tear film, the conjunctival epithelium is part of the ocular surface.[2] Both corneal and conjunctival epithelial cells produce different mucins, but the main mucin-secreting cells are conjunctival goblet cells.

Mucins are highly glycosylated glycoproteins, consisting of a protein core and multiple side chains.[3] In the human, up to 20 mucin genes have been identified. Mucins are classified into two different types: membrane-spanning mucins and secretory mucins.[4] One of the most studied mucins is MUC5AC, a large gel-forming secretory mucin found in the conjunctiva. MUC5AC or a similar mucin MUC5B is located in airway mucosa and the gastrointestinal tract.[5] Conjunctival goblet cells are the only cells secreting MUC5AC onto the ocular surface, and therefore, MUC5AC is one of the best markers for goblet cell identification.

In this review, we analyzed what techniques have been developed to identify goblet cells and analyze their changes in different conditions. We focused on dry eye and allergic conjunctivitis because of the high prevalence of these diseases. In addition, we reviewed the literature studying goblet cell variations in contact lens (CL) wearers, with special attention to patients with giant papillary conjunctivitis (GPC). Unfortunately, conjunctival goblet

Eye & Contact Lens. 2015 May 19. [Epub ahead of print]

ESTUDIO 3

Las células caliciformes conjuntivales son esenciales para mantener una superficie ocular sana. Se sabe que el número de células caliciformes se encuentra aumentado o disminuido en algunas enfermedades, lo que implica cambios en la secreción de mucinas que afectan significativamente a la salud de la superficie ocular. Sin embargo, no hay muchos estudios que vayan más allá del cambio en las densidades de células caliciformes y que analicen en profundidad su interacción con estímulos inflamatorios.

En este estudio se lleva a cabo una revisión bibliográfica de los últimos 30 años sobre el efecto que tienen sobre las células caliciformes el uso de lentes de contacto, así como la presencia de distintas citoquinas.

STUDY 3

Conjunctival goblet cells are essential to maintain a healthy ocular surface. Goblet cell numbers are increased or decreased in several pathologies, what leads to changes in mucin secretion that alter ocular surface health. However, most studies are just focused on the number of goblet cells, but they do not analyze in detail their interaction with inflammatory stimuli.

In this study we have reviewed published literature in the last 30 years to write a revision about the effects of contact lens wear and inflammatory cytokines on conjunctival goblet cell function.

Conjunctival Goblet Cell Function: Effect of Contact Lens Wear and Cytokines

ABSTRACT

This review focuses on conjunctival goblet cells and their essential function in the maintenance of eye health. The main function of goblet cells is to produce and secrete mucins that lubricate the ocular surface. An excess or a defect in those mucins leads to several alterations that makes goblet cells central players in maintaining the proper mucin balance and ensuring the correct function of ocular surface tissues. A typical pathology that occurs with mucous deficiency is dry eye disease, whereas the classical example of mucous hyperproduction is allergic conjunctivitis. In this review we analyze how goblet cell number and function can be altered in these diseases and in contact lens wearers. We found that most published studies focused exclusively on goblet cell number. However, recent advances have demonstrated that, along with mucin secretion, goblet cells are also able to secrete cytokines and respond to them. We describe the effect of different cytokines on goblet cell proliferation and secretion. We conclude that it is important to further explore the effect of contact lens wear and cytokines on conjunctival goblet cell function.

Keywords

goblet cell; contact lens; cytokines; dry eye; giant papillary conjunctivitis

INTRODUCTION

Goblet cells are highly specialized epithelial cells, present in mucosal tissues along the body. The main function of these cells is to produce and secrete mucins, which hydrate and lubricate mucosal surfaces.[1] Under non-pathologic conditions in the eye, goblet cells are confined to the conjunctival epithelium.

Along with the corneal epithelium and the tear film, the conjunctival epithelium is part of the ocular surface.[2] Both corneal and conjunctival epithelial cells produce different mucins, but the main mucin-secreting cells are conjunctival goblet cells.

Mucins are highly glycosylated glycoproteins, consisting on a protein core and multiple side chains.[3] In the human, up to 20 mucin genes have been identified. Mucins are classified into two different types: membrane-spanning mucins and secretory mucins.[4] One of the most studied mucins is MUC5AC, a large gel-forming secretory mucin found in conjunctiva. MUC5AC or a similar mucin MUC5B are located in airway mucosa and the gastrointestinal tract.[5] Conjunctival goblet cells are the only cells secreting MUC5AC onto the ocular surface, and for that reason, MUC5AC is one of the best markers for goblet cell identification.

In this review we analyzed what techniques have been developed to identify goblet cells and analyze their changes in different conditions. We focused on dry eye and allergic conjunctivitis due to the high prevalence of these diseases. In addition, we reviewed literature studying goblet cell variations in contact lens wearers, with special attention to patients suffering giant papillary conjunctivitis (GPC). Unfortunately, conjunctival goblet cells have not been studied as much as goblet cells in other mucosal tissues. For that reason, we summarized the effect that several cytokines have on either conjunctival or nonconjunctival goblet cells. Cytokines are immunomodulatory agents that are altered in immune diseases. Altered levels of these molecules in ocular diseases and in contact lens wearers have been broadly studied. For that reason, we aimed at drawing a global picture of the main effects exerted by cytokines in goblet cells. Bearing all this in mind, and knowing which cytokines are altered in which conditions, we hope this review will guide further research.

IDENTIFICATION OF GOBLET CELLS

Because of the impact of goblet cell alteration in ocular surface diseases, several techniques to identify these cells have been developed. Goblet cells are usually identified in biopsies or cytologies (mainly, conjunctival impression cytology[6]) using a classical chemical staining that interacts with mucopolysaccharides, namely periodic acid-Schiff (PAS) staining (Figure 1a). With this method, filled goblet cells are identified, but since the staining is based in the reaction with their mucous content, it is not possible to detect those cells that have already released their products.

Goblet cells release their products in an apocrine manner. When they receive the appropriate stimuli, they secrete all the contents of their secretory granules at once. As a consequence, those cells that have secreted their mucins are completely empty and, remain invisible to the PAS staining. The same problem remains if MUC5AC or lectins such as UEA-1 or HPA are used to identify goblet cells. In 1997, Krenzer and Freddo described that cytokeratin (CK) 7 stained the cell body of goblet cells,[7] allowing the identification of all goblet cells, regardless whether they had already secreted their contents or not. Thus, it is possible to identify the total number of goblet cells, filled and empty, using co-immunolocalization with CK7 and HPA or MUC5AC[8] (Figure 1b).

Another technique to identify goblet cells is *in vivo* laser scanning confocal microscopy.[9] This technique allows evaluation of tissue structure by *in vivo* identification of goblet cells[10] (Figure 1c). The main advantage compared to conjunctival impression cytology, is that confocal microscopy is not invasive at all, whereas cytology is a minimally invasive method. Furthermore, cytologies or biopsies need histological processing that can introduce artifacts, what can invalidate the study or diagnosis; whereas *in vivo* confocal microscopy does not.

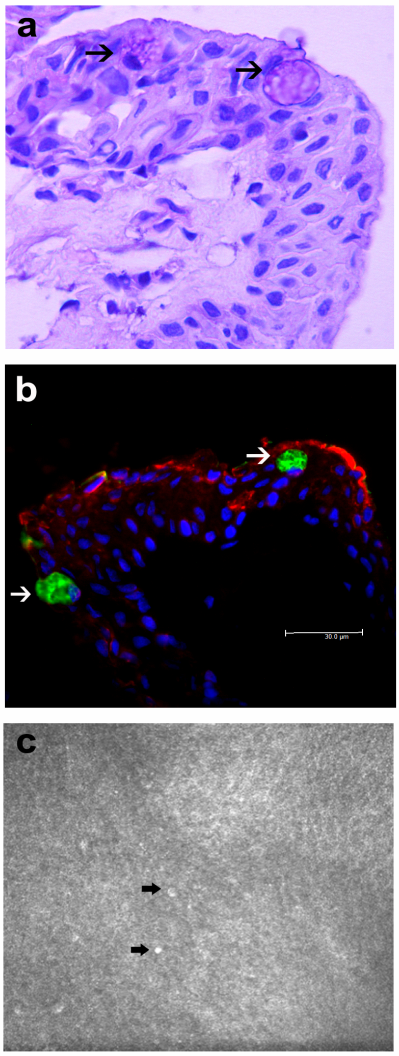

Figure 1. Goblet cell identification by several techniques. (a) PAS staining in a human conjunctiva. (b) Goblet cells identified by cytokeratin 7 (red) immunostaining and HPA lectin binding (green). (c) *In vivo* laser scanning confocal microscopy image showing possible goblet cells, identified by its rounded shape and brightness (black arrows).

IN VITRO MODELS TO STUDY CONJUNCTIVAL GOBLET CELL FUNCTION

A generally accepted theory some decades ago was that the only function of goblet cells was to secrete mucins and that those mucins acted only in lubricating the ocular surface. Recent studies have demonstrated that goblet cells have more functions, and that they also produce more substances apart from mucins. Cytokine secretion has been described and an immunomodulatory function of conjunctival goblet cells has been identified based on their ability to modulate dendritic cell phenotype.[11] Moreover, it has recently been proven that intestinal goblet cells play a role in presenting food antigens to dendritic cells[12] which opens a new field of

potential treatments against inflammatory bowel disease or celiac disease. Thus, we now know that goblet cells are much more than just mucin-secretory cells and that their mucins have more important functions than simply lubrication.

In fact, the ocular surface depends in part on the levels of mucins present in the tear film to keep its integrity, and in turn, this surface depends largely on goblet cell number and their rate of production and secretion of mucins. These mucins protect the ocular surface against desiccation, but also against pathogen access. Thus, conjunctival goblet cells are one of the first lines of defense of the ocular surface and the entire eye.[13]

Recent discoveries of unexpected functions of goblet cells makes it imperative to study further the physiology of these cells in both health and disease. However, goblet cells are slow-cycling cells, so it is difficult to culture and expand them *in vitro*. For that reason, few *in vitro* studies were performed until 10 years ago. In 2001, Shatos et al. published a method to culture primary goblet cells from rat conjunctiva.[14] Later, in 2003, this technique was developed for human cells.[15] Since then, the number of reported *in vitro* studies using goblet cell cultures has experienced a significant increase.

The lack of human tissue sometimes makes it difficult to advance the study of goblet cell pathophysiology. However, parallel studies using both rat and human cultured goblet cells have demonstrated that rat cells are a good model for the human ones.[16,17] The main difference between rat and human goblet cells is distribution within the conjunctiva. Human goblet cells usually occur as single cells, mainly in the external layers of the epithelium, whereas rat cells are often associated in clusters. Regarding signaling pathways and celular functions, results in the species are similar.

ROLE OF GOBLET CELLS IN OCULAR SURFACE DISEASE

As previously mentioned, goblet cells are altered in several diseases (Figure 2). While their specific role in pathologies affecting the gastrointestinal tract or the airway mucosa has been widely studied, the study of their function in ocular surface diseases is at an earlier stage.

Ocular surface diseases are often associated with inflammation.[18,19] Several cell types are involved in the inflammatory reaction. An important cellular participant

is the T helper (Th) cell. Depending on the pattern of signals these cells receive, different types of Th cells develop, the most studied being Th1, Th2, Th17, and regulatory cells (Treg).[20,21] Each Th cell subtype produces a specific profile of molecules that modulate the immune response. Cytokines are one of these immunomodulatory molecules, and are also classified as Th1, Th2, or Th17 cytokines, among others. The predominant Th response pattern varies between diseases.

From the wide range of pathologies affecting the ocular surface, the role of goblet cells has been most extensively studied in two main conditions dry eye and ocular allergy. These two diseases have been classically associated with different Th responses. Dry eye is a predominantly Th1-mediated disease[22] and allergic diseases are typically Th2 inflammatory responses.[23]

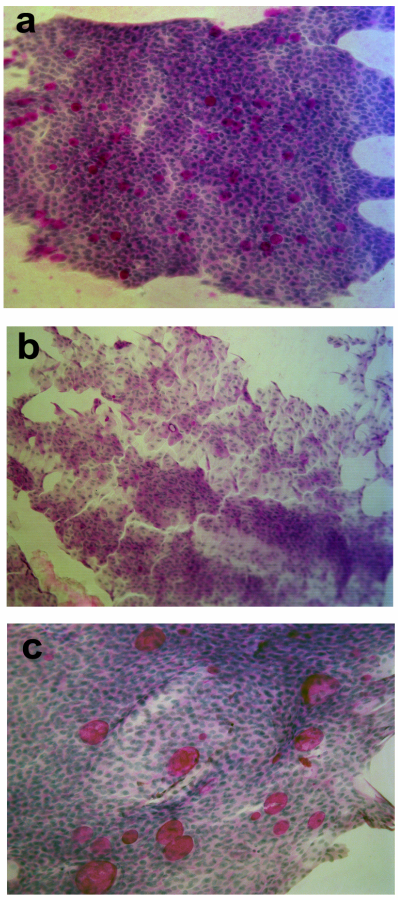

Figure 2. Conjunctival impression cytologies (CIC) obtained from different patients and stained with PAS. (a) CIC from a patient with no alterations in the conjunctiva. Goblet cells can be identified by the PAS staining and are distributed along all the cytology. (b) CIC from a patient with dry eye disease. No goblet cells were found in the CIC. (c) CIC from a patient with allergic conjunctivitis showing goblet cell hyperplasia.

Dry Eye Disease

Dry eye affects millions of people worldwide.[24,25] In this inflammatory disease the lacrimal functional unit is altered.[26] Increased levels of several cytokines, such as IFN-γ, TNF-α, or IL-6, along with goblet cell loss have been reported in this disease (Figure 2b). In fact, the lack of goblet cells has been the topic of numerous studies on dry eye.[27–31]

Ocular Allergic Diseases

Allergic diseases of the cornea and conjunctiva are typically associated with mucus hypersecretion. Higher goblet cell densities and/or hyperplasia are usually found in this type of disease (Figure 2c). Allergy has been classically associated with the presence of Th2 cytokines. They are not the only type of cytokines involved in these diseases, but Th2 cytokines, such as IL-4 or IL-13, are especially important in allergy. There are four types of allergic diseases affecting the eye, namely allergic conjunctivitis (seasonal or perennial), vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), and giant papillary conjunctivitis (GPC).[32] The inclusion of GPC in this group of allergic diseases is controversial because the pathophysiological features are quite different from the other diseases. For that reason, and because of its special relation with contact lenses, we will comment separately on this disease.

Giant Papillary Conjunctivitis

GPC is an adverse ocular reaction to contact lenses, and can occur with both soft and rigid contact lenses.[33,34] Although it is predominantly associated with contact lenses, additional etiologies such as sutures, corneal foreign bodies, or prostheses have been reported.[33,34] Common symptoms usually reported by GPC patients are decrease comfortable CL wearing time, excessive lens movement, foreign body sensation, and blurred vision.

The inclusion of GPC in the group of ocular surface allergy is controversial because this disease is a non-IgE mediated hypersensivity.[35] The incorrect inclusion of GPC as an allergic disease was also pointed out by the mechanical theory that suggested an irritative and mechanical etiology for GPC, rather than an allergic

etiology.[36,37] This hypothesis is also supported by reported cases due to sutures or foreign bodies. For these reasons, GPC is now included in the group of non-allergic hypersensivity disorders.[38] However, for many years, GPC was considered an allergic disease. This mistake was probably due to the clinical symptoms that are similar to those observed in allergic diseases: itching, tearing, mucous hyperproduction, and an increase of symptoms during spring pollen season.[39] In addition, higher levels of IL-4 and IL-13 have also been found in this pathology. GPC is characterized by papillae on the upper tarsal conjunctiva. There is also a significantly greater number of inflammatory cells in the conjunctiva of these patients, especially in the epithelium.[34]

In summary, GPC is an ocular surface disease, presumably induced by contact lens wear, with mucous hyperproduction as one of its main symptoms. As we previously explained, the main mucous-producers cells in the ocular surface are goblet cells, so GPC links contact lens with goblet cell function.

CONTACT LENS WEAR AND GOBLET CELL FUNCTION

The influence that contact lenses have on the ocular surface mucous system started to be explored in the 1980s. The Allansmith group published several studies regarding non-goblet epithelial cells in human conjunctiva, and concluded that there were more secretory vesicles in non-goblet epithelial cells in contact lens users.[40–42]

An extensive review about contact lens wear and goblet cells was published in 2011 by Doughty.[43] In that review, the author highlighted the contradictory results found in the literature regarding the effect of contact lens wear on conjunctival goblet cells. The majority of authors found a large decrease in goblet cell numbers in contact lens users,[44,45] several others described the opposite,[46,47] and two studies found no differences in goblet cell density.[48,49] As Doughty explains in his review, the reason for this variability could be inconsistency in the methodologies used.

More recently, the Tear Film and Ocular Surface society (TFOS) International Workshop on Contact Lens Discomfort also summarized the changes found in goblet cell density induced by contact lens wear.[50] As well as Doughty, they also remarked on the variations in the methodology as the potential cause for the diversity of results. Another possibility for the diverse results is that the number of

goblet cells per unit area varies with location on the conjunctiva.[51,52] If goblet cells are not sampled from the same area in each study participant, the results of a study are not valid. In addition, since the contact lens interacts differently with each area of the conjunctiva, if studies use different areas of the conjunctiva they could obtain diverse results. In fact, the TFOS study concluded that the location at which the cytology sample is obtained is likely the main reason of the variations in study results.

However, all the studies described in the previous paragraph focused on the effect of contact lens wear on goblet cell density, but not on other possible influences on goblet cell function. Surprisingly, the authors have not yet explained the reason for changes in goblet cell density. Interestingly, several recent studies have shown the effect of a variety of cytokines on goblet cells, and changes in cytokine levels (mainly increased or decreased) have been reported in contact lens wearers.[53–57] Research investigating the effect of contact lens wear on goblet cell function is critically needed.

CYTOKINES AND CONJUNCTIVAL GOBLET CELL FUNCTION

Cytokines are immunomodulatory agents that are secreted by different types of cells. Every cell, except the red blood cell, can produce and respond to cytokines.[58] In addition there is a wide range of cytokines, with different biochemical characteristics and biologic functions. However, all of them play an important role in cell signaling. As regulators of the immune response cytokines can exert pro-inflammatory actions, anti-inflammatory actions, or both. These compounds can be classified into different subtypes, depending on the type of T helper (Th) cell that produces them.[20] The main subtypes are: Th1, Th2, and Th17.[59]

Th1-derived cytokines

Interferon gamma (IFN-γ)

IFN-γ is a key cytokine coordinating immune defense.[60] It is probably the most studied cytokine in relation to conjunctival goblet cell function. The main reason for this focus is the clear implication of IFN-γ in dry eye disease, as well as the extensive interest in understanding this quality of life-threatening condition.

Increased levels of IFN-γ in tears of dry eye patients have been reported by several authors.[61,62]

IFN-γ binds to its receptor IFN-γ-R to trigger a cell signaling cascade.[60,63] The presence of IFN-γRs in goblet cells has been confirmed in C57/BL6 mice,[64,65] and we have recently described it in cultured rat and human goblet cells (manuscript under review). The main signaling pathway used by IFN-γ is the JAK-STAT cascade. However, some other alternative pathways have been described.[66]

IFN-γ has an apoptotic effect on goblet cells.[30,64,65] This would explain the loss of goblet cells typically found in dry eye patients. In addition to finding a decrease in goblet cell proliferation by IFN-γ, we have also demonstrated that the secretion of the remaining cells is also altered in the presence of IFN-γ when rat cells were investigated. We showed that this cytokine by itself induced an increase in $[Ca2+]_i$, which relates to high molecular weight glycoconjugate secretion. In addition to increasing $[Ca^{2+}]_i$, IFN-γ blocked the effect of a cholinergic agonist on inducing goblet cell secretion in rat goblet cells.[67] Similar results were obtained in cultured mouse goblet cells.[65]

Tumor Necrosis Factor alpha (TNF-α)

TNF-α is another Th1 cytokine that is also involved in dry eye disease. This cytokine is upregulated in conjunctival epithelial cells of patients with Sjögren's syndrome-associated aqueous-deficient dry eye, but not in those with non-Sjögren's syndrome.[68] Due to its central role in the inflammatory state of dry eye disease, TNF-α blockers have been proposed as a treatment for dry eye disease.[69]

The effect of TNF-α on conjunctival goblet cells has not been extensively explored, but it has been shown that TNF-α inhibits MUC5AC secretion induced by cholinergic agonists and increases goblet cell apoptosis.[65] These effects are similar to the ones exert by IFN-γ.

Th2-derived cytokines

Th2 cells are characterized by the production of IL-4, IL-5, and IL-13.[70] These cytokines have a critical role in allergic diseases affecting the mucosa, such as asthma or allergic conjunctivitis.

Interleukin 4

The presence of IL-4 is essential for a naïve Th cell to become a Th2 cell. IL-4 is necessary for Th2 lymphocyte differentiation and it is later secreted by these Th2 cells.[71] This pleiotropic cytokine binds to IL-4 receptors to activate signal transduction, mainly through Jak1 and Jak2 kinases. We have demonstrated the presence of IL-4 receptor in cultured human goblet cells.[72]

The effect of IL-4 on goblet cells has been studied in the airway epithelium.[73] These authors showed that IL-4 induced the differentiation of epithelial cells into mucous-producing goblet cells, and that after IL-4 exposure there was an increase in glycoconjugate secretion. These experiments were performed *in vitro* with a human pulmonary mucoepidermoid cell line and *in vivo* with BALB/c mice. We showed results consistent with those of Dabbagh K. et al in rat cultured conjunctival goblet cells.[67] We found that IL-4 increased conjunctival goblet cell proliferation by 1.94 fold over basal. In addition, intracellular [Ca^{2+}] was also increased by IL-4 exposure. Intracellular [Ca^{2+}] measurement after different stimuli is especially relevant in goblet cells, since it is directly related with mucin secretion.[74,75]

Interleukin 13

Interleukin 13 is, along with IFN-γ, the most extensively studied cytokine in relation to conjunctival goblet cells. IL-13 is a small glycoprotein with a broad spectrum of actions, such as promotion of eosinophil migration, upregulation of adhesion molecules, and goblet cell differentiation and mucous hypersecretion. IL-13 induces mucin synthesis and hyperplasia in airway goblet cells.[76] The role of this cytokine in asthma is well known, where it is a critical mediator in the pathology of this disease.[77] In fact, a vaccine that neutralized endogenous IL-13 has been proposed as a therapy to attenuate allergic inflammation in asthma.[78]

In the eye, it has been demonstrated that IL-13 promotes conjunctival goblet cell differentiation and proliferation.[65,79] These authors confirmed the expression of IL-13 receptor in mouse goblet cells, and we have done the same in human cultured goblet cells.[72]

Interleukin 5

Interleukin 5 is an important cytokine in allergic processes. Its main role is to enhance eosinophilic accumulation and activation during allergen-induced

inflammation.[80] Although it has been extensively studied in allergy, where it is found elevated, the interaction of IL-5 with goblet cells is not clear. Lee et al. stated that IL-5 expression in the lung epithelium of mice leads to several pulmonary changes, including goblet cell hyperplasia.[81] However, it is unclear if this hyperplasia is produced directly by IL-5, or if it is eosinophils-mediated.[82,83]

Th17-derived cytokines

The Th17 cell subtype was described in 2005.[59,84–86] These cells act as regulators of the immune response and produce IL-17, IL-6, IL-21, or IL-22, among other cytokines.[87]

Interleukin 17

The prototypic member of the IL-17 family is IL-17A, a cytokine with high homology among mouse, rat, and human.[88] It has been associated with ocular inflammatory diseases, such as uveitis, scleritis, and dry eye syndrome.[89] IL-17 along with IL-6 is able to increase MUC5AC levels in primary human tracheobronquial epithelial cells.[90] Regarding the conjunctiva, Contreras-Ruiz et al. Have recently shown an increase in proliferation of cultured goblet cells when cells were exposed to IL-17A.[65]

Interleukin 6

Interleukin 6 is another well studied Th17-type cytokine. IL-6 acts as an activator of the immune system, and is elevated in most inflammatory states.[91] This cytokine is secreted mainly by monocytes/macrophages and epithelial cells, including conjunctival epithelial cells.[92,93] Several authors have determined levels of IL-6 in tears.[57,94,95] Boehm et al. described a 2.1 fold increase of IL-6 levels in dry eye patients compared to healthy controls. Others have studied expression levels of this cytokine on conjunctival epitelial cells.[92,96] However, in most of these studies, goblet cells have not been analyzed.

Other cytokines

Recently, interest in the study of IL-22 and IL-33 in mucosal pathologies has increased.[97–99] Turner et al. suggested a key role for IL-22 in intestinal goblet cells, showing IL-22-induced mucin secretion and goblet cell activation.[99] Luzina et al.

reported that full-length IL-33 isoforms induced goblet cell hyperplasia in lungs[97] and Tanabe et al. showed that IL-33 acts directly on goblet cells stimulating IL-8 secretion, what may maintain mucus hypersecretion in asthma patients.[98] However, the effect of these cytokines on conjunctival goblet cells is still unexplored. The similarity previously found in goblet cells among different mucosa suggests that IL-33 would probably play an important role in the ocular surface that still needs to be discovered.

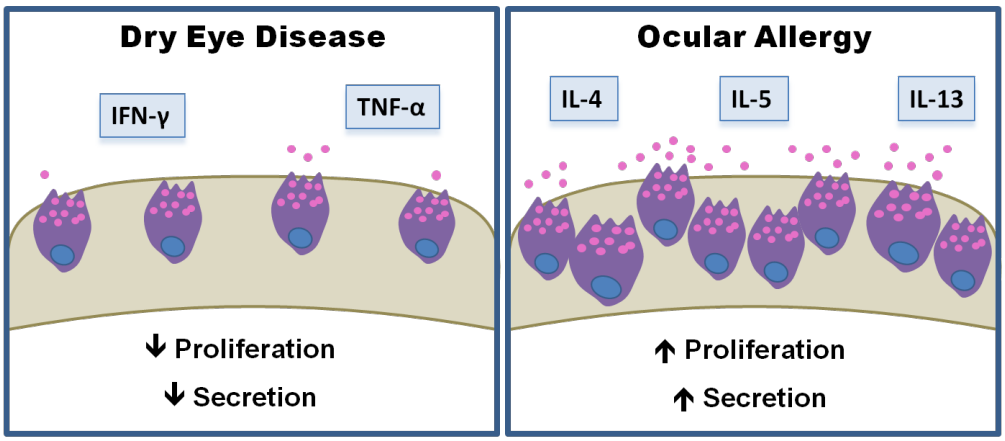

Figure 3. Schematic illustrating the effect of cytokines on goblet cells in dry eye disease and ocular allergy.

SUMMARY

Goblet cells have been studied in several diseases due to their mucin-secreting capacity. It is known that the number of these cells is lower in dry eye diseases, and that higher goblet cell densities are found in allergic diseases (Figure 3). The cytokine balances in these two diseases is also opposite. While a Th1 profile predominates in dry eye disease with special involvement of the Th1-cytokine IFN-γ, a Th2 profile predominates in allergic diseases that are associated with mucous hyperproduction. Recent findings on the effect of cytokines on goblet cells have shown that Th1 cytokines have pro-apoptotic effects on goblet cells, whereas Th2 cytokines promote goblet cell proliferation. A summary of the effect that cytokines have on conjunctival goblet cells is shown in Table 1.

Interestingly, several authors have studied goblet cell density in contact lens wearers. Most of studies have found a decrease in goblet cell number in these patients. However, the biological processes that lead to this decrease are not fully understood.

TABLE 1. Summary of cytokines effect on conjunctival goblet cells

Cytokine	Effect	Specie	Authors
IFN-γ	↑ apoptosis	Mouse	Zhang et al. (2011, 2014)[30, 64]; Contreras-Ruiz et al. (2013)[65]
	↓ proliferation	Rat	García-Posadas et al. (ARVO 2013)[67]
	↑ secretion	Rat	García-Posadas et al. (ARVO 2013) [67]
TNF-α	↑ apoptosis	Mouse	Contreras-Ruiz et al. (2013) [65]
IL-4	↑ proliferation	Rat; Human	García-Posadas et al. (ARVO 2013, 2014)[67, 72]
	↑ secretion	Rat; Human	García-Posadas et al. (ARVO 2013, 2014) [67, 72]
IL-13	↑ proliferation	Mouse	De Paiva et al. (2011)[79]; Contreras-Ruiz et al. (2013) [65]
	↑GC differentiation	Mouse	De Paiva et al. (2011) [79]
IL-17	↑ proliferation	Mouse	Contreras-Ruiz et al. (2013) [65]
IL-6	↑ proliferation	Mouse	Contreras-Ruiz et al. (2013) [65]

CONCLUSIONS

Recent studies about the influence of cytokines on goblet cells open a new field of research. It is important to observe how different types of Th cytokines seem to have opposite effects on conjunctival goblet cells, especially when comparing Th1 versus Th2-derived cytokines.

Goblet cells are becoming increasingly more important in the study of all types of pathologies affecting the ocular surface, from dry eye to allergies. It is surprising that, until now, most clinical studies regarding goblet cells were only focused on goblet cell number, but not on goblet cell function. The latest advances in this area are revealing previously unknown functions of these important cells and we know now that it is not only an issue of numbers.

ACKNOWLEDGEMENTS

Authors thank José Carlos López and Nieves Fernández, from the IOBA Ocular Pathology Laboratory, for providing CIC pictures, and Inmaculada Pérez for her help in the obtaining of *in vivo* laser scanning confocal microscopy images.

Financial support: FPI Scholarship Program BES-2011-046381 and CICYT Grant MAT2010-20452- C03-01(Ministry of Science and Innovation, Spain), NIH R01 EY019470.

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Study 4

Interaction of IFN-γ with Cholinergic Agonists to Modulate Rat and Human Goblet Cell Function

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Accepted in *Mucosal Immunology*

ACCEPTANCE LETTER

Asunto	**Mucosal Immunology - Decision on Manuscript ID MI-14-347.R1**
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May 5, 2015

Dear Mrs. García-Posadas:

I apologize again for the delay, we were unable to get a review from the second reviewer, however, it is a pleasure to accept your manuscript entitled "Interaction of IFN-γ with Cholinergic Agonists to Modulate Rat and Human Goblet Cell Function" in its current form for publication in Mucosal Immunology. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

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Thank you for your fine contribution. On behalf of the Editors of Mucosal Immunology, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Marsha Wills-Karp
Editor, Mucosal Immunology
mwkarp@jhsph.edu

ESTUDIO 4

Tal y como se vio en el estudio anterior, las células caliciformes pueden responder a varios estímulos, tales como lentes de contacto o la presencia de distintas moléculas.

En este estudio se lleva a cabo un análisis detallado del efecto del IFN-γ en las células caliciformes. Esta citoquina está elevada en distintas enfermedades y es especialmente relevante en el síndrome de ojo seco. Además, se estudia la interacción del IFN-γ con agonistas colinérgicos y cómo dicha interacción es capaz de modular la función de las células caliciformes en la secreción de mucinas.

STUDY 4

In the previous study we reviewed the goblet cell response to several stimuli such as contact lens or cytokines.

In this study, we further investigate the effect of IFN-γ on goblet cell function. This cytokine is elevated in several ocular surface inflammatory diseases, including in dry eye. In addition, we study the interaction of IFN-γ with cholinergic agonists and how this interaction modulates goblet cell function in terms of mucin secretion.

Interaction of IFN-γ with Cholinergic Agonists to Modulate Rat and Human Goblet Cell Function

ABSTRACT

Goblet cells populate wet-surfaced mucosa including the conjunctiva of the eye, intestine, and nose, among others. These cells function as part of the innate immune system by secreting high molecular weight mucins that interact with environmental constituents including pathogens, allergens, and particulate pollutants. Herein we determined whether IFN-γ, a Th1 cytokine increased in dry eye, alters goblet cell function. Goblet cells from rat and human conjunctiva were cultured. Changes in intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$), high molecular weight glycoconjugate secretion, and proliferation were measured after stimulation with IFN-γ with or without the cholinergic agonist carbachol. IFN-γ itself increased $[Ca^{2+}]_i$ in rat and human goblet cells and prevented the increase in $[Ca^{2+}]_i$ caused by carbachol. Carbachol prevented IFN-γ-mediated increase in $[Ca^{2+}]_i$. This cross-talk between IFN-γ and muscarinic receptors may be partially due to use of the same Ca^{2+}_i reservoirs, but also from interaction of signaling pathways proximal to the increase in $[Ca^{2+}]_i$. IFN-γ blocked carbachol-induced high molecular weight glycoconjugate secretion and reduced goblet cell proliferation. We conclude that increased levels of IFN-γ in dry eye disease could explain the lack of goblet cells and mucin deficiency typically found in this pathology. IFN-γ could also function similarly in respiratory and gastrointestinal tracts.

INTRODUCTION

The wet-surfaced mucosa including the conjunctiva of the eye, the intestine, colon, nose, bronchioles, Eustachian tube, and vagina contain goblet cells. These cells function as part of the innate immune system by secreting high molecular weight mucins that directly interact with environmental constituents including pathogens, allergens, and particulate pollutants. Substantial experimental evidence demonstrates that goblet cells function in mucosal epithelial protection and disease pathogenesis in respiratory and gastrointestinal tracts. [1,2]

In the ocular surface goblet cells are found in the epithelial layer of the conjunctiva, the mucous membrane that surrounds the cornea and lines the eyelids. These goblet cells are specialized cells that produce and secrete mucins, most notably the mucin (MUC) MUC5AC that lubricates and protects the ocular surface, maintaining its health. [3,4] Goblet cells are also integral participants in diseases of the ocular surface including allergic conjunctivitis, bacterial keratitis and conjunctivitis, and dry eye.

MUC5AC is a high molecular weight glycoconjugate that forms the mucous layer of the tear film. [5] The amount of MUC5AC found in the ocular surface is tightly controlled by goblet cell number, MUC5AC synthesis, and MUC5AC secretion. In inflammatory disorders such as dry eye, Sjögren's syndrome or ocular cicatricial pemphigoid goblet cells die or are non-functional. [6-8] On the other hand, in diseases such as allergic conjunctivitis, higher goblet cell numbers are found. As early as in 1992, Lemp [9] suggested that either an increase or a decrease in the number of filled goblet cells was associated with ocular surface pathology.

Under normal conditions, goblet cell secretion is under neural control by the efferent parasympathetic nervous system. Cholinergic, muscarinic mediators that are analogs of the parasympathetic neurotransmitter acetylcholine are major stimuli. [10] Cholinergic agonists transmit their signal by activating the G protein $G_{\alpha q/}11$ that activates phospholipase C, which breaks down phosphatidylinositol 4,5 bisphosphate (PIP_2) producing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. The increase in IP_3 binds to its receptor in the endoplasmic reticulum to release Ca^{2+} from intracellular stores thereby elevating the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). [11] The increase in $[Ca^{2+}]_i$ leads to activation of extracellular regulated kinase (ERK)1/2 (also known as p44, p42 mitogen activated protein kinase (MAPK)), and

secretion of high molecular weight glycoconjugates including MUC5AC. [12] In airway epithelium, mucin secretion is dependent upon the calcium sensors Munc13-2 and synaptotagmin2.[13] These proteins have not yet been identified in the conjunctiva. Cholinergic agonists mediate goblet cell secretory responses to environmental changes under normal conditions. When inflammation develops in the ocular surface as occurs in dry eye, these responses may be altered. This alteration would lead to a change in goblet cell mucin production. In early disease mucus production can be increased as a protective response, but later in the disease goblet cell mucin production can be decreased leading to ocular surface pathology.

Interferon gamma (IFN-γ) is the major Th1-derived cytokine. This cytokine is implicated in several different immune responses, such as inflammation or graft rejection. IFN-γ is secreted by cytotoxic T cells, Th1 cells, and natural killer cells. [14] It binds to its receptor, IFN-γ-R, that is ubiquitously expressed at the cell surface on all cells except erythrocytes. [15] The main signaling pathway induced by IFN-γ is through the JAK-STAT intracellular signal transduction pathway leading to activation of STAT-1 target genes. [16,17] IFN-γ-R can also recruit and activate phosphatidylinositol-3 kinase (PI-3K), Src, or MyD88, that initiate signaling cascades involving ERK1/2, Akt or NF-κB.[13]

In the conjunctival epithelium, IFN-γ induces squamous metaplasia, which leads to progressive goblet cell loss. Both changes are related to dry eye disease. Recently Zhang et al. [18] showed that IFN-γ caused goblet cell apoptosis in a mouse model of dry eye. However, the signaling pathways activated by this cytokine and its role regulating in goblet cell function remain unclear.

The purpose of this study was to determine if IFN-γ, a mediator of dry eye, directly regulates mucin production in rat and human conjunctival goblet cells. Thus, presence of IFN-γ-R-1, as well as effect on intracellular Ca^{2+} signaling, mucin secretion, and goblet cell proliferation were measured after stimulation with IFN-γ alone or in the presence of a normal stimulus of secretion, a cholinergic agonist. We found that IFN-γ blocked the cholinergic agonist-stimulated increase in $[Ca^{2+}]_i$, mucin secretion, and decreased goblet cell proliferation. Thus, IFN-γ leads to decreased goblet cell mucous production and contributes to the mucin deficiency found in dry eye disease.

RESULTS

Goblet Cell Characterization

Identity of cells cultured from both rat and human conjunctiva was confirmed by immunofluorescence microscopy using the following markers: cytokeratins (CK) 4 (specific for stratified squamous non-goblet epithelial cells) and 7 (goblet cell specific keratin), [19,20] and lectins from *Ulex europaeus agglutinin* type 1 (UEA-1) (rat) or *Helix pomatia agglutinin* (HPA) (human). Lectins identify high molecular weight glycoconjugates including MUC5AC synthesized and secreted by goblet cells.

The majority of rat cells in culture were positive for both lectin UEA-1 (green) and CK7 (red) as shown in **Figure 1a.** To assure that lectins were identifying goblet cells, immunocytochemistry against UEA-1 and MUC5AC was performed in cultured cells (**Figure 1b**). We observed that UEA-1 and MUC5AC staining co-localized, showing that there were identifying the same mucous products. Human cultured cells were positive for lectin HPA (red) and CK7 (green), as shown in **Figure 1c,** and positive for MUC5AC (**Figure 1d**). Cultured cells did not express CK4 (data not shown). Additionally, the expression of MUC5AC in these cells and in conjunctival tissue from rat and human was confirmed by semi-quantitative real time RT-PCR (**Figure 1e**). MUC5AC was detected in both rat (**left**) and human (**right**) conjunctival tissue and in cultured goblet cells. MUC5AC mRNA levels were 277.7 times higher in cultured rat goblet cells that in rat conjunctiva, and 170.7 times higher in human goblet cells than in human conjunctiva. Therefore the overwhelming majority of cells in culture were goblet cells.

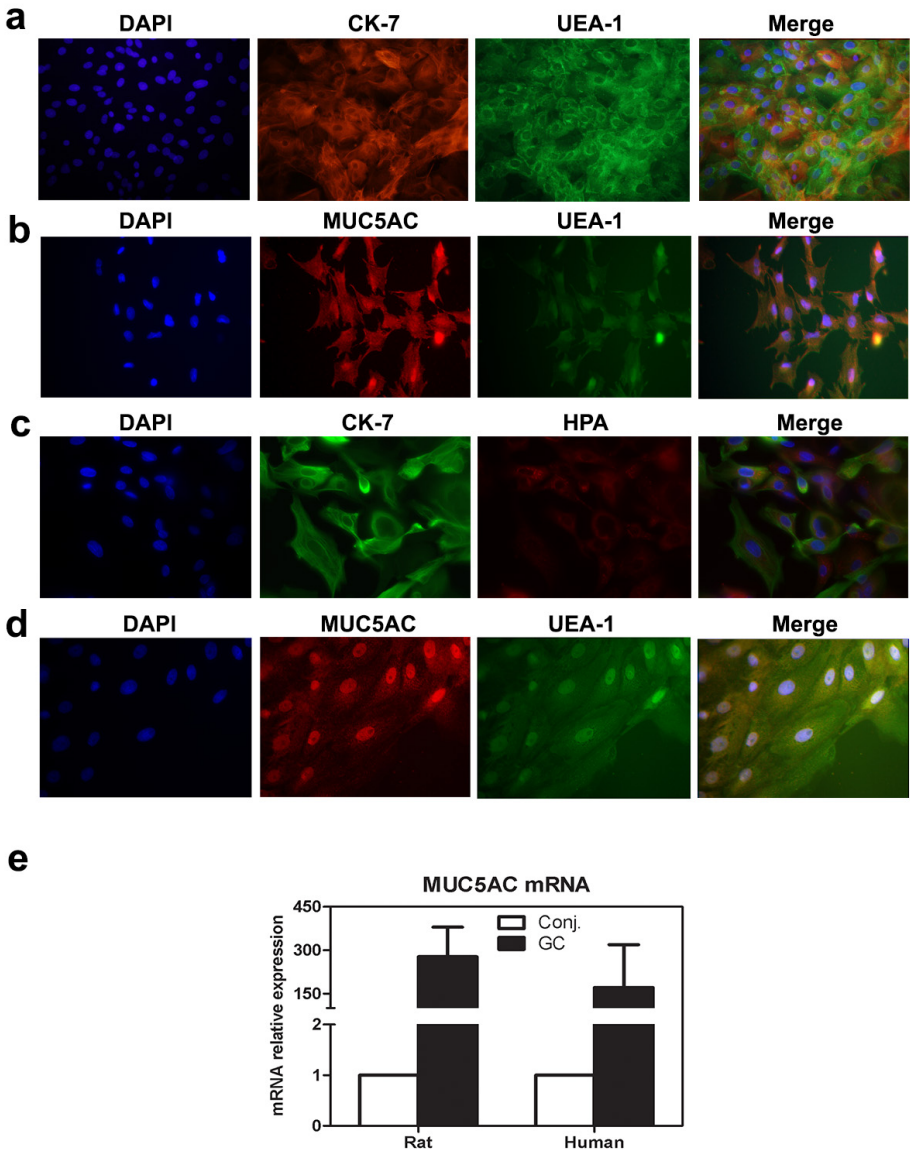

Figure 1. Cultured cells are goblet cells as they contain CK7, lectin binding domains, and MUC5AC. Representative images of rat (a-b) and human (c-d) cultured goblet cells. Nuclei were stained in blue with DAPI. Cells expressed CK7 (a, c) lectins UEA-1 (rat cells, a) and HPA (human cells, c), and MUC5AC (b, d) as seen in merged images. Magnification X100 for a, X400 for b and d, and X200 for c. MUC5AC mRNA expression was measured by Real Time RT-PCR (e). Values are expressed as mean ± SEM (n=2). CK, cytokeratin; DAPI, 6-diamidino-2-phenylindole; HPA, *Helix pomatia agglutinin*; MUC, mucin; RT-PCR, reverse transcription polymerase chain reaction; UEA-1, *Ulex europaeus agglutinin* type 1; Conj., conjunctiva; GC, goblet cells.

Goblet Cells Express IFN-γ-R1

To determine if conjunctival goblet cells were able to directly respond to IFN-γ, the presence of IFN-γ-R1, the receptor for this cytokine, was determined by immunofluorescence microscopy and by real time RT-PCR. IFN-γ-R1 protein expression was identified in both rat and human cultured cells (**Figure 2a-b**). In addition, IFN-γ-R1 was detected in conjunctival tissue and cultured goblet cells (**Figure 2c**). We confirmed that both rat and human cultured cells expressed the receptor needed for IFN-γ to be effective.

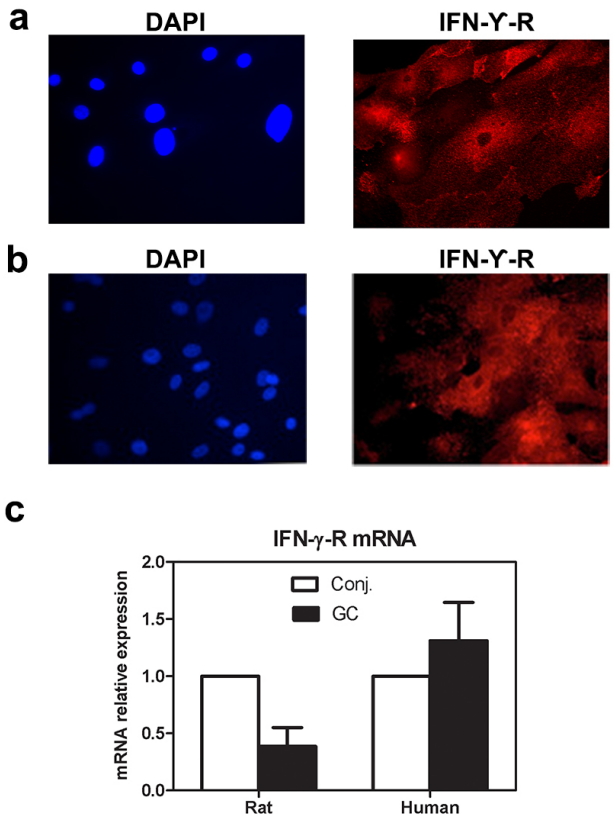

Figure 2. Conjunctival goblet cells express the IFN-γ receptor. Images of rat (a) and human (b) cultured goblet cells, where IFN-γ-R is stained in red. Nuclei were stained in blue with DAPI. IFN-γ-R mRNA expression was analyzed by Real Time RT-PCR (c). Values are expressed as mean ± SEM (n=2). DAPI, 6-diamidino-2-phenylindole; IFN-γ, interferon-gamma; RT-PCR, reverse transcription polymerase chain reaction; Conj., conjunctiva; GC, goblet cells.

IFN-γ Increases $[Ca^{2+}]_i$ in Goblet Cells

Due to its role in goblet cell signaling, $[Ca^{2+}]_i$ was measured in cultured cells after addition of different stimuli. The effect of IFN-γ was compared with the cholinergic agonist carbachol, an agonist of parasympathetic nerve mediated responses, that are known to increase $[Ca^{2+}]_i$ in conjunctival goblet cells. [21] Concentration-dependency assays were first performed in cultured rat cells to select optimal concentrations for IFN-γ and carbachol. IFN-γ caused a rapid increase in $[Ca^{2+}]_i$ to a peak value that was maintained at a lower plateau level over time (**Figure 3a**). IFN-γ at 0.1, 1.0, and 3.0 ng/ml significantly increased the peak $[Ca^{2+}]_i$ in a concentration dependent manner to 68.8 ± 29.6, 115.6 ± 37.3, and 239.5 ± 52.3 (n=6), respectively (**Figure 3b**). The highest concentration of IFN-γ 10 ng/ml did not produce any change. Thus IFN-γ itself increases $[Ca^{2+}]_i$. IFN-γ at 3 ng/ml was chosen as the maximal concentration for use in subsequent experiments.

When three different concentrations (10^{-6} M, 10^{-5} M, and 10^{-4} M) of carbachol were used, all these concentrations significantly increased $[Ca^{2+}]_i$ over time with a peak response followed by an elevated plateau (**Figure 3c**). When peak values were analyzed, the highest response of 271.4 ± 64.2 nM (n=4) was obtained at 10^{-4} M carbachol (**Figure 3d**). This result is in agreement with previous studies, [22] that found the plateau for carbachol was at 10^{-4} M. Therefore, carbachol at 10^{-4} M was chosen as the concentration to be used in subsequent experiments. Comparison of carbachol and IFN-γ responses shows that IFN-γ caused a $Ca^{2+}{}_i$ response comparable to that induced by the cholinergic agonist.

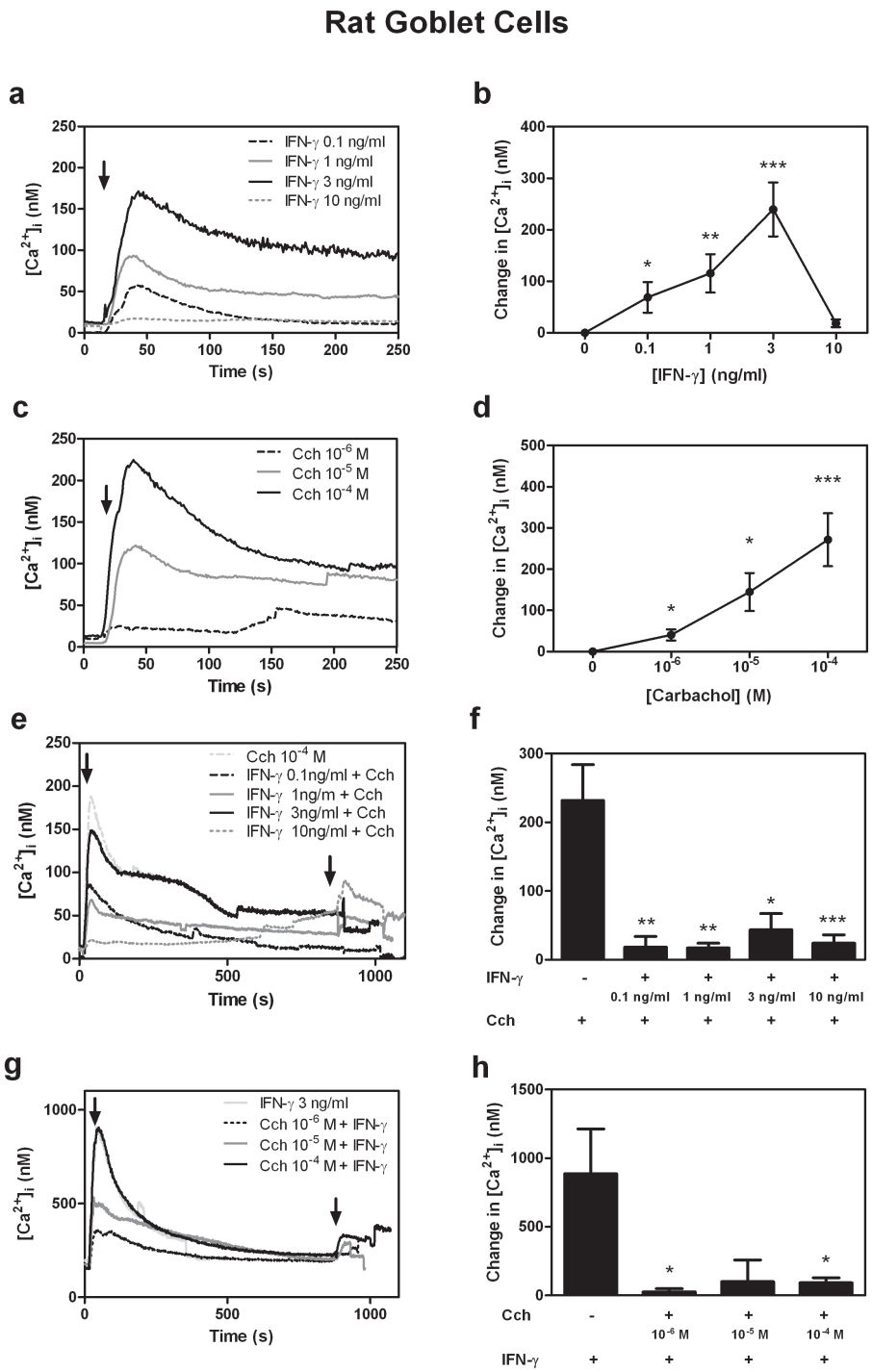

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Figure 3. IFN-γ and Cch cause a concentration dependent increase in intracellular [Ca^{2+}] and desensitize each others Ca^{2+} response in cultured conjunctival goblet cells. Concentration dependency of IFN-γ (a-b) and Cch (c-d) of $[Ca^{2+}]_i$ in rat conjunctival goblet cells. Mean intracellular Ca^{2+} response over time after addition of increasing concentrations of IFN-γ from 0.1 to 10 ng/ml (n=6) (a) and of Cch from 10^{-6} -10^{-4} M (n=5) (c). Peak $[Ca^{2+}]_i$ over basal is shown in (b) and (d) for IFN-γ and Cch, respectively. Mean intracellular Ca^{2+} response over time (e) or peak value over basal (f) in response to Cch at 10^{-4} M alone or the effect of addition of increasing concentrations of IFN-γ (indicated by first arrow) on the Cch response 15 min (indicated by second arrow) after IFN-γ addition (n=5). Mean intracellular Ca^{2+} response over time (g) or peak value over basal (h) in response to IFN-γ (3 ng/ml) alone or the effect of increasing concentrations of Cch (indicated by first arrow) on the IFN-γ response 15 min (indicated by second arrow) after Cch addition (n=4). * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.005$. Cch, carbachol; IFN-γ, interferon-gamma.

IFN-γ and a Cholinergic Agonist Block Each Others' Increase in $[Ca^{2+}]_i$ in Goblet Cells

The effect of a 15 min pretreatment with IFN-γ on the carbachol $Ca^{2+}{}_i$ response was next studied. Carbachol 10^{-4} M significantly increased $[Ca^{2+}]_i$ to 232.0 ± 52.1 nM (n=5) (**Figure 3e and f**). IFN-γ at 0.1, 1.0, 3.0, and 10 ng/ml significantly inhibited the carbachol-stimulated peak $[Ca^{2+}]_i$ response decreasing it to 18.4 ± 15.6, 17.1 ± 7.1, 43.2 ± 24.0, and 24.2 ± 11.9 nM (n=5), respectively. This was a 92.07%, 92.63%, 81.36%, and 89.57% inhibition, respectively. All four IFN-γ concentrations blocked the carbachol-induced increase in $[Ca^{2+}]_i$ (**Figure 3f**).

Conversely, the effect of 15 min pretreatment with carbachol on the IFN-γ response was also studied. IFN-γ (3ng/ml) significantly increased peak $[Ca^{2+}]_i$ to 886.4 ± 326.7 nM (n=4). Carbachol at 10^{-6} M and at 10^{-4} M significantly decreased IFN-γ stimulated $[Ca^{2+}]_i$ response to 27.3 ± 10.9 and 91.6 ± 19.0 nM (n=4), respectively, with 96.92 and 89.67 % inhibition (**Figure 3g and h**). Although not a significant decrease, carbachol at 10^{-5} M decreased the IFN-γ response by 88.6 %.

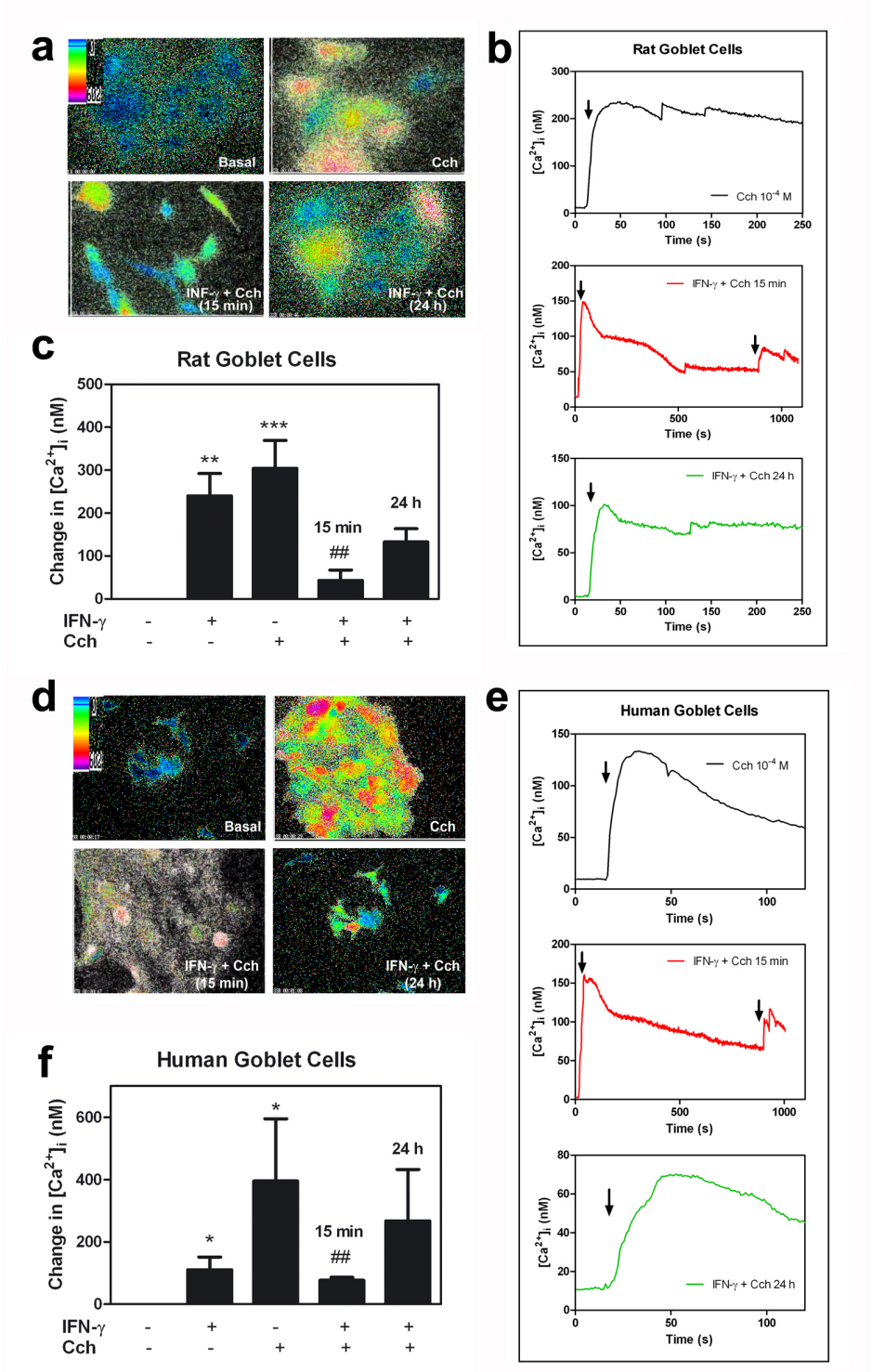
IFN-γ and the cholinergic agonist carbachol each block the intracellular $Ca^{2+}{}_i$ response of the other agonist.

IFN-γ Causes a Time-dependent Inhibition of a Cholinergic Agonist-Induced Increase in $[Ca^{2+}]_i$ in Goblet Cells

The effect of IFN-γ on carbachol-induced increase in $[Ca^{2+}]_i$ was evaluated at two different incubation times. The first time, 15 min, is a short treatment that indicates the immediate effect on intracellular Ca^{2+} levels and the use of the different cellular Ca^{2+} pools. The effect of a 15-min treatment was shown in **Figure 3** and is used as a control in **Figure 4**. The second time, 24 h, is used to study longer term activation of cytokine dependent signaling pathways probably involving synthesis of signaling mediators. In rat goblet cells, IFN-γ (3 ng/ml) increased peak $[Ca^{2+}]_i$ to a mean of 240.4 ± 52.0 nM (n=5) (p = 0.009, **Figure 4 a-c**). IFN-γ treatment for 15 min significantly reduced the effect of carbachol (10^{-4} M) from 304.5 ± 64.7 nM to 43.2 ± 23.9 nM (p = 0.009), whereas with the 24 h treatment no statistically significant difference was found when comparing the carbachol response before with the response after IFN-γ (p = 0.06, **Figure 4a-c**).

In human cells, IFN-γ (3 ng/ml) also induced a significant increase in peak $[Ca^{2+}]_i$ (**Figure 4 d-f**), to an average of 110.7 ± 40.8 nM compared to basal (n=7) (p = 0.019; **Figure 4f**). The 15 min treatment with IFN-γ blocked carbachol-induced increase from 395.3 ± 199.8 nM to 76.9 ± 9.0 nM (p = 0.009, **Figure 4f**). In contrast, the 24 h treatment with IFN- γ showed no significant effect on the carbachol-induced $[Ca^{2+}]_i$ response before compared to after IFN-γ.

Figure 4 (next page). A fifteen minutes, but not a 24 h incubation with IFN-γ blocks the increase in $[Ca^{2+}]_i$ stimulated by Cch in rat and human conjunctival goblet cells. A pseudo color image of $[Ca^{2+}]_i$ from fura-2 loaded single goblet cells in rat (a) and human (d) shows the increase in $[Ca^{2+}]_i$ under basal conditions, after stimulation with Cch (10^{-4} M), or Cch after 15 min or 24 h incubation with IFN-γ. Shown in rat (b) and human (e) goblet cells is the mean intracellular Ca^{2+} response over time, after addition of Cch (Cch- arrow black line), IFN-γ (first arrow red line), Cch (second arrow red line) after a 15 min treatment with IFN-γ or Cch (first arrow green line) after a 24 h treatment with IFN-γ. Peak $[Ca^{2+}]_i$ over basal for each condition is shown in (c) and (f) and is expressed as mean ± SEM (n=5) in rat cells (b and c) and (n=7) in human cells (e and f). * indicates statistical significance compared to basal values, and $^{\#}$ is compared to Cch values. * or $^{\#}$ $p \leq 0.05$; ** or $^{\#\#}$ $p \leq 0.01$; *** or $^{\#\#\#}$ $p \leq 0.005$. Cch, carbachol; IFN-γ, interferon-gamma.



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Effect of Extracellular and Intracellular Ca^{2+} Store Depletion on IFN-γ and Cholinergic Agonist Ca^{2+}_i Responses in Goblet Cells

We explored the cellular Ca^{2+} pools used by IFN-γ and carbachol to increase $[Ca^{2+}]_i$. To determine the role of extracellular Ca^{2+} (Ca^{2+}_o) we removed Ca^{2+}_o for 3 min before adding 3 ng/ml IFN-γ or 10^{-4} M carbachol. In rat goblet cells, IFN-γ increased $[Ca^{2+}]_i$ to 368.1 ± 109.4 nM (n=6) (**Figure 5 a and c**). In the absence of Ca^{2+}_o this response was significantly decreased to 92.6 ± 41.8 nm. When Ca^{2+}_o was re-added, a significant increase in $[Ca^{2+}]_i$ to 531.9 ± 123.2 nM was observed showing that the intracellular Ca^{2+} stores were not altered in these experiments. Similar results were obtained with carbachol (**Figure 5 a and c**). Carbachol increased $[Ca^{2+}]_i$ to 1061.2 ± 356.1 nM (n=6), which was significantly decreased to 258.7 ± 57.7 nM in the absence of Ca^{2+}_o. Re-addition of Ca^{2+}_o increased the carbachol $[Ca^{2}_{+}]_i$ response to 618.9 ± 200.7 nM.

A similar experiment was conducted using human goblet cells (**Figure 5 b and d**). In the presence of extracellular Ca^{2+}, IFN-γ increased Ca^{2+} to 398.6 ± 222.7 nM (n=5), and that increase was reduced to 21.2 ± 10.6 nM in the absence of Ca^{2+}_o. When Ca^{2+}_o was re-added, $[Ca^{2+}]_i$ increased to 380.5 ± 225.0. Carbachol increased $[Ca^{2+}]_i$ to 394.4 ± 77.9 nM, and in the absence of Ca^{2+}_o it was decreased to 100.7 ± 34.8 nM. Re-addition of Ca^{2+}_o increased $[Ca^{2+}]_i$ to 284.2 ± 215.4 nM.

To explore the role of intracellular Ca^{2+} stores, rat goblet cells were treated for 15 min with 10^{-5} M thapsigargin or for 30 min with 10^{-5} M 2-amino-ethoxydiphenylborane (2-APB). Thapsigargin depletes intracellular Ca^{2+} stores and 2-APB blocks store-operated calcium release. [23] After treatment, cells were stimulated with 3 ng/ml IFN-γ or 10^{-4} M carbachol (**Figure 5 e-h**). IFN-γ increased $[Ca^{2+}]_i$ to 961.4 ± 308.1 nM (n=6) (**Figure 5 e and g**). The IFN-γ-induced increase in $[Ca^{2+}]_i$ after treatment with thapsigargin was significantly reduced to 110.5 ± 66.6 and with 2-APB was decreased to 303.3 ± 146.7 nM. Carbachol increased $[Ca^{2+}]_i$ to 1128.9 ± 348.6 nM (n=6) (**Figure 5 f and h**). After treatment with thapsigargin the carbachol increase in $[Ca^{2+}]_i$ was significantly depressed to 212.1 ± 131.2 and after 2-APB was decreased to 422.5 ± 217.5 nM.

Both IFN-γ and carbachol responses were significantly lowered with prior treatment with thapsigargin. 2-APB also blocked both IFN-γ and carbachol responses, but the decrease was not statistically significant. IFN-γ and cholinergic agonists use similar cellular Ca^{2+} stores in goblet cells. These stores have both

intracellular and extracellular components as previously demonstrated for cholinergic agonists. [24]

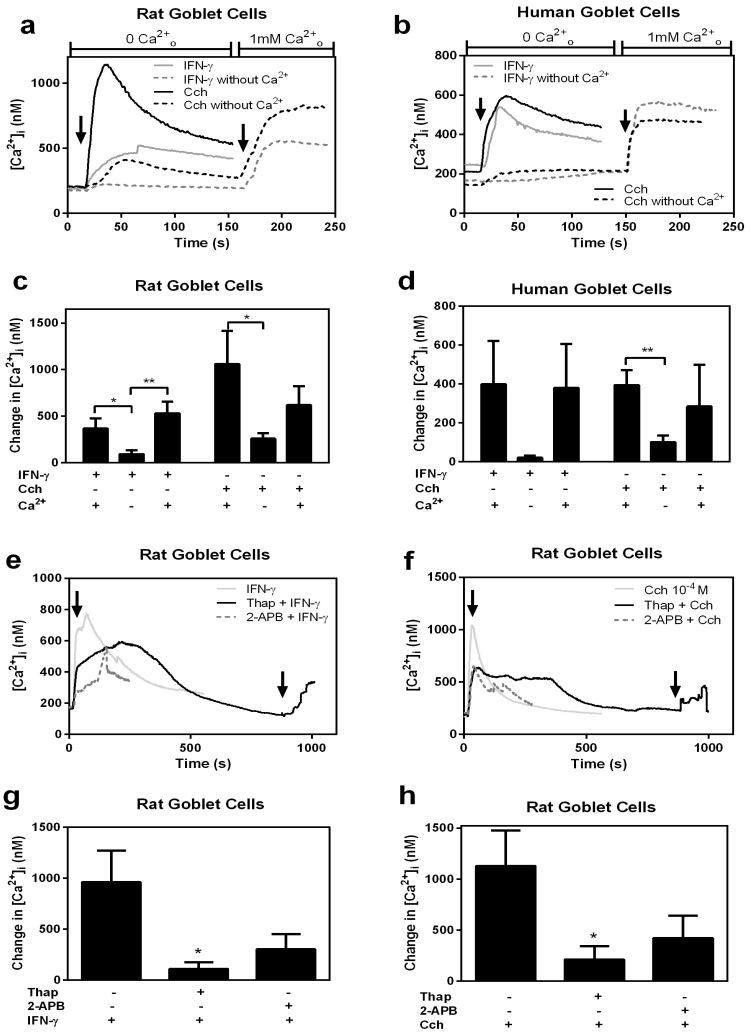

Figure 5. The IFN-γ and Cch stimulated increases in $[Ca^{2+}]_i$ are dependent upon both extracellular Ca^{2+} and intracellular Ca^{2+} stores in rat and human conjunctival goblet cells. The mean $[Ca^{2+}]_i$ over time is shown for 3 ng/ml IFN-γ or 10^{-4} M Cch in the presence or absence of extracellular calcium (first arrow), or the re-addition of extracellular Ca^{2+} (second arrow) was studied in rat (a) and human (b) goblet cells in (n=6 in rat cells) or (n=5 in human cells). Peak $[Ca^{2+}]_i$ over basal for each condition is shown in (c) and (d) and is expressed as mean ± SEM. The $[Ca^{2+}]_i$ over time is shown for 3 ng/ml IFN-γ (e) or 10^{-4} M Cch (f) alone (first arrow) or after a 15 min treatment with thapsigargin (10^{-5} M) (second arrow) or 30 min after addition of 2-APB (10^{-5} M) in rat goblet cells (n=6). Peak $[Ca^{2+}]_i$ over basal for each condition is shown in (g) and (h) and is expressed as mean ± SEM. * $p \leq 0.05$; ** $p \leq 0.01$. 2-APB, 2-amino-ethoxydiphenylborane; Cch, carbachol; IFN-γ, interferon-gamma.

Effect of Inhibition of PI-3K and ERK1/2 on IFN-γ- and Cholinergic Agonist-Stimulated Increase in $[Ca^{2+}]_i$ in Goblet Cells

Phosphoinositide-3 kinase (PI-3K) and mitogen-activated protein kinase kinase (MEK) 1/2 form part of an alternative IFN-γ signaling cascade compared to STAT-1.[13] Rat goblet cells were preincubated with the PI-3K inhibitor LY294002 or the MEK1/2 inhibitor U0126 at 10^{-5} M (prevents activation of ERK1/2) for 30 min and then stimulated with IFN-γ (3 ng/ml) or carbachol (10^{-4} M).

IFN-γ increased $[Ca^{2+}]_i$ to 453.7 ± 82.9 nM (n=5) (**Fig 6a and b**). Addition of LY294002 before IFN-γ decreased $[Ca^{2+}]_i$ to 259.3 ± 130.3 nM, but the effect was not significant. When U0126 was added, goblet cell response to IFN-γ was significantly reduced to 133.9 ± 68.5 nM. This is a 70.49 % inhibition.

Carbachol increased $[Ca^{2+}]_i$ to 942.9 ± 253.4 nM (n=5) (**Fig 6 c and d**). After pretreatment with LY294002, the response to carbachol was significantly decreased to 114.3 ± 10.7 nM, an 87.88% inhibition. In contrast, preincubation with U0126 did not significantly inhibit carbachol-mediated increase in $[Ca^{2+}]_i$, which was 309.0 ± 197.4 nM.

These data suggest that in goblet cells IFN-γ induces ERK1/2, but not PI-3K to increase $[Ca^{2+}]_i$. In contrast, cholinergic agonists activate PI-3K, but not ERK1/2 to increase $[Ca^{2+}]_i$. These results confirm results from a previous study. [25]

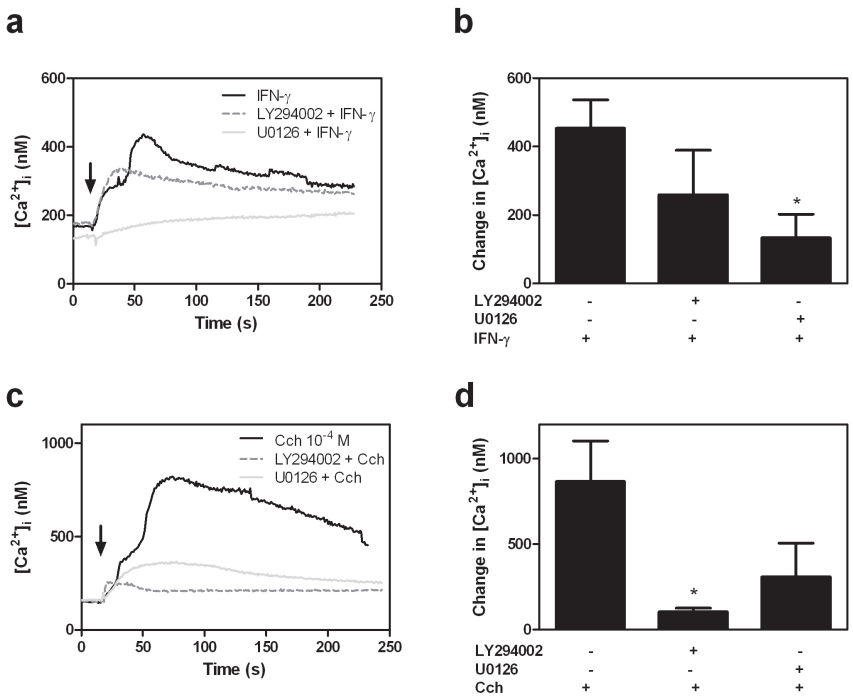

Figure 6. Blockage of PI-3K or ERK1/2 differentially inhibits IFN-γ and Cch stimulation of $[Ca^{2+}]_i$. The mean $[Ca^{2+}]_i$ over time is shown for 3 ng/ml IFN-γ (a) or 10^{-4} M Cch (c) alone or after a 30 min treatment with the PI-3K inhibitor LY294002 (10^{-5} M) or the MEK inhibitor U0126 (10^{-5} M) in rat goblet cells (n=3). Arrow indicates addition of agonist. Peak $[Ca^{2+}]_i$ over basal for each condition is shown in (b) and (d) and is expressed as mean ± SEM. * $p \leq 0.05$. Cch, carbachol; ERK, extracellular regulated kinase; IFN-γ, interferon-gamma; MEK, mitogen-activated protein kinase kinase; PI-3K, phosphatidylinositol-3 kinase.

IFN-γ Induces Mucin Secretion and Blocks Cholinergic Agonist-Stimulated Mucin Secretion from Goblet Cells

Goblet cell secretion was evaluated after stimulation with 3 ng/ml IFN-γ or 10^{-4} M carbachol. IFN-γ by itself did not have a significant effect on rat goblet cell secretion, and did not stimulate secretion from human cells (n=3) (**Figure 7a and d**). As a positive control carbachol induced high molecular weight glycoconjugate secretion from both rat and human goblet cells to 1.60 ± 0.08 and 2.01 ± 0.20 fold increase over basal (n=3), respectively. This response is consistent with previous results.[26] The effect of IFN-γ on carbachol-induced secretion was determined at two

times of incubation 15 min and 24 h. A 15 min treatment with IFN-γ did not have any significant effect on carbachol-induced secretion, in either rat or human goblet cells. In contrast, IFN-γ incubated for 24 h significantly blocked carbachol-induced secretion to 0.88 ± 0.06 and to 1.21 ± 0.23 in rat (p = 0.002, **Figure 7b**) and human cells (p = 0.05, **Figure 7c**), respectively. In rat cells a 120.0 % inhibition was obtained, whereas in human cells a 79.2 % inhibition was obtained.

These data suggest that activation of a long-term pathway such as JAK-STAT by IFN-γ can prevent cholinergic agonist stimulated secretion, but that stimulating a short-term pathway such as increasing the $[Ca^{2+}]_i$ cannot.

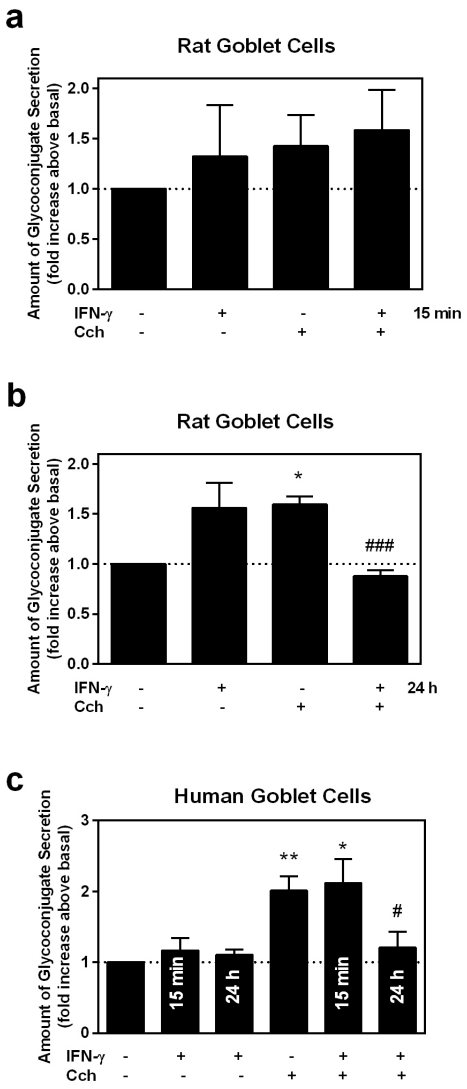

Figure 7. IFN-γ blocks Cch stimulated secretion of high molecular weight glycoproteins in rat and human goblet cells. Secretion from rat (a, b) and human (c) goblet cells is shown after a 2 h incubation with Cch (10^{-4} M), a 24 h incubation with IFN-γ (3 ng/ml), or a 15 min or 24 h treatment with IFN-γ followed by a 2 h incubation with Cch. Data are mean ± SEM (n=3). * means statistical significance compared to basal values, and # is compared to Cch values. * or # $p \leq 0.05$; ** or ## $p \leq 0.01$; *** or ### $p \leq 0.005$. Cch, carbachol; IFN-γ, interferon-gamma.

IFN-γ Decreases Goblet Cell Proliferation

Cell proliferation was measured in rat conjunctival goblet cells. A 24 h treatment with IFN-γ at 3 ng/ml decreased proliferation to 0.8 compared to basal value set as 1 (n=3) (p = 0.00018) (**Figure 8**). As expected carbachol 10^{-4} M did not have a significant effect on goblet cell proliferation after a 2 h treatment and IFN-γ added 24 h before carbachol did not alter proliferation either.

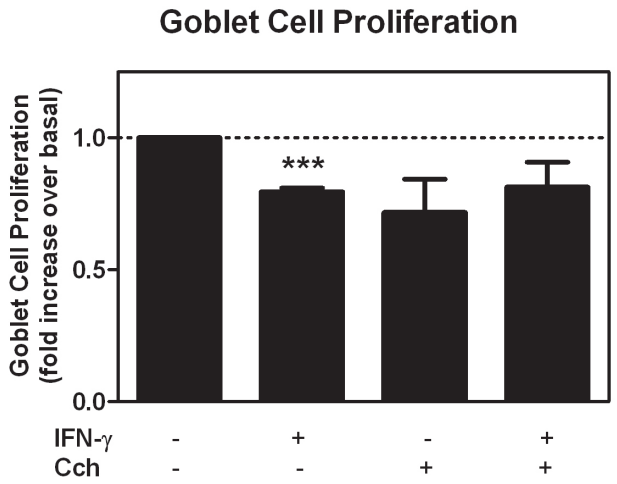

Figure 8. IFN-γ inhibits proliferation of cultured rat conjunctival goblet cells. Proliferation of goblet cells in response to no addition, IFN-γ (3 ng/ml) for 24 h, Cch (10^{-4} M) for 2 h, alone or after IFN-γ for 24 h. Data are mean ± SEM (n=3). *** p ≤ 0.005. Cch, carbachol; IFN-γ, interferon-gamma.

DISCUSSION

The role of the Th1 cytokine IFN-γ has been widely studied in a number of diseases, such as dry eye, Steven-Johnson syndrome, or Sjögren's syndrome, where elevated levels of IFN-γ have been found. [27-29] In humans, IFN-γ correlates with disease severity. [30] Our results demonstrate that IFN-γ, which is found to be increased in several inflammatory diseases of the conjunctiva, has a direct effect on conjunctival goblet cell function. We found that IFN-γ increases $[Ca^{2+}]_i$, but did not stimulate goblet cell secretion. As a decrease in goblet cell mucin production plays a

critical role in ocular surface inflammation [31] the most important finding of the present study is that IFN-γ blocks two of the three processes used by goblet cells to increase mucin production: mucin secretion and goblet cell proliferation. That is, IFN-γ prevented the increase in $[Ca^{2+}]_i$ and stimulation of secretion caused by cholinergic agonists. In addition, IFN-γ itself reduced goblet cell proliferation. These *in vitro* studies using isolated goblet cells were performed to determine the effect of IFN-γ on goblet cells not contaminated by other cell types. *In vivo*, is likely that cross talk between goblet and non-goblet cells occurs, involving multiple signaling cascades that could alter goblet cell signaling pathways. Therefore, further research *in vivo* is warranted.

Cultured goblet cells responded to IFN-γ in a concentration-dependent manner. IFN-γ from 0.1 ng/ml to 3 ng/ml showed progressive increase in $[Ca^{2+}]_i$, whereas the highest concentration (10 ng/ml) did not. This suggests that at the highest concentration of IFN-γ, a Ca^{2+} inhibitory signaling pathway is being activated. Interestingly, all four concentrations of IFN-γ blocked the effect of cholinergic agonists in a very similar way. This result suggests that IFN-γ at high concentrations is not using a Ca^{2+}-dependent mechanism to block cholinergic agonist-mediated Ca^{2+} responses. IFN-γ is known to activate other short-term signaling pathways [16] that will be investigated in future.

The mechanisms involved in short- and long-term responses induced by IFN-γ are likely to be different. Short-term blockade could be due to depletion of intracellular calcium reservoirs. Our results suggest that cholinergic agoinsts and IFN-γ are using, at least in part, the same intracellular Ca^{2+} reservoirs. Major Ca^{2+} reservoirs are the intracellular stores located in the endoplasmic reticulum [32] that are linked by Orai-1 and STIM1 to Ca^{2+} influx. [33,34] This intracellular store is depleted by thapsigargin. The findings that: 1) intracellular Ca^{2+} responses caused by cholinergic agonists and IFN-γ are both decreased after the addition of thapsigargin, and 2) the recovery of the intracellular Ca^{2+} response after the re-addition of extracellular Ca^{2+} supports this hypothesis. In agreement with our hypothesis is the finding that cholinergic agonist treatment for 15 min also inhibited IFN-γ induced Ca^{2+} responses and that a similar IFN-γ treatment inhibited the cholinergic Ca^{2+} response.

Cholinergic agonists bind to muscarinic receptors and IFN-γ binds to its own receptor, IFN-γ-R. The main signaling pathway activated by IFN-γ-R is JAK-STAT,[17] a pathway probably not activated by muscarinic receptors. However, several of the

alternative pathways activated by IFN-γ involve two kinases that form part of the muscarinic pathways, PI-3K and MEK1/2,[16] suggesting that an interaction based on these pathways may be occurring. For that reason we used specific inhibitors of these two kinases. We found that when PI-3K was blocked the response to cholinergic agonists was inhibited, but the response to IFN-γ was not altered. In contrast, when MEK1/2 was inhibited, goblet cell response to cholinergic agonists remained unaltered, while response to IFN-γ was significantly blocked. Even though cholinergic agonists and IFN-γ use the same cellular Ca^{2+} stores, the mechanism by which they use these stores appears to be different. The short-term effect could also be independent of Ca^{2+}, occur before the rise in intracellular Ca^{2+}, or involve a direct effect on the muscarinic receptors. Because both IFN-γ and cholinergic agonists blocked one another's responses, a cross desensitization of both receptors may be occurring.

A hallmark of dry eye disease is reduced mucin secretion. Although the levels of $[Ca^{2+}]_i$ were not significantly blocked after 24 h incubation with IFN-γ alone, secretion induced by cholinergic agonists was reduced when there was a previous 24 h incubation with IFN-γ. This result is similar to the report of Contreras-Ruiz et al. of a reduction of cholinergic agonist-mediated mucin secretion of mouse goblet cells exposed to IFN-γ for 24 h. [35] As a 24 hr treatment of IFN-γ does not alter cholinergic agonist increase in $[Ca^{2+}]_i$ it is possible that activation of the JAK-STAT pathway that involves stimulation of transcription factors and synthesis of proteins could be responsible for the inhibition of secretion.

We showed in this study that IFN-γ decreases cultured goblet cell proliferation. This conclusion is supported by published results that associated increased IFN-γ levels with low numbers of goblet cells and increased apopotosis. [18,36] Interestingly, in some cells, IFN-γ stimulation led to JAK2-dependent transactivation of epidermal growth factor receptor (EGFR).[13] As activation of the EGFR is known to stimulate conjunctival goblet cell proliferation, it is unlikely that the JAK-STAT pathway plays a role in the IFN-γ blockade of cell proliferation. Blockade of goblet cell proliferation induced by IFN-γ could use another long-term mechanism or signaling pathway different from that used to decrease secretion.

A decrease in goblet cell proliferation could result from inhibition of the cell cycle or stimulation of apoptosis. When IFN-γ is present in the goblet cell environment, the proliferation of these cells is blocked (current study) and the

apoptotic processes begin, as demonstrated by Zhang et al.[18] IFN-γ has been involved in goblet cell apoptosis through both extrinsic and intrinsic apoptosis pathways.[18] The main organelles that regulate Ca^{2+} homeostasis are also the main sites of apoptotic regulation.[37] Thus, there could be an interaction between apoptotic and other Ca^{2+} pathways that explain the long-term effect on IFN-γ on proliferation.

In previous studies, rat conjunctival goblet cells were found to behave in a similar manner as human goblet cells.[38-40] Similarly, in the current study in both rat and human goblet cells, IFN-γ blocked carbachol-mediated increase in $[Ca^{2+}]_i$ and secretion in both species. Thus rat conjunctival goblet cells are an excellent model for human goblet cells, especially considering the difficulty in obtaining human conjunctiva.

The main limitation of using primary cultures is the high variability between experiments. We observed that especially in calcium experiments, where only a few cells are analyzed, increasing the risk of higher variation. In addition, in the case of human cells, that variability may be increased due to the use of cells from both male and females, as well as from elderly donors. However, even with those limitations, we found several significant effects.

We conclude that IFN-γ affects multiple processes that control the amount of mucin produced by goblet cells. IFN-γ itself decreases goblet cell number by blocking proliferation and potentially by stimulating apoptosis. In addition, we have shown that the normal goblet cell increase in $[Ca^{2+}]_i$ and mucin secretion, usually mediated by cholinergic agonists, is blocked by this Th1 cytokine. Taking all these data together, we conclude that the presence of IFN-γ could explain the mucin deficiency typically found in dry eye disease [6] since goblet cell number is diminished and the secretion of the remaining cells is blocked.

METHODS

Materials

RPMI-1640 cell culture medium, penicillin/streptomycin, and L-glutamine were purchased from Lonza (Walkerville, IL). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA).

Antibodies against CK4, CK7, and MUC5AC were from Abcam (Cambridge, MA). The antibody against IFN-γ receptor was from Novus Biologicals (Littleton, CO). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA). Lectins UEA-1 and HPA were from Sigma-Aldrich (St. Louis, MO).

Primers for human IFN-γ receptor were from OriGene Technologies, Inc. (Rockville, MD), and those for rat were from SABioscience-Qiagen (Frederick, MD). The Superscript First-Strand Synthesis system for reverse transcription polymerase chain reaction (RT-PCR) was from Invitrogen, (Carlsbad, CA). SYBR Green PCR Master Mix was from Applied Biosystems (Carlsbad, CA).

Recombinant Rat IFN-γ and recombinant Human IFN-γ (carrier-free) were purchased from BioLegend Inc. (San Diego, CA). Carbachol was purchased from Sigma (St. Louis, MO). Fura-2/AM was from Life Technologies (Grand Island, NY). The 35mm Glass Bottom Culture Dishes were from MatTek Corporation (Ashland, MA). Thapsigargin and 2-APB were from Sigma-Aldrich, and LY294002 and U0126 were from Tocris Bioscience (Minneapolis, MN).

Enzyme-linked lectin assay (ELLA) was from Pierce Biotechnology (Rockford, IL) and Amplex Red was from Invitrogen. The Cell Counting Kit-8 was purchased from Dojindo Molecular Technologies (Gaithersburg, MD).

Animals

Male Sprague-Dawley rats between 4 and 5 weeks of age were obtained from Taconic Farms (Germantown, NY). All experiments followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Schepens Eye Research Institute Animal Care and Use Committee. Rats were anesthetized with CO_2 for 2 min and then euthanized by decapitation. Forniceal and bulbar conjunctival tissues were removed from both eyes.

Human Tissue

Human conjunctival tissues were obtained from Heartland Lions Eye Bank (Columbia, MO) and Michigan Eye Bank (Ann Arbor, MI). This study adhered to the Tenets of the Declaration of Helsinki and was approved by the Schepens Eye Research Institute Human Studies Internal Review Board. Received tissues were normal bulbar and forniceal conjunctival tissues.

Cell Culture

Tissue samples were obtained from 29 rats and from 12 human donors. Rat and human conjunctival goblet cells were grown in organ culture as previously described. [38,41] Briefly, conjunctival tissue was carefully minced into small pieces, and placed in six-well plates. When cell outgrowth was observed, tissue explants were removed. Conjunctival cells were cultured from every sample, although goblet cells were not obtained from each piece. As early as 24 h after establishment of organ culture, cell outgrowth from the explant was observed. Cells were fed with RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and 100 µg/ml penicillin-streptomycin. Cells were maintained at 37º C in 5% CO_2, and the medium was changed every other day. After 7-10 days, cells were trypsinized and passaged. Cells in passage 1 were used for all experiments.

Immunofluorescence microscopy

First-passage cultured cells were grown on glass cover slips and fixed in methanol or formaldehyde. To confirm that the cultured cells were goblet cells, these cells were stained with antibody against CK7, CK4, MUC5AC, and the lectins UEA-1 conjugated to FITC or HPA conjugated to tetramethylrhodamine (TRITC). Cells were incubated for 2 h with a blocking solution. Thereafter, primary antibody anti-CK7, anti-CK4, or anti-MUC5AC at 1:100 dilution was added. After one hour, cover slips were washed with PBS, and then secondary antibody conjugated with Cy2 or Cy3 (at 1:200 dilution), and UEA-1 or HPA (at 1:500 dilution) were added for one hour. To detect cell nuclei, 6-diamidino-2-phenylindole (DAPI) was added to the mounting medium. The same protocol was used to determine the presence of IFN-γ receptor, using an anti-IFN-γ-R antibody. Negative controls included the omission of primary antibodies. Specificity of primary antibodies and lectins had been previously tested. Cells were viewed by fluorescence microscopy (Eclipse E80i, Nikon, Tokyo, Japan). Micrographs were taken with a digital camera (Spot, Diagnostic Instruments, Inc, Sterling Heights, MI).

RNA Isolation and Real Time Reverse Transcript PCR (RT^2-PCR)

Briefly, RNA was extracted with TRIzol and total RNA was isolated according to manufacturer's instructions. One microgram of purified total RNA was

used for complementary DNA (cDNA) synthesis using the Superscript First-Strand Synthesis system for RT-PCR.

RT^2-PCR reaction was performed with 10 ng cDNA, 1 µl primers (**Table 1**) and 10 µl SYBR Green PCR Master Mix in a final volume of 20 µl. Conditions of the PCR reaction were: denaturation at 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 60s. After the 40 cycles there was a final cycle of 95°C for 90 s. All reactions were performed in duplicate. The levels of GAPDH for each sample were used as endogenous controls. Non template controls (NTC) included the omission of cDNA. To assure the specificity of the PCR products, a melting curve analysis was performed. For the posterior analysis of mRNA expression levels, the $2^{-\Delta\Delta Ct}$ method was used. [42]

Table 1. Primers sequences

Primer	Primer sequence/Reference	Source
GAPDH	Sense: 5'-GAACGTGAAGGTCGGAGTCAAC-3' Antisense: 5'-CGTGAAGATGGTGATGGGATTTC-3'	Sigma-Aldrich (St.Louis, MO)
MUC5AC	Sense: 5'-CCCACAGAACCCAGTACAA -3' Antisense: 5'-AATGTGTAGCCCTCGTCT -3'	Sigma-Aldrich
Human IFN-γ-R1	Ref: HP200396	OriGene Technologies, Inc. (Rockville, MD)
Rat IFN-γ-R1	Ref. PPR06409A	Qiagen (Frederick, MD)

Measurement of $[Ca^{2+}]_i$

First-passage cultured goblet cells were grown on 35 mm glass-bottom culture dishes for 1 day. Cells were then incubated in KRB buffer (containing 120 mM NaCl, 25 mM $NaHCO_3$, 10 mM HEPES, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1 mM $CaCl_2$) with 0.5% BSA, 8 µM pluronic acid F127, 250 µM sulfinpyrazone, and 0.5 µM of Fura-2/AM for 1 h at 37º C. Fura-2/AM is a fluorescent molecule that indicates the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). After incubation, cells were washed with KRB buffer containing sulfinpyrazone, and the dishes were observed using a Ca^{2+} imagining system, InCyt Im2 (Intracellular Imaging, Cincinnati, OH). This system allows measuring the ratio of Fura-2 using excitation

wavelengths of 340 and 380 nm, and an emission wavelength of 505 nm. A mean of 10 cells per dish was selected, and $[Ca^{2+}]_i$ was measured in each individual cell. A basal reading was done for at least 15 seconds before addition of agonists or inhibitors. Data are presented as the change in peak $[Ca^{2+}]_i$, that was calculated by subtracting the basal value from the $[Ca^{2+}]_i$ peak.

High Molecular Weight Glycoconjugate Secretion

For secretion assays, first-passage goblet cells were cultured in 24-well plates and grown to confluence. After serum starving for 24 h, cells were incubated with buffer alone (basal), carbachol (10^{-4} M), or IFN-γ (3 ng/ml) for 24 h in serum-free RPMI 1640 supplemented with 0.5% bovine serum albumin. Goblet cell secretion was measured using ELLA. UEA-1 lectin conjugated to horseradish peroxidase was used to detect high molecular weight glycoconjugates, including the mucin MUC5AC produced by rat and human goblet cells. After incubation, the culture medium was collected and the amount of lectin-detectable glycoconjugates was measured. After collection of supernatant for the ELLA, cells in the 24-well plate were removed and sonicated. The cell homogenate was analyzed for total amount of protein using the Bradford protein assay. Bovine submaxillary mucin was used for the standard curve.

To perform the ELLA, standards and supernatants were placed into 96-well microplates and dried overnight at 60º C. The manufacturer's protocol was followed. UEA-1 was detected using Amplex Red. In the presence of hydrogen peroxide, Amplex Red is oxidized producing a fluorescent molecule. Fluorescence was then quantified using a fluorescence ELISA reader (Bio-Tek, Winooski, VT), using 530 nm and 590 nm excitation and emission wavelengths, respectively. The amount of high molecular weight glycoconjugate secretion was normalized to total protein in the homogenate, and expressed as fold increase over basal. Basal value was set at 1.

Proliferation

Proliferation was measured using Cell Counting Kit-8. Briefly, cells were serum starved for 24 h, treated with IFN-γ (3 ng/ml), for 24 h, and then stimulated with carbachol (10^{-4} M) for 2 h. After stimulation, WST-8 product was added to the

wells and after a 45 min incubation, absorbance was read in a spectrophotometer, following manufacturer's instructions.

Data Presentation and Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM). Student's *t*-test was performed to analyze data and $p \leq 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS

The authors thank Dr. Wendell Scott, Heartland Lions Eye Bank, and Michigan Eye Bank for providing human conjunctival tissues.

This study was supported by NIH EY019470 Grant, FEDER-CICYT Grant MAT2010-20452-CO3-01, FPI Scholarship Program BES-2011-046381, and EEBB-I-12-05371 (Ministry of Economy and Competitiveness, Spain).

DISCLOSURE

No conflict of interest to declare.

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Chapter 2

ACQUIRING MATERIAL RESOURCES TO BUILD A NEW 3D MODEL OF HUMAN CONJUNCTIVA

¿Por qué repetir los errores antiguos habiendo tantos errores nuevos que cometer?

"*Why repeat the old errors, if there are so many new errors to commit?*"

— Bertrand Russell

Study 5

A new human primary epithelial cell culture model to study conjunctival inflammation

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Cornea

A New Human Primary Epithelial Cell Culture Model to Study Conjunctival Inflammation

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Submitted: July 19, 2013
Accepted: September 26, 2013

Citation: García-Posadas L, Arranz-Valsero I, López-García A, Soriano-Romaní L, Diebold Y. A new human primary epithelial cell culture model to study conjuctival inflammation. *Invest Ophthalmol Vis Sci.* 2013;54:7143–7152. DOI:10.1167/iovs.13-12866

Purpose. To develop a complete and optimized method to expand in culture human conjunctival epithelial cells from cadaveric donor samples.

Methods. Epithelial cells were obtained from cadaveric conjunctival tissue ($n = 47$). Preplating and differential trypsinization were optimized to eliminate stromal contamination. Epithelial cells were grown with five different media: control, epithelial growth factor (EGF)-enriched, H_2O_2-supplemented, fibroblast-conditioned, and human serum media. Adhesion, proliferation, colony forming efficiency (CFE), and percentage of $CK19^+$ and $Ki67^+$ cells were determined with the five different media. Cells were characterized by immunofluorescence and/or Western blotting techniques for the expression of CK4, CK7, CK19, MUC5AC, vimentin, FSP-1, Ki67, E-cadherin, and zonula occludens (ZO)-1 markers. In addition, cells were treated with TNF-α and levels of secreted IL-6 were measured by enzyme-linked immunosorbent assay.

Results. Pure epithelial cell cultures were obtained. Human serum medium showed the best properties in proliferation and CFE, while maintaining epithelial phenotype. Cells with this medium were passaged up to five times, although they maintained all epithelial characteristics only through passage 3. Cultured cells expressed epithelial markers, but not stromal ones. The number of $MUC5AC^+$ cells increased throughout the passages, whereas $Ki67^+$ cell numbers decreased. Cells in culture maintained adherens and tight junctions, and responded to TNF-α treatment by releasing more IL-6, showing that they can be used for inflammation assays.

Conclusions. We have developed a complete protocol to expand conjunctival epithelial cells from cadaveric tissue. This culture system responded to an inflammatory stimulus, so it could be used to develop a more complex in vitro model of inflammation.

Keywords: conjunctival epithelial cells, cell culture, cadaveric donor

Investigative Ophthalmology & Visual Science. 2013;54:7143-7152.

ESTUDIO 5

En los estudios que constituyen el Capítulo 1 de esta memoria se pudo ver que las líneas celulares no son adecuadas para realizar todo tipo de ensayos, ya que su fenotipo difiere notablemente del que expresan en realidad las células a las que representan. Es por ello, que trabajar con cultivos primarios resulta esencial. En el estudio 4 se utilizaron cultivos primarios de células caliciformes, siguiendo el protocolo publicado por el grupo de la Profesora Darlene A. Dartt, del *Schepens Eye Research Institute.*

Se ha considerado necesario desarrollar y optimizar algún protocolo que permita también tener cultivos primarios de otros dos tipos celulares conjuntivales: fibroblastos y células epiteliales no caliciformes. El desarrollo de dicho protocolo se describe en el estudio 7.

STUDY 5

The study 1 of this thesis report showed that cell lines are not appropriate to perform some type of studies, because their phenotype differs from the one of primary cells that originate them. For that reason, research done using primary cultures is essential. In the Study 4, primary goblet cell cultures were performed following the protocol published by Prof. Darlene A. Dartt group, from the Schepens Eye Research Institute.

We consider necessary to optimize a protocol to obtain primary cultures from conjunctival epithelial non-goblet cells and conjunctival fibroblasts. This study 7 presents the development of that protocol.

A New Human Primary Epithelial Cell Culture Model to Study Conjunctival Inflammation

ABSTRACT

Purpose. To develop a complete and optimized method to expand in culture human conjunctival epithelial cells from cadaveric donor samples.

Methods. Epithelial cells were obtained from cadaveric conjunctival tissue (n = 47). Preplating and differential trypsinization were optimized to eliminate stromal contamination. Epithelial cells were grown with five different media: control, epithelial growth factor (EGF)-enriched, H_2O_2-supplemented, fibroblast-conditioned, and human serum media. Adhesion, proliferation, colony forming efficiency (CFE), and percentage of $CK19^+$ and $Ki67^+$ cells were determined with the five different media. Cells were characterized by immunofluorescence and/or Western blotting techniques for the expression of CK4, CK7, CK19, MUC5AC, vimentin, FSP-1, Ki67, E-cadherin, and zonula occludens (ZO)-1 markers. In addition, cells were treated with TNF-α and levels of secreted IL-6 were measured by enzyme-linked immunosorbent assay.

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Conclusions. We have developed a complete protocol to expand conjunctival epithelial cells from cadaveric tissue. This culture system responded to an inflammatory stimulus, so it could be used to develop a more complex *in vitro* model of inflammation.

Keywords: conjunctival epithelial cells; cell culture; cadaveric donor

INTRODUCTION

The conjunctiva is the mucous membrane that covers the ocular surface from the limbus to the posterior surface of the eyelids. It is composed of stroma and epithelium, which consists of 2 to 7 layers of stratified squamous epithelial cells. In the more external layers, goblet cells, which are specialized epithelial cells, produce mucin.[1] Epithelial conjunctival cells are essential for maintaining a healthy ocular surface, having themselves an immunomodulatory role[2] that makes them an important target of study. To elucidate the effect of cytokines or drugs in the conjunctiva, a good *in vitro* system resembling the in situ epithelium is needed; however, a good model for this has not yet been developed.

Most of the reported *in vitro* studies of conjunctiva are done using established cell lines. To the best of our knowledge, the only human conjunctival cell lines that have been previously described are the Chang conjunctival cell line,[3] the Wong-Kilbourne derivative of Chang conjunctival epithelial cells (American Type Culture Collection 20.2, Manassas, VA), the IOBA-normal human conjunctiva (NHC) cell line,[4] and the ConjEp-1/p53DD/cdk4R/TERT (HCjE) cell line.[5] These lines have many advantages, such as availability and homogeneous results. However, in many ways they are not a fair representation of the original tissue.[6,7] This fact makes primary cell cultures a more valuable *in vitro* working tool.

Conjunctival primary cell cultures of different species have been established, such as rat,[8] rabbit,[9] and mouse and monkey,[10] among others. There are obvious interspecies differences, so it is not always possible to use them because the results cannot be readily extrapolated to the human situation.

The main source of human tissue for conjunctival cell culture is conjunctival biopsy,[6,11–13] which can be done for patients undergoing cataract or other scheduled surgeries.[14] Although the procedure of the biopsy is not very aggressive, some ethical concerns exist when doing biopsies for research alone, even if donors have signed informed consent releases. Additionally, tissue availability may be limited. For those reasons, the use of cadaveric tissues provides a promising alternative to biopsy tissues.

Independent from the source of tissues, epithelial primary cell cultures are very often established with the aide of feeder layers, usually murine 3T3 fibroblasts[15]

because they improve culture performance. However, this strategy has some risks, especially if the final destination of the culture cells is for transplantation.[16] Thus, the optimization of the culture procedure and the culture medium is the main concern when culturing epithelial cells without using feeder layers. Several supplements have been described to promote epithelial proliferation. The main one is epithelial growth factor (EGF),[17] but there are some others. For instance, Pan et al.[18] reported higher adhesion, migration, and proliferation levels in corneal cells when adding small amounts of H_2O_2 to the medium. Finally, the main source of growth factors is serum, and for that reason its inclusion in the culture system is an issue of great importance.

Notwithstanding some obvious problems such as elderly donors and the elapse of days between eye extraction and cell culture, the aim of this research was to establish an optimized protocol for expanding human conjunctival cells derived from cadaveric donors. Here, we demonstrate optimized procedures for isolating and culturing conjunctival epithelial cells from cadaveric tissue and efficiently expanding the cells in the absence of feeder layers. Our results provide a good *in vitro* model to deepen the knowledge of human conjunctiva and may eventually provide abundant cells for transplantation to save vision in diseased and traumatized eyes.

MATERIALS AND METHODS

Human Conjunctival Tissues

Healthy human conjunctival tissues were obtained from corneoscleral buttons from cadaveric donors (n = 47, from 40 donors). Fifty-seven and half percent of donors were male and 42.5% were female, with a mean age and SEM of 80.5 ± 1.23 years. The average interval between the death of the donor and harvesting of the conjunctival tissue was 2 to 3 days. Corneoscleral buttons were obtained with informed research consent from Barraquer Eye Bank of Barcelona (Spain). This study was in strict accordance with the tenets of the Declaration of Helsinki and Spanish Regulations concerning the use of human tissues for biomedical research, and had the approval of institutional review board of the University of Valladolid.

Human bulbar conjunctiva was carefully isolated from the rest of tissues, and used for research purposes.

Isolation and Culture of Conjunctival Cells

Human fibroblasts were obtained from conjunctival tissue. The epithelium was gently scraped, and then the underlying stroma was cut in small pieces (approximately 2 mm2/piece), that were plated in 12-well plastic plates (Nunc, Roskilde, Denmark). Fibroblast culture medium was composed by Dulbecco's modified Eagle's Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS), 2.5 µg/mL fungizone, and 5000 units/mL penicillin/streptomycin (all from Invitrogen, Inchinnan, UK).

Human conjunctival epithelial cells were obtained by disaggregation of the conjunctivas using either an enzymatic method or a combined enzymatic and mechanical method. Briefly, conjunctival tissue was incubated with dispase (1.2 U/mL; Invitrogen) for 2 hours, at 37°C in 5% CO2. After that, loosened epithelial cells were recovered with the help of a pipette. In the combined method, additional mechanical scraping was performed. The dispase solution containing the cells was then centrifuged for 5 minutes at 900 rpm. The recovered cells were left in 0.25% trypsin/EDTA composed by 2500 mg/L trypsin and 380 mg/L sodium EDTA (Invitrogen) for 5 minutes at 37°C, and then centrifuged and resuspended in five different culture media. The control medium was composed of DMEM/F12 supplemented with 2.5 µg/mL fungizone, 5000 units/mL penicillin/streptomycin, 1 µg/mL insulin, 0.5 µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO), 0.1 µg/mL cholera toxin (Gentaur, Brussels, Belgium), 2 ng/mL EGF, and 10% FBS (all from Invitrogen unless otherwise indicated). The four other media were based on the control medium, with the following variations: EGF-enriched medium contained 10 ng/mL EGF; H2O2-supplemented medium contained 20 µM hydrogen peroxide solution (Sigma-Aldrich); fibroblast-conditioned medium was prepared as described below (in the “Preparation of Fibroblast-Conditioned Medium” section); and human serum medium had 10% of human serum (Lonza Group Ltd., Basel, Switzerland), instead of FBS. In addition, epithelial cells were also obtained using the explant technique.[11] Conjunctival tissue was carefully cut in small pieces, and plated in 12-well plates, using the same culture media described above. Explants were fed by

superficial tension until cell growth was observed. After that, more culture medium was added.

Cells were maintained in standard conditions (humidified atmosphere of 5% CO_2 at 37°C), and the medium was changed every other day. Cell viability was evaluated with 0.4% trypan blue solution (Sigma-Aldrich).

Purification Techniques

To assure the purity of the epithelial culture, two combined techniques were used to eliminate contaminating fibroblasts: preplating and differential trypsinization with 0.25% trypsin/EDTA. Different times of preplating (1, 2, and 3 hours) and trypsinization (2, 5, and 10 minutes) were assayed for effectiveness in eliminating all contaminating stromal cells. To determine if cultured cells were epithelial or stromal cells, immunostaining for the epithelial marker cytokeratin (CK) 19 and against the stromal marker vimentin was performed.

Preparation of Fibroblast-Conditioned Medium

Fibroblasts isolated as described above were grown until confluence in 80-cm^2 flasks, and they were mitotically inactivated using 10 µg/mL mitomycin C (Sigma-Aldrich). Then, 15 mL control culture medium were added. After 24 hours, the medium was collected, centrifuged at 1000 rpm for 3 minutes, and the supernatant was frozen until used. This procedure was repeated for 7 days. Recovered media from the same flask were mixed and filtered through a 0.20-µm filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany).

Cell Adhesion Assays With Different Media

To evaluate adhesion, 20,000 cells/cm^2 were seeded in 96-well plates (Nunc) and allowed to adhere for 24 hours. After that, medium with nonattached cells was removed, and the recovered cells were counted in a hemocytometer (Bright-Line Hemacytometer, Sigma-Aldrich). Plating efficiency was calculated using the following equation:

$$Plating\ efficiency\ (\%) = \frac{100x(Nº\ of\ seeded\ cells - Nº\ of\ recovered\ cells)}{Nº\ of\ seeded\ cells}$$

Additionally, the DNA content of the attached cells after 24 hours was measured. After removing the media, cells were washed with PBS and Hoechst 33342 dye (Sigma-Aldrich) was added. After 3 minutes of incubation, cells were washed with PBS, and fluorescence was measured at 355 nm excitation and 465 nm emission wavelengths, using a fluorescence plate reader (SpectraMax M5; Molecular Devices, Sunnyvale, CA). That measure is proportional to the amount of DNA present in the sample. Three independent experiments were performed in triplicates.

Cell Proliferation Assays With Different Media

To evaluate proliferation, the Alamar Blue (R) colorimetric indicator assay (AbD Serotec, Oxford, UK) was used. Alamar Blue is a nontoxic fluorescent dye that does not affect viability or proliferation. For proliferation assays, 20,000 cells/cm^2 were seeded in 96-well plates. The Alamar Blue assay was performed at days 1, 2, 3, 4, 7, 11, and 14. Cells were incubated in medium with 10% vol/vol Alamar Blue for 4 hours. After incubation, that medium was recovered, and its fluorescence was measured at 560 nm excitation and 590 nm emission wavelengths, using the SpectraMax M5 fluorescence plate reader (Molecular Devices). Four independent experiments were performed in triplicates.

Colony Forming Efficiency

For the colony forming efficiency (CFE) assay, 500 cells were seeded in each well of a 6-well plates, with the five different culture media. After 7 days, the number of colonies with more than eight cells in each well was counted. Additionally, the colony size was measured by counting the number of cells in each colony.

Characterization of Conjunctival Cells by Immunocytochemistry

Fibroblasts and epithelial cells from the established conjunctival cultures (passages 1–3) were grown in 8-well multichamber Permanox slides (Nunc). When cells reached confluence, they were fixed with ice cold methanol. Immunocytochemistry for different epithelial cells (E-cadherin, CK19, and CK7), goblet cells (MUC5AC), stromal cells (vimentin and FSP-1), and proliferating cells (Ki67) markers (Table 1) was then performed on the fixed cells. For MUC5AC and Ki67 staining, cells were blocked in PBS with 4% donkey serum (Sigma-Aldrich) and

0.3% Triton X-100 (Sigma-Aldrich) for 1 hour at room temperature (RT). For MUC5AC, Ki67, and zonula occludens (ZO)-1, the slides were incubated with primary antibodies (Table 1) for 1 hour at 37°C. For E-cadherin, CK19, CK7, vimentin, and FSP-1, the slides were incubated with primary antibodies (Table 1) overnight at 4°C. Alexa Fluor-conjugated secondary antibodies were applied for 1 hour at RT. Cell nuclei were counterstained with Hoechst dye. The preparations were viewed under an epifluorescence microscope (Leica DMI 6000B; Leica Microsystems, Wetzlar, Germany). Negative controls included the omission of primary antibodies. All antibodies had been previously tested in our laboratory. The number of positive cells for each marker was counted in five different photographs from at least three independent experiments.

Characterization of Conjunctival Cells by Western Blotting

Cultured conjunctival epithelial cells from passages 1 to 5 and conjunctival fibroblasts were homogenized in ice cold radioimmunoprecipitation assay (RIPA) buffer supplemented with the protease inhibitors 100 µL/ml phenylmethylsulfonyl fluoride, 6 µL/ml aprotinin, and 100 nM sodium orthovanadate (all from Sigma-Aldrich). After incubation on ice for 30 minutes, samples were centrifuged at 14,000 rpm for 30 minutes at 4°C. The supernatants were collected, and the protein concentration was measured using the BCA assay (Pierce, Rockford, IL).

Proteins in each sample were separated by SDS-PAGE on 10% acrylamide gels (Bio-Rad Laboratories, Hercules, CA) according to the method of Laemmli.[19] The separated proteins were transferred to nitrocellulose membranes.[20] Membranes were blocked in Tris-buffered saline (TBS; Bio-Rad Laboratories) containing 0.05% Tween-20 BioXtra (Sigma-Aldrich), 5% milk, and 4% FBS for 1 hour at RT. Membranes were incubated with primary antibodies (CK19, CK4, CK7, MUC5AC, vimentin, E-cadherin, and ZO-1; Table 1) at 4°C overnight. Proteins recovered as described above from human colorectal adenocarcinoma HT29-MTX cells (a kind gift by Thécla Lesuffleur, PhD) served as positive controls for CK19 and MUC5AC expression.21 Then the membranes were washed with TBS and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno-Research Laboratories, Inc.) for 1 hour at RT. Immunoreactive bands were visualized by a chemiluminescence method using the ChemiDoc gel documentation system (Bio-Rad Laboratories), and images were analyzed with the Quantity One

software (Bio-Rad Laboratories). Quantitation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assured equal gel loading. Two independent experiments were performed.

Table 1. Antibody Sources and Concentrations

Antibody	Host and Type	Dilution for IMF	Dilution for WB	Code	Source
CK19	Mouse mo.	1:50	1:1000	MO888	Dako, Glostrup, Denmark
CK4	Mouse mo.	-	1:250	C-5176	Sigma-Aldrich
CK7	Mouse mo.	1:100	1:200	MS-1352-P0	ThermoScientific, Rockford, IL
MUC5AC	Mouse mo.	1:500	1:500	MAB-2011	EMD Millipore Corporation, Billerica, MA
Vimentin	Mouse mo.	1:500	1:200	SC-6260	Santa Cruz Biotechnology, Santa Cruz, CA
FSP-1	Mouse mo.	1:100	-	Ab11333	Abcam, Cambridge, UK
Ki67	Mouse mo.	1:50	-	M7240	Dako
E-cadherin	Mouse mo.	1:100	1:1000	610151	BD Bioscience, Franklin Lakes, NJ
ZO-1	Rabbit po.	1:50	1:250	617300	Invitrogen
AlexaFluor 448 anti-mouse IgG	Donkey	1:150	-	A21202	Invitrogen
AlexaFluor 647 anti-mouse IgG	Goat	1:200	-	A21236	Invitrogen
AlexaFluor 448 anti-rabbit IgG	Donkey	1:100	-	A21206	Invitrogen
Texas Red anti-mouse IgG	Donkey	-	1:5000	715-075-150	Jackson Immuno-Research Laboratories, Inc., West Grove, PA
Goat anti-rabbit IgG	Goat	-	1:2000	c-2004	Santa Cruz Biotechnology

mo., monoclonal; po., polyclonal.

In Vitro Inflammation Model

Cells from passages 0 to 3 were cultured in 24-well plates at a cell density of 25,000 cells/cm^2. When cells reached confluence they were stimulated with 25 ng/mL TNF-α (PeproTech, London, UK) for 24 hours. After that, supernatants were

collected, and levels of IL-6 were measured with an enzyme-linked immunosorbent assay (IL-6 ELISA; Diaclone, Besançon, France), following the manufacturer's instructions. Nonstimulated cells were used as controls for each experiment. Three independent experiments were performed for each cell passage.

Statistical Analysis

Statistical analyses were done using Statistical Procedures for the Social Sciences software (SPSS 15.0; SPSS Inc., Chicago, IL). Data were expressed as means ± SEM. Levene's test was used to evaluate the equality of variance. When it existed, a one-way ANOVA was performed, followed by pairwise comparisons (Tukey test). When there was no equality of variance, a robust test (Brown-Forsythe test) was performed, followed by pairwise comparisons with Games-Howell test. Differences were considered to be significant when P was less than or equal to 0.05.

RESULTS

Acquisition and Purification of Conjunctival Epithelial Cells

Epithelial cells were efficiently expanded from single cell suspensions and from explants. There were no significant differences in cell numbers obtained between the two disaggregation methods. However, recovered cell viability was significantly higher with the enzymatic method than with the combined enzymatic and mechanical one (P = 0.0024, Fig. 1A). Explants promoted more rapid expansion of cells. After 11 days, a mean of 98,707 ± 12,967 cells/explant were obtained. In addition, this technique was easier and faster to perform. For those reasons, expansion from explants was used for the rest of the studies, except for adhesion and colonies assays.

Purification of the cultures by either preplating or differential trypsinization produced epithelial cells with no contaminating fibroblasts. By discarding cells attached after preplating for 2 hours or by removing cells after 5 minutes of trypsinization, we obtained pure epithelial cell cultures (Fig. 1B).

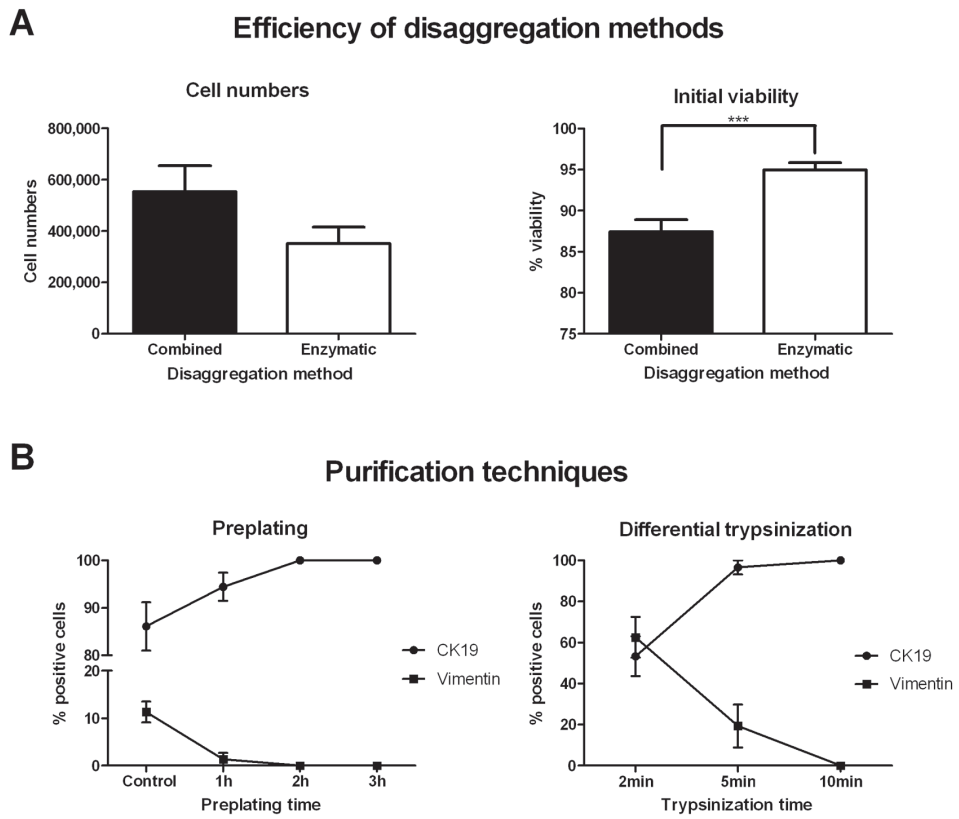

Figure 1. Establishment of epithelial cell culture. Two disaggregation methods were studied (A). Briefly, the enzymatic method consisted of a 2 hour incubation with dispase and 5 minutes incubation with trypsin/EDTA. The combined method included a mechanical scraping between both incubations. Viability of recovered cells was significantly higher with the enzymatic method (P = 0.0024) than with the combined one (n = 9). Two purification techniques were assayed (B). After 2 hours of preplating all the fibroblasts were attached. After 5 minutes of contact with trypsin, all the fibroblasts were detached, whereas most of the epithelial cells still remain attached to the culture surface (n = 3). ***P ≤ 0.005.

Evaluation of Five Different Cell Culture Media

To evaluate the suitability of the five different culture media in maintaining epithelial cell cultures, different parameters were analyzed: (1) cell adhesion to the plastic culture surface, (2) cell growth and proliferation, (3) colony-forming efficiency, and (4) maintenance of the epithelial phenotype (CK19+ cells) and proliferative capacity (Ki67+ cells).

Cell Adhesion

Cell adhesion was measured in passage 0 cells at 24 hours, using two different indicators: seeding efficiency and DNA content of attached cells (Fig. 2). Fibroblast-conditioned medium resulted in better promotion of cell attachment. Seeding efficiency of cells cultured with this medium was significantly higher when compared with that of cells cultured with control (P = 0.023), EGF-enriched (P = 0.035), and human serum (P = 0.005) media. There were no significant differences in DNA content among the cells in the different media.

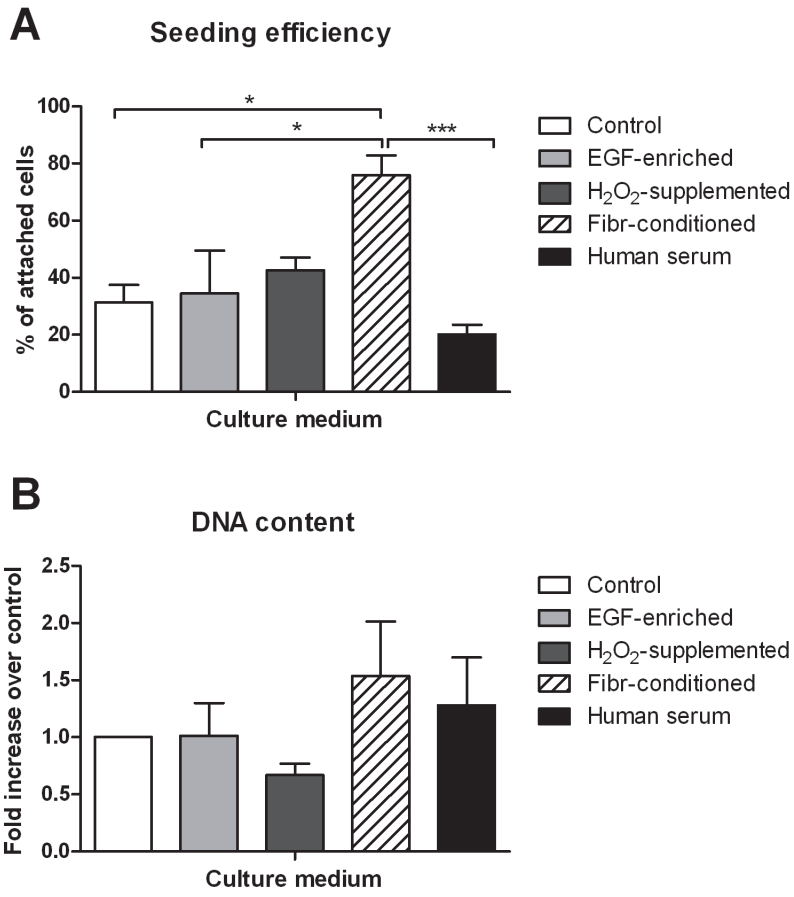

Figure 2. Cell adhesion. The suitability of five different culture media in promoting cellular attachment was measured in passage 0 cells with two procedures. (A) Seeding efficiency was higher in cells cultured with the fibroblast-conditioned medium than in those of the control (P = 0.023), EGF-enriched (P = 0.035), and human serum (P = 0.005) media. (B) No significant differences were found in the DNA content of attached cells cultured with the different media. *P ≤ 0.05; ***P ≤ 0.005.

Cell Proliferation

Cell proliferation with the five different media was assayed at different time points from days 1 to 14 (Fig. 3) in passage 1 cells. At day 11, proliferation rates with fibroblast-conditioned and human serum media were significantly greater than that of the control medium (P = 0.015 and P = 0.008, respectively). Cells cultured with H2O2-supplemented medium did not survive for 14 days. Cell proliferation rate was significantly higher with human serum medium after 14 days when compared with that of cells cultured with control (P < 0.001), EGF-enriched (P < 0.001), and fibroblast-conditioned (P = 0.002) media. Fibroblast-conditioned medium also allowed higher proliferation rates compared with control and EGF-enriched media (P < 0.001 and P < 0.001, respectively) at day 14.

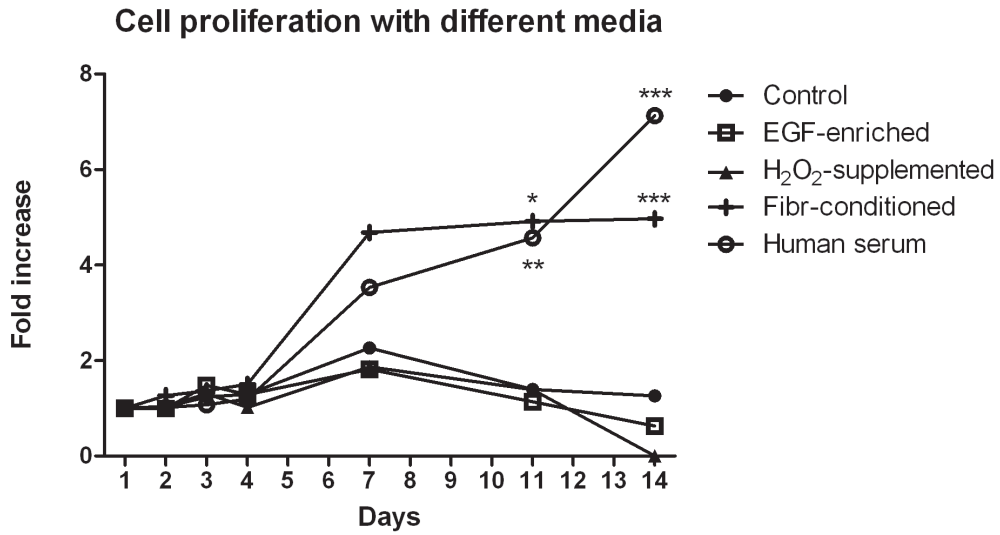

Figure 3. Cell proliferation. The effectiveness of different media in inducing cell proliferation was evaluated with Alamar Blue assay in passage 1 cultured cells. The highest proliferation rates were reached with the human serum medium, followed by the fibroblast-conditioned medium. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005, compared with control.

Colony Forming Efficiency and Colony Size.

Passage 0 cells grown in human serum medium showed the highest CFE (control, P = 0.049; H_2O_2-supplemented, P = 0.028; Fig. 4A). Furthermore, colonies formed using human serum medium were significantly larger than those formed using the four other culture media (control, P = 0.001; EGF-enriched, P = 0.001; H2O2-supplemented, P = 0.001; fibroblast-conditioned, P = 0.006; Fig. 4B).

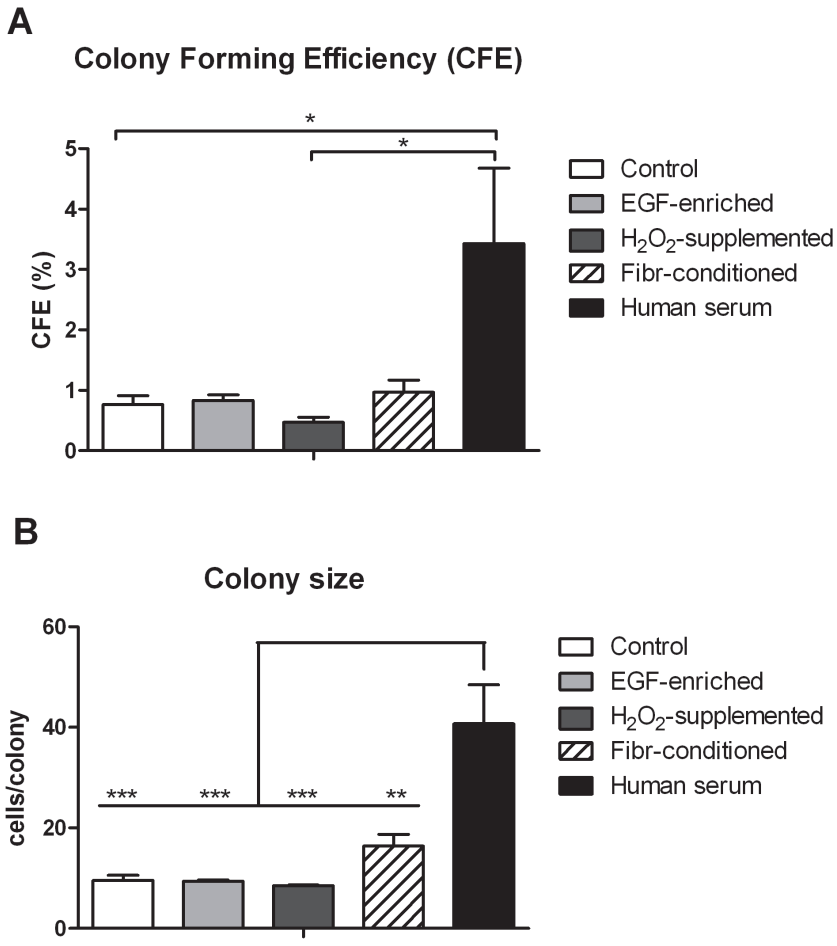

Figure 4. Colony forming efficiency and colony size. The number and size of colonies was measured in passage 0 cells cultured with the different media. Cells cultured in human serum medium showed the highest CFE (A). These colonies were also the largest ones (B). *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005.

Epithelial Phenotype and Proliferation Capacity

Cells from passage 1 grown in the different media were evaluated for CK19 expression (n = 3). There were no significant differences among the five media (Fig. 5). Evaluation of Ki67 expression (n = 3), a marker for proliferative capacity, was also performed. Fibroblast-conditioned and human serum media maintained the proliferative capacity of cultured cells significantly better than the control medium (P = 0.002 and P = 0.019, respectively; Fig. 5).

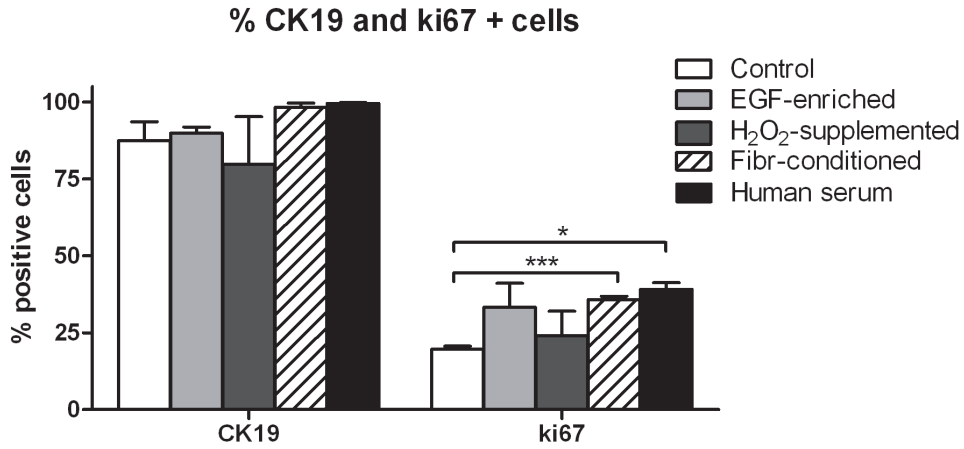

Figure 5. Percentage of positive cells for CK19 and Ki67. There were no significant differences in CK19+ cell numbers between cells cultured in the different media. Fibroblast-conditioned and human serum media showed a higher percentage of Ki67+ cells than the control medium. *P ≤ 0.05; ***P ≤ 0.005.

Characterization of Cells Grown in Human Serum Medium

As the human serum medium showed better results than the others, cells at different passages cultured in this medium were characterized by immunocytochemistry and Western blotting (Fig. 6). Cells from passages 1, 2, and 3 grown in human serum medium were evaluated by immunofluorescence assay for six different markers (Table 2). In addition, epithelial cells from passages 0, 1, 2, 3, and 5, and human conjunctival fibroblasts were also evaluated by Western blotting (Fig. 6). Cells from passages 0 to 3 were positive for epithelial markers CK4, CK19, and CK7, and negative for stromal markers vimentin and FSP-1, whereas the opposite was true for fibroblasts. Epithelial cells from passage 5 showed an intermediate phenotype characterized by some expression of both epithelial and

stromal markers and by different morphology. By immunofluorescence, some cells expressing MUC5AC were detected, but it was not detected by Western blotting. There was a significant increase in the amount of cells expressing MUC5AC from passage 1 to 3 (P = 0.05), and a decrease in the percentage of cells expressing Ki67 from passage 1 to 2 (P = 0.021) and passage 3 (P = 0.003).

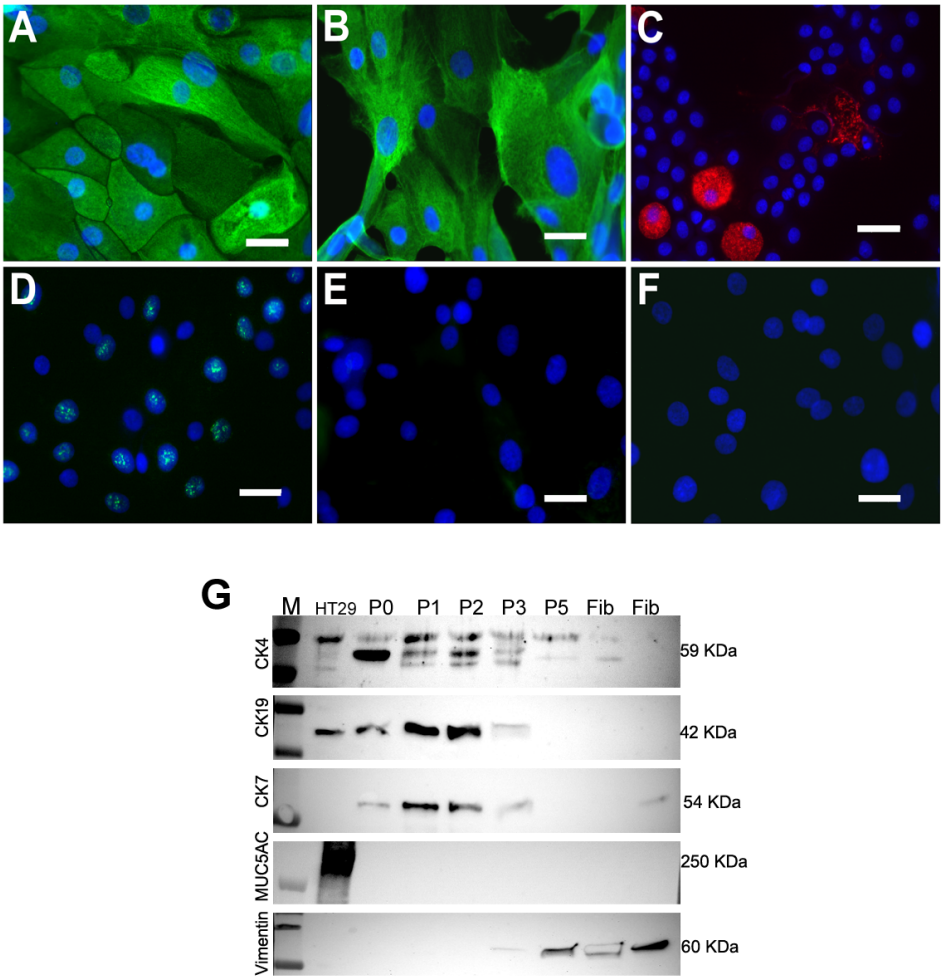

Figure 6. Characterization of cells cultured in human serum medium. Representative images of the different markers. All cells were positive for CK19 and CK7 ([A, B], respectively), whereas few MUC5AC-expressing cells were detected (C). Cells with proliferative capacity were identified by Ki67 staining (D). Cultured epithelial cells did not show staining for the stromal markers vimentin (E) or for FSP-1 (F). Scale bars: 30 μm (A–F). Also, some markers were evaluated by Western blotting (G). HT-29 cells were used as control for CK19 and MUC5AC. P0-P5: epithelial cells from passages 0 to 5. Fib: human conjunctival fibroblasts.

Table 2. Percentage of Positive Cells for Each Marker in Passages 1 Through 3

Marker	Specificity	% P1 + Cells	% P2 + Cells	% P3 + Cells
E-cadherin	Epithelium; adherens junctions	99.9 ± 0.1	99.57 ± 0.43	100 ± 0
CK19	Stratified epithelium	99.6 ± 0.23	100 ± 0	99.75 ± 0.2
CK7	Secretory epithelium	99.73 ± 0.14	99.9 ± 0.06	99.9 ± 0.08
MUC5AC	Mucin, goblet cells	0 ± 0	5.82 ± 2.91	8.82 ± 0.41
Vimentin	Stromal cells	8.72 ± 2.92	7.2 ± 2.75	18.05 ± 3.63
FSP-1	Fibroblasts	0.27 ± 0.27	0.32 ± 0.19	0.46 ± 0.38
Ki67	Proliferating cells	39.12 ± 2.16	27.36 ± 2.08	17.68 ± 1.99

Evaluation of Intercellular Junctions

Cells cultured in human serum medium expressed E-cadherin and ZO-1, which means that they maintained adherens and tight junctions, respectively (Fig. 7). Immunofluorescence assays confirm that both proteins were expressed in the plasma membranes between neighboring cells. In Western blots, immunoreactive bands appeared for each protein at the appropriate molecular weight (120 KDa for E-cadherin and 225 KDa for ZO-1). Both proteins were expressed in epithelial cells from passage 0 to 3. Epithelial cells in passage 5 and fibroblasts did not express either E-cadherin or ZO-1.

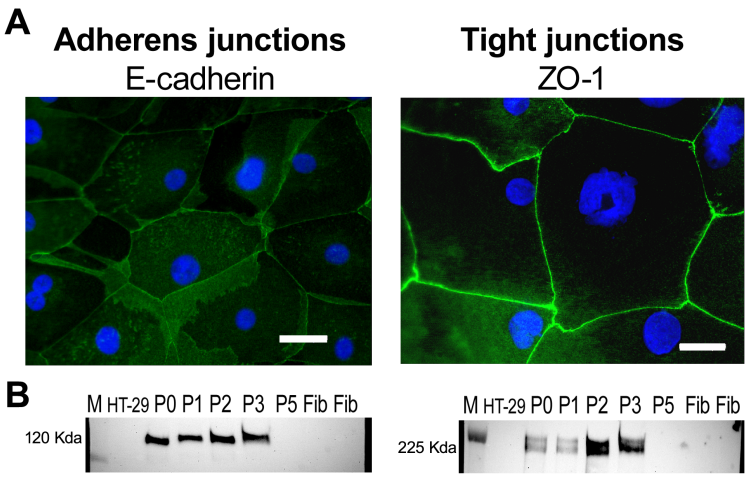

Figure 7. Adherens and tight junctions in cultured conjunctival epithelial cells. (A) Representative image of E-cadherin (left) and ZO-1 (right) expression in cultured cells. Scale bars: 30 μm. (B) The same proteins were also detected by Western blotting in epithelial cells from different passages.

In Vitro Inflammation Assay

Cells treated with TNF-α secreted higher amounts of IL-6 than non-stimulated cells (Fig. 8). This increase of IL-6 secretion in cultured cells was statistically significant in passages 0 ($P < 0.001$), 1 ($P = 0.009$), and 3 ($P < 0.001$). Cells in passage 2, also tended to secrete more IL-6, but the increase was not significant ($P = 0.089$).

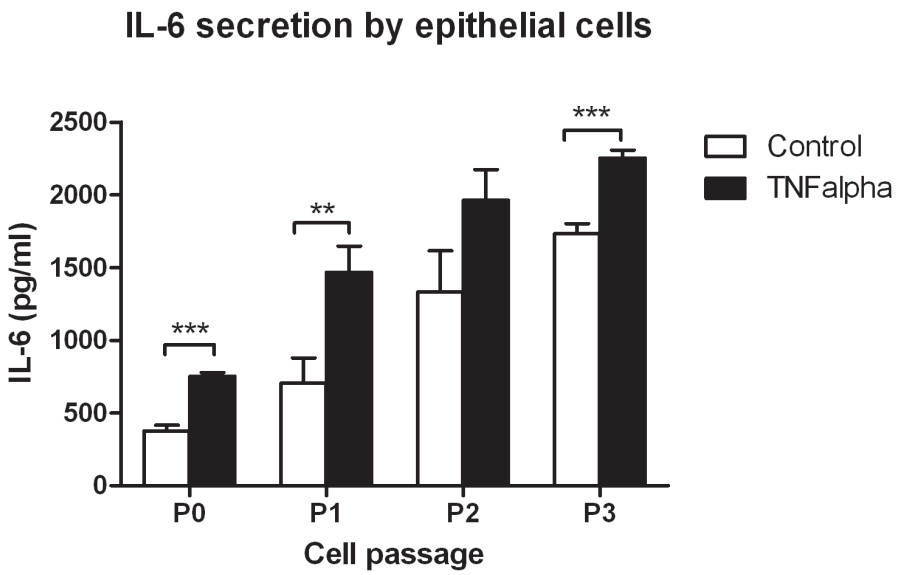

Figure 8. IL-6 secretion by epithelial cells treated with TNF-α. Treated cells from passages 0, 1, and 3 showed a significant increase in the secretion of IL-6 compared with untreated cells ($P < 0.001$, $P = 0.009$, and $P < 0.001$, respectively). **$P \leq 0.01$; ***$P \leq 0.005$.

DISCUSSION

In this study we developed an optimized method for expanding human conjunctival epithelial cells from cadaveric donors for at least 3 passages. Others have used cadaveric samples as a source for conjunctival primary cultures.[22] However, they pooled tissues from 8 to 10 eyes to produce enough cells for culture. In contrast, with our method we obtained enough cells from each eye to establish one culture per eye, obviating the need to pool samples from different eyes. Some other authors also used cadaveric samples, but they only required a small amount of

cells for their studies, so they did not optimize cell expansion nor did they establish long-term cultures.

One of the most common problems that researchers face when working with primary epithelial cell cultures is stromal cell contamination, which has huge implications.[23] This problem is not usually reflected in the literature, but it produces a high number of failed experiments, which means a waste of resources. We decided to try two combined techniques to eliminate fibroblasts from our epithelial cultures: preplating and differential trypsinization. Usually, researchers remove stromal cells from cultures by scraping away the contaminating cells. However, this procedure is quite subjective, and it is used only when the contamination by the stromal cells is big enough to be detected. The influence of the cocultured stromal cells, until they are removed, can alter some results.[24] Recently, it has been demonstrated that the effects of coculture can persist for weeks after epithelial cell isolation.[25] In addition, in some experiments a coculture situation can exist without knowing.

With our two simple strategies of preplating and trypsinization, we avoided the risk of fibroblast contamination. Neither procedure is based on morphologic evaluation; rather we used the inherent differential properties of epithelial and stromal cells, which make the procedures more objective. These procedures may result in the loss of some epithelial cells; however, the improved reliability of the outcomes achieved by almost completely eliminating the possibility of stromal contamination is worth it. Something similar happens with feeder layers. While we may have obtained higher efficiency rates using feeder layers, without them we avoided zoonosis and some other risks, such as transmission of prions and animal viruses.[16,26] Another important advantage of our culture procedure without feeder layers is the simplicity of it.

As the initial cell numbers are being limited with the purification techniques, and feeder layers are not being used, we consider the use of an optimized culture medium of paramount importance. We started by using the culture medium that maintains an immortalized conjunctival epithelial cell line.[4] With this medium as a starting point, the effect of different supplements and growth factors in growth and differentiation was evaluated. With EGF and H_2O_2 we did not obtain the expected results. Contrary to the description by Pan et al.[18] in corneal epithelial cells, even small amounts of H_2O_2 adversely affected the viability of our conjunctival epithelial cells.

Several studies have revealed the importance of the signals that cells receive in their native tissues. In mimicking the stem cell niche in culture, epithelial cells better maintain progenitor cell-like *in vivo* characteristics.[27] Most of the signals received by conjunctival epithelial cells come from conjunctival fibroblasts.[28] For that reason we used fibroblast-conditioned medium. As expected, the results with this medium were better compared with control medium. Proliferation rates and Ki67 levels were significantly higher, and there was an increase in cell attachments at 24 hours. These results are in accordance with a published report.[29] However, the best results came from human serum medium. Although cell adhesion with this medium was worse than with the control one, the promotion of growth as shown by Ki67 proliferation assay and the CFE was good enough to compensate for that inconvenience. These results are in contrast with some others.[30,31] With human serum, we not only obtained better results, but we also avoided the use of animal serum, such as FBS. Although FBS is widely accepted, when using cultured cells for tissue engineering or transplants it is better to avoid the use of animal products so as to reduce the risk of zoonosis and possible rejection.[16,30] Great efforts are being made to design a medium without animal products. Recently, Ang et al.[31] proposed the use of cord-blood serum as an alternative to adult human serum and FBS. They reached proliferation rates similar to those obtained with FBS, while their results were worse when using adult human serum. Although we think that cord blood is an interesting source of serum, its use in our cultures was not justified. Our results with human serum were very good and cord-blood serum availability is lower, and its price is higher than that of human serum. In addition, with this culture medium we could see that cells began to stratify when maintained in culture after reaching confluence (data not shown). Thus, human serum medium is the better choice for our purposes. Moreover, in the future it may be possible to use autologous serum to culture human cells for transplantation.

With the selected human serum culture medium, we analyzed different markers to determine the resulting phenotype of the cultured cells. Cells from passages 0 to 3 expressed all of the epithelial markers analyzed, and they did not show stromal characteristics. However, a small percentage of cells positive for cytokeratins were also positive for vimentin, although immunofluorescence staining intensity was weak. The absence of detectable FSP-1 protein suggested that these cells were not fibroblasts. Some authors suggested that cells expressing CK19 and

vimentin at the same time could be epithelial stem cells.[32,33] Further studies with this population of cells could help to test this hypothesis.

Cells from passage 5 showed an intermediate morphology and phenotype between epithelial and stromal cells. A possible explanation for this observation is that the cells were undergoing an epithelial-mesenchymal transition,[34] since they began to express vimentin at the same time that E-cadherin expression was reduced. In our cells, we observed by immunofluorescence assay a small amount of MUC5AC that increased throughout the passages through passage 3. This could be due to an increase in cell differentiation that occurred concurrently with the decrease in cell proliferation shown by the number of cells expressing Ki67+. However, in contrast with immunofluorescence microscopy, MUC5AC was not detected by Western blotting. The small percentage of MUC5AC-producing cells was probably not enough to allow immunoblot detection. Also, the homogenization process to isolate the protein could damage the MUC5AC. These results indicate the importance of characterizing the cultures by both immunohistochemistry and Western blotting.

In situ, the conjunctiva functions as a barrier for small particles and microorganisms, although the conjunctival barrier is not as tight as corneal barrier.[35] The effectiveness of this barrier depends not only on mucous production, but also on intercellular junctions.[36] We showed that the cultured conjunctival epithelial cells maintained tight junctions (ZO-1) and adherens junctions (E-cadherin). These results indicate that the cultured cells could potentially act *in vitro* as a biological barrier.

Finally, we performed an *in vitro* inflammation assay to determine if the cultured cells were responsive to a well-known inflammatory stimulus. We analyzed the secretion of IL-6 because it is one of the most important molecules in conjunctival inflammation. Tumor necrosis factor-α is reported to induce IL-6 secretion by conjunctival epithelial cells *in vitro*.[37] The measured increase in IL-6 secretion by the TNF-α–treated cells in our culture system indicates that cultured cells respond to cytokines. We expected these results because TNF-α is one of the major stimuli for the secretion of IL-6. We observed an increased release of IL-6 while the cells passaged in culture. This may have been due to increased cellular differentiation (as suggested by increased numbers of MUC5AC-positive cells and decreased numbers of Ki67 positive cells) that could make them more sensitive to

several signals. Although the study of only one cytokine is not enough to represent the complexity of ocular surface inflammation, the ability of our cultured conjunctival epithelial cells to respond to TNF-α and secrete IL-6 opens the possibility of using them in other more complex inflammation assays.

Optimizing the culture of human conjunctival cells is an issue of great importance, not only to perform *in vitro* experiments, but also for tissue engineering. We have developed, to the best of our knowledge, the first complete and optimized protocol to expand human conjunctival cells from cadaveric donors. We have shown the feasibility of using this source of tissue for cell culture. Moreover, we have demonstrated that it is possible to subculture these cells up to three times without losing the unique characteristics of the native epithelia. We obtained good performances despite the disadvantages of this underestimated source of tissue. Our results suggest that an even higher efficiency could be reached in the future if small cell samples from living donors are used for further transplantation purposes.

ACKNOWLEDGMENTS

The authors thank Thécla Lesuffleur, from National Institutes of Health and Medical Research INSERM U843, Paris, France, for kindly providing the HT29MTX cell line.

Supported by European Regional Development Fund - Interministerial Commission on Science and Technology (FEDER-CICYT) Grant MAT2010-20452-C03-01 and Research Staff Training (FPI) Scholarship Program BES-2011-046381 (Ministry of Economy and Competitiveness), and by Regional Government of Castile and Leon Grant VA132A11-2. Additional financial support was provided by Biomedical Research Networking Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN).

Disclosure: L. García-Posadas, None; I. Arranz-Valsero, None; A. López-García, None; L. Soriano-Romaní, None; Y. Diebold, None

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Study 6

Selection of the appropriate scaffold to culture conjunctival cells

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Manuscript in preparation

ESTUDIO 6

Hasta el momento se ha demostrado cómo obtener los tres tipos celulares necesarios para el desarrollo de un modelo conjuntival completo: células epiteliales, células caliciformes y fibroblastos. Pero dicho modelo debe ser, necesariamente, tridimensional.

Para elaborar el modelo tridimensional se emplearán técnicas de ingeniería de tejidos. Además de las células, es necesario un andamiaje polimérico. Este estudio aborda el análisis de distintos biomateriales para su uso como andamiaje de células conjuntivales y concluye con la elección de uno que resulta adecuado para el cultivo de las células conjuntivales: las matrices de fibrina.

STUDY 6

The procedures to obtain the different cell types needed to develop a new and complete in vitro model of human conjunctiva have been shown until now. But our purpose is to achieve the construction of a three-dimensional model.

Tissue engineering techniques will be used to construct the three-dimensional model. Apart from cells, a polymeric scaffold is also needed. In this study, we address the analysis of several biomaterials to be used as scaffolds for conjunctival cells. The study finishes with the election of the most appropriate biomaterial in our hand: fibrin-based matrices.

Selection of the Appropriate Scaffold to Culture Conjunctival Cells

ABSTRACT

This study analyzed two formulations of gellan gum/spermidine hydrogels and fibrin-based matrices made with human plasma as scaffolds for human conjunctival cells.

The conjunctival epithelial cell line IOBA-NHC was used to study cell adhesion, proliferation, and viability to the different biomaterials.

Gellan gum hydrogels were more transparent than fibrin matrices, whereas fibrin matrices handling was easier. Conjunctival epithelial cells adhered better to the surface of fibrin matrices than to gellan gum hydrogels. The highest cell proliferation rate measured with alamarBlue® reagent was found when cells were cultured on the surface of fibrin matrices. To further investigate the negative results obtained in the gellan gum hydrogels, cell viability was measured with the *In Vitro* Toxicology assay, XTT based. With that experiment we could corroborate that gellan gum hydrogels were cytotoxic to conjunctival cells, because viability levels were under 25%, similar to the levels obtained with the toxicity positive control benzalkonium chloride (BAK).

In summary, in this study we found that gellan gum/spermidine hydrogels were not compatible with the studied cells. On the contrary, fibrin-based matrices allowed conjunctival epithelial cell growth not only on the surface of the matrices but also inside them. In conclusion, fibrin-based matrices can be use as scaffolds to culture conjunctival epithelial cells.

INTRODUCTION

The experimental systems that have been used to study conjunctiva are based in two-dimensional (2D) cultures. However, they do not recapitulate the three-dimensional (3D) organization of tissues. (Shamir and Ewald, 2014) Then, the information that can be extracted from 2D cultures is incomplete to understand the biology of the tissue. To develop a 3D model that recapitulates the human conjunctival tissue, a scaffold that represents the stroma is needed.

The term scaffold is used in regenerative medicine to describe the non-cellular component of the engineered tissue. It gives support to cells and adds a 3D structure. Usually, natural or synthetic biomaterials that are biocompatible and biodegradable are used as scaffolds. Some of the most promising scaffolds are hydrogels, which are 3D networks formed by highly hydrophilic polymers that imbibe great amounts of aqueous fluids.

In this research, two types of hydrogels were studied to determine their convenience to be used as scaffolds in an engineered conjunctiva: 1) hydrogels made from gellan gum and spermidine, and 2) fibrin-based matrices. Gellan gum is a polysaccharide produced by the bacteria *Sphingomonas elodea* (Prajapati et al., 2013). It is water-soluble, and is widely used as a food aditive. Spermidine is a natural polyamine, which exerts crucial roles in cell survival and anti-aging properties (Ramot et al., 2011; Minois, 2014). Fibrin is a natural protein present in the serum. When an injury occurs, thrombin converts fibrinogen to fibrin, forming a clot. Fibrin has been extensively used as a scaffold for tissue engineering (Ahmed et al., 2008).

The purpose of this study is to find an appropriate biomaterial to be used as a scaffold for human conjunctival cells.

METHODS

Gellan Gum/Spermidine Hydrogels

Two different formulations of gellan gum/spermidine hydrogels were obtained from the Department of Pharmacy and Pharmaceutical Technology,

Faculty of Pharmacy, University of Santiago de Compostela. Formulation 1 (F1) composition was 5 mg/ml gellan gum and 2 mg/ml spermidine. Formulation 2 (F2) composition was 5 mg/ml gellan gum, 2 mg/ml spermidine, 6 mg/ml condroitin sulphate, and 20 mg/ml albumin.

Fibrin Matrix Preparation

Fibrin matrices were prepared from human fresh frozen plasma that was obtained from Centro de Hemoterapia y Hemodonación de Castilla y León (CHEMCYL, Valladolid, Spain). Matrices were produced by mixing plasma (400 µl/ml) with 40 µl/ml tranexamic acid (Rottapharm, Valencia, Spain) and 40 µl/ml calcium chloride (Braun, Barcelona, Spain), all diluted in DMEM/F12 culture medium (Invitrogen-GIBCO, Inchinnan, UK).

Cell Culture

IOBA-NHC conjunctival cells (Diebold et al. 2003) from passages 70 to 75 were used in all the experiments. Cells were grown in DMEM/F-12 medium supplemented with 10% FBS, 5,000 U/ml penicillin, 5 mg/ml streptomycin, 2.5 µg/ml fungizone, 2 ng/ml human EGF, 1 µg/ml bovine insulin, hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), and 0.5 µg/ml (all from Invitrogen unless otherwise indicated). Cells were maintained at 37º C in a 5% CO_2-95% air atmosphere and the medium was changed every other day.

Biomaterials Analysis under Phase Contrast Microscope

The structure of the biomaterials and the morphology of the cells in contact with them was evaluated by phase contrast microscopy, using the Eclipse TS100 microscope from Nikon (Tokyo, Japan). Micrographs were taken every other day from day 1 to day 19.

Sample Processing and Staining

Gellan gum/spermidine hydrogels and fibrin matrices were washed with PBS and fixed with 4% formaldehyde. Fixed samples were processed and paraffin-embedded. Then, five µm sections were cut and collected in poly-L-lysine coated slides. Sections were then stained with hematoxylin and eosin (H/E) and viewed under the light microscope Eclipse TS100.

Cell Proliferation Measurement with the Cell Proliferation Assay Reagent alamarBlue®

To evaluate proliferation, the non-toxic fluorescence dye alamarBlue® (AbD Serotec, Oxford, UK) was used. Cells grown on the surface of gellan hydrogels or fibrin matrices were incubated in medium with 10% alamarBlue® for 6 hours. Then, the medium was recovered, and its fluorescence was measured at 560 nm excitation and 590 nm emission wavelengths, using the SpectraMax M5 fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). Cells grown in plastic were used as control. The fluorescence measure obtained for the control at 24 hours was set as 1. Then, fold increase was calculated.

Cell Viability Measurement with the *In Vitro* Toxicology Assay kit, XTT based

Viability of cells in contact with gellan gum hydrogels was evaluated with the XTT test (Sigma-Aldrich). This is a colorimetric assay based on the tetrazolium 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT). Cells were incubated with XTT diluted in fresh phenol red-free RPMI medium (Invitrogen-GIBCO) for 17 hours at 37º C. As a control of cytotoxicity 0.001% benzalkonium chloride (BAK) was used. After incubation, absorbance of each well was measured at 450 nm (reference wavelength: 620 nm). The absorbance value obtained is proportional to the number of viable cells.

Statistical Analysis

Data were shown as mean ± standard error of the mean (SEM). To analyze differences between means a Student *t test* was performed. Differences were considered to be significant when $p \leq 0.05$.

RESULTS

Macroscopic Evaluation

The macroscopic appearance of the different biomaterials differs notably. Both formulations of gellan gum/spermidine hydrogels were completely transparent, while fibrin matrix was opaque.

The manipulation was also very different depending on the composition. Gellan gum/spermidine hydrogels were hard to manipulate, because they broke easily. On the contrary, fibrin matrix could be holded with forceps without damaging it, as observed in Figure 1.

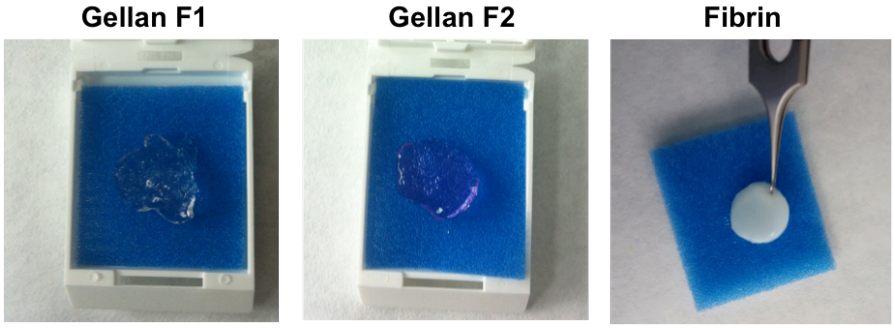

Figure 1. Photographs from the two formulations of gellan hydrogels and from a fibrin matrix. The transparency of gellan hydrogels can be observed.

Conjunctival Cells Adhered to Fibrin Matrices Better than to Gellan Gum/Spermidine Hydrogels

A small amount of the conjunctival epithelial cells added to gellam gum hydrogels were able to adhere to their surface, as seen in Figure 2. Most of the seeded cells showed rounded morphology and do not adhere to the surface of the hydrogels after 24 hours, as observed by phase contrast microscopy. On the contrary, cells seeded on to fibrin matrices showed good adhesion.

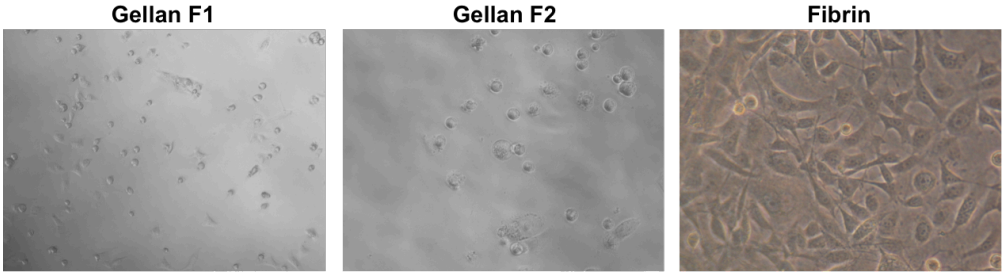

Figure 2. Rounded cells that are not attached can be seen in F1 and F2 gellan gum formulations micrographs (left and middle). Cells growing over a fibrin matrix are shown in the right micrograph.

Gellan gum/spermidine hydrogels and fibrin matrices were fixed, processed, and stained with hematoxylin/eosin to check if conjunctival cells were able to cover the surface of the materials.

In the two different formulations of gellan gum/spermidine hydrogels no cells were observed (Figure 3a, b). By contrast, conjunctival cells added above fibrin matrices (Figure 3c) or incorporated inside them (Figure 3d) remained viable and proliferate.

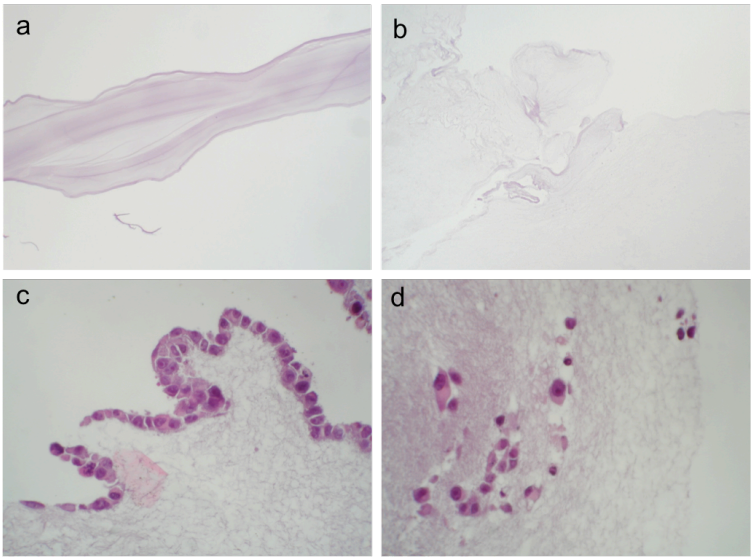

Figure 3. Sections of seeded gellan hydrogels (a, b) and fibrin matrices (c, d) stained with hematoxylin/eosin. No cells were observed in F1 (a) or F2 (b) hydrogels. Conjunctival cells grew on the surface of fibrin matrices (c) and inside them (d). Magnification: X10 (a, b), and X20 (c, d).

Cells Cultured on the Surface of Fibrin Matrices Showed the Highest Proliferation Rates

The proliferation rate of conjunctival epithelial cells grown on the surface of gellan gum hydrogels and fibrin matrices, and inside fibrine matrices was evaluated periodically up to 21 days. Cells grown on standard plastic cell culture surface were used as control. The two gellan gum hydrogel formulations studied showed similar results. A small increase in proliferation was observed until day 9. After that day, proliferation rate decreased and maintained lower levels up to day 21. However, that proliferation rate was always significantly lower than that of those cells grown in plastic (Figure 4).

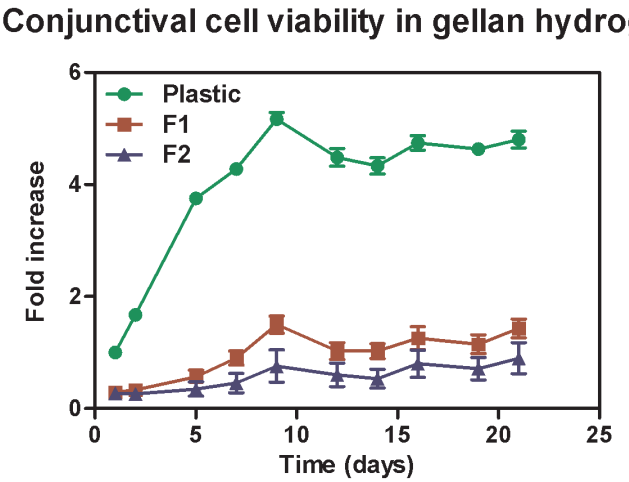

Figure 4. Cell proliferation rates were measured with alamarBlue® assay. Significantly lower levels were observed when the cells were cultured on the surface of gellan gum hydrogels (red and blue lines).

The proliferation in fibrin matrices was evaluated using the fibrin matrices in two different ways: as scaffolds (culturing the cells inside the matrix) and as supports (culturing the cells on the surface of the matrix). Conjunctival epithelial cells proliferate in both conditions, although the proliferation rates were higher when cells were grown on the surface of the matrices (Figure 5, blue line).

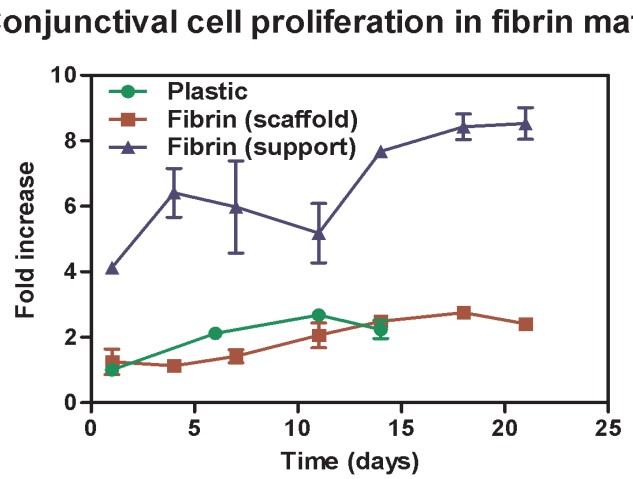

Figure 5. Cell proliferation rates were measured with alamarBlue® assay. Significantly higher levels were observed when the cells were cultured on the surface of fibrin matrices (blue line).

Gellan Gum/Spermidine Hydrogels Decreased Conjunctival Cell Viability

The low cell adhesion and cell proliferation rates obtained when conjunctival epithelial cells were seeded in gellan hydrogels could be due to cytotoxicity. To address this issue, cell viability was measured with the *in vitro* toxicology assay XTT. The values obtained for cells growing in plastic (positive control) were set as 1. The conjunctival epithelial cell viability percentage significantly decreased when cells were seeded on both gellan gum hydrogel formulations compared to that of cells seeded on plastic (Figure 6). Moreover, viability levels were similar to those obtained with BAK, the negative control.

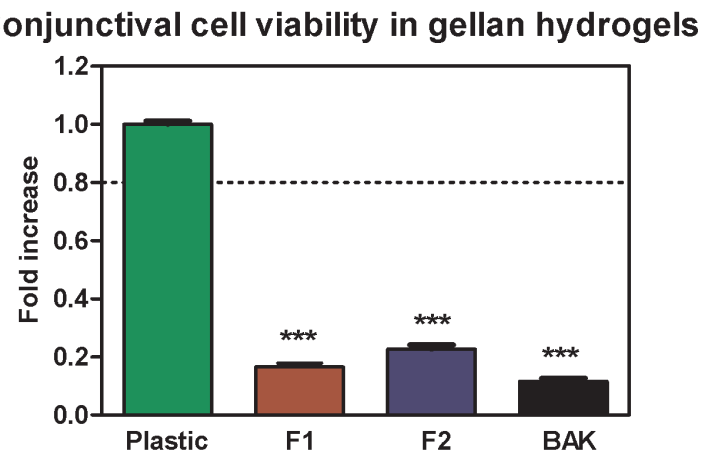

Figure 6. Cell viability measured with the XTT test. BAK, benzalkonium chloride.

DISCUSSION

The identification of a good scaffold is an important and difficult issue that must be set up before starting the development of an engineered tissue. In this research we analyzed the feasibility of two potential scaffolds for conjunctival modelling: gellan gum/spermidine hydrogels and fibrin matrices.

The epitheliall cell line IOBA-NHC derived from human conjunctiva was used to perform the first assays, because primary cultures are not always available.

Gellan gum hydrogels have been proposed for several clinical applications (Cencetti et al., 2011; Lee et al., 2012; Douglas et al., 2014), including drug delivery to the posterior segment of the eye (Chen et al., 2012; Duan et al., 2015). Despite the good results showed by other authors, we observed cytotoxicity and we were not able to maintain human conjunctival viable cells on the gellan gum/spermidine hydrogels. Therefore, we renounced to use these hydrogels as scaffolds for conjunctival cells.

On the contrary, fibrin matrices allowed conjunctival epithelial cell culture. IOBA-NHC cells attached to the matrices, and they were able to proliferate for 21 days on the surface of them. Moreover, cells on the surface of fibrin matrices exhibited the best proliferation rates, even higher than those in plastic. Fibrin has excellent properties that led researchers and clinicians to extend its use. It is routinely used as glue in different surgical procedures, including conjunctival grafts after pterygium excesion (Sandra et al., 2014; Malhotra et al., 2015). In addition, fibrin has also been used as a scaffold for different engineered tissues, such as dermis (De la Puente et al., 2011), cornea (Han et al., 2002; Alaminos et al., 2006), and even for rabbit conjunctiva (Safinaz et al., 2014). Therefore, our results are in agreement with published reports.

In summary, in this study we analyzed two types of biomaterials that could be used in conjunctival tissue engineering. Our results led us to conclude that fibrin matrices are a good scaffold to develop a 3D model of human conjunctiva.

ACKNOWLEDGEMENTS

This research was funded by CICYT Grant MAT2010-20452- C03-01 (Ministry of Science and Innovation, Spain).

Authors thank Javier Iglesias (CHEMCYL) for technical support with fibrin matrices preparation.

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Chapter 3

DEVELOPMENT OF A THREE-DIMENSIONAL MODEL OF HUMAN CONJUNCTIVA

"No vayas donde te lleve el camino,
ve donde no hay camino y deja un rastro"

"*Do not go where the path may lead,*
go instead where there is no path and leave a trail"

— Ralph Waldo Emerson

Study 7

Development of a three-dimensional model of normal human conjunctiva

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Manuscript in preparation

ESTUDIO 7

En el estudio anterior se analizaron distintos biomateriales para obtener uno que resultara adecuado para desarrollar un modelo tridimensional de conjuntiva humana. Previamente habían sido optimizados los protocolos de cultivo de las células a partir de las muestras disponibles. Por lo tanto, ya se dispone de todos los materiales necesarios para elaborar un modelo tridimensional: células y andamiaje.

En este estudio se lleva a cabo el desarrollo y la caracterización de un nuevo modelo tridimensional de conjuntiva humana, elaborado con células primarias y matrices biocompatibles preparadas a partir de fibrina humana.

STUDY 7

The previous study analyzed different biomaterials to select the most appropriate one to culture human conjunctival cells. Formerly, we developed the protocols to isolate and expand conjunctival cells from available sources. Therefore, the materials needed to develop a three-dimensional model, cells and scaffolds, are now available.

This study presents the development and characterization of a new three-dimensional model of human conjunctiva made with primary cells and biocompatible fibrin matrices.

Development of a Three-Dimensional Model of Normal Human Conjunctiva

ABSTRACT

Aim: The aim of this study was to develop a three-dimensional model of human conjunctiva that can be used to perform physiology and pathophysiology experiments within one week.

Methods: Fibrin-based matrices (derived from human plasma or cryoprecipitate) were used as scaffolds to develop the three-dimensional model. Cells were obtained from conjunctival tissue obtained from cadaveric donors. Immunofluorescent staining against CK19, HPA, and Ki67 was performed on the cells cultured in fibrin matrices. Proliferation was measured with alamarBlue® assay. The structure of the matrices and the morphology of the cells were studied with scanning electron microscopy (SEM).

Results: Fibrin-based matrices supported conjunctival cell growth. Epithelial cells grew on the surface of the scaffold and stratified in different layers, which numbers increased over time. Epithelial cells retained epithelial markers and some of them had mucins. Fibroblasts showed better proliferation rates on plasma matrices, whereas epithelial cells proliferated more on cryoprecipitate matrices. In addition, the number of Ki67 positive epithelial cells was higher in cryoprecipitate matrices. SEM micrographs showed that epithelial cells covered the surface of the scaffold and had microvilli. Fibroblasts were integrated in the scaffold and showed elongated shape.

Conclusions: A new three-dimensional model of human conjunctiva has been developed. The different results obtained with the two types of fibrin-based matrices showed that this model is versatile. The results also suggested that epithelial cells grown on the surface of fibrin scaffolds are polarized, indicating functionality.

INTRODUCTION

The conjunctiva is the mucous membrane that lines with the limbal cornea and covers the inner surface of the eyelids. Although this tissue has been less studied than the cornea, it is essential for the maintenance of the ocular surface.

The conjunctiva is in the cause and/or suffers the consequences of several pathologies, such as dry eye disease, Sjögren's syndrome, and allergic conjunctivitis, among others. These are high-prevalent diseases, what makes imperative its study.

However, there are not complete *in vitro* models to study human conjunctiva. *In vitro* studies are often performed in immortalized cell lines, what can be very useful for initial approaches. Nevertheless, researchers should be cautious when extrapolating these results. It has been demonstrated that cell line behavior can differs significantly from primary cells directly obtained from the tissue.

In addition to this limitation, *in vitro* models are usually cell monolayers that do not recapitulate the three-dimensional complexity of the native tissue. Consequently, there is a large gap between our knowledge of cellular processes and our incomplete understanding of the biology at the tissue level (Shamir y Ewald, 2014). The conjunctiva is comprised by the epithelium anchored to the underlying stroma. Therefore, a good model of conjunctiva should contain, at least, a three-dimensional scaffold representing the stroma with fibroblasts inside, and a stratified epithelium anchored to that scaffold. In addition, the epithelium should maintain mucus-secretion capacity.

Tissue engineering has provided the necessary knowledge to combine biomaterials and different cell types to produce tissue equivalents that can be used for research or transplantation. In the last years, some attempts to elaborate conjunctival equivalents have been reported, most of them focused to clinical use (Schrader et al., 2009; Zhou et al., 2014). There are a few three-dimensional models of conjunctiva that can be used to perform *in vitro* experiments, although not all of them include stroma and fibroblasts (Chung et al., 2007). One main disadvantage of these models is the time needed to obtain them. Usually, researchers must wait for at least three weeks to use them. This is time-consuming and often it is very expensive.

Different biomaterials have been used in tissue engineering, such as collagen, agarose, gelatin (Rosellini et al., 2007), or fibrin (Chung et al., 2015). Fibrin is a natural biopolymer that is derived from the plasma protein fibrinogen. It is biocompatible, degradable, and promotes cell adhesion, what makes it an excellent material for tissue engineering (Li et al., 2015).

Hence, the aim of this research is to develop a three-dimensional model of human conjunctiva that can be used within a week, using for that purpose a biocompatible scaffold based on fibrin, and primary cells obtained from human cadaveric conjunctival tissue.

MATERIALS AND METHODS

Human Conjunctival Tissues

Human conjunctival tissues were obtained from corneoscleral buttons from cadaveric donors (n = 11). Corneoscleral buttons were obtained with informed research consent from Barraquer Eye Bank of Barcelona (Spain). This study was in strict accordance with the tenets of the Declaration of Helsinki and Spanish Regulations concerning the use of human tissues for biomedical research, and had the approval of Institutional Review Board of the University of Valladolid. Human bulbar conjunctiva was carefully isolated from the rest of tissues, and used for research purposes.

Isolation and Culture of Conjunctival Cells

Conjunctival cells were cultured as previously described (García-Posadas et al., 2013). Briefly, conjunctival tissue was carefully cut in small pieces, and plated in 12-well plates. Fibroblasts and epithelial cells were isolated by preplating and differential trypsinization. Fibroblasts were cultured in supplemented DMEM/F12 (with 2.5 µg/ml fungizone, 5000 units/ml of penicillin/streptomycin, and 10% human serum), and used in passage 3. Epithelial cells culture medium was composed of DMEM/F12 supplemented with 2.5 µg/ml fungizone, 5000 units/ml of penicillin/streptomycin, 1 µg/ml insulin, 0.5 µg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA), 2 ng/ml EGF, and 10% human serum (Ref. 14-498E, Lonza Group Ltd., Basel, Switzerland), all from Invitrogen-GIBCO (Inchinnan, UK) unless

otherwise indicated. Primary epithelial cells at passage 1 were used for all the experiments.

Cells were maintained in standard conditions (humidified atmosphere of 5% CO_2 at 37ºC), and the medium was changed every other day.

Fibrin-Based Matrices Preparation

Fibrin-based matrices were used as scaffolds to develop the three-dimensional model. They were prepared from human fresh frozen plasma or cryoprecipitate, obtained from Centro de Hemoterapia y Hemodonación from Castille and Leon (CHEMCYL, Valladolid, Spain). Matrices were produced by mixing plasma (400 µl/ml) or cryoprecipitate (333 µl/ml) with 40 µl/ml tranexamic acid (Rottapharm, Valencia, Spain) and 40 µl/ml calcium chloride (Braun, Barcelona, Spain), all diluted in fibroblast culture medium (described in the previous section, "Isolation and culture of conjunctival cells").

Cell Seeding in Fibrin Scaffolds

Fibroblasts were incorporated inside the scaffolds by adding 100,000 cells/ml to the plasma or cryoprecipitate at the preparation time.

Epithelial cells were seeded on the surface of fibrin scaffolds 24 hours after fibrin polymerization, with a cell density of 100,000 cells/cm^2.

Histological Processing

At different time points, complete constructs of fibrin matrices with conjunctival cells were fixed with 4% formaldehyde after rinsing in PBS for three times. Fixed samples were processed and embedded in paraffin. Five µm sections were cut and collected in poly-L-lysine coated slides. Slides were kept at -80ºC until use. Sections were stained with hematoxylin and eosin (H/E) and viewed under the light microscope Eclipse TS100 (Nikon, Tokyo, Japan).

Immunofluorescence Assays

Paraffin slides were deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was performed in 0.01% trypsin for 10 minutes. Sections were rinsed in PBS and blocked with 4% donkey serum (Sigma-Aldrich) and 0.03% Triton X-100. A primary antibody against CK19 (Dako, Glostrup,

Denmark; 1:50 dilution) was applied overnight at 4º C, and a primary antibody against Ki67 (Abcam, Cambridge, UK; 1:50 dilution) was applied for 1 hour at 37º C. After washing 3 times with PBS, AlexaFluor® 488 or AlexaFluor® 647 secondary antibody (Invitrogen) were applied for 1 hour at room temperature.

To identify glycoconjugates, a lectin staining was used. The agglutinin obtained from *Helix pomatia* (HPA) was used at 1:500 dilution, for 30 minutes at room temperature. HPA lectin binds to high molecular weight glycoconjugates secreted by goblet cells. Nuclei were counterstained with Hoechst dye (Sigma-Aldrich) in all the slides. The preparations were viewed under an epifluorescence microscope (Leica DMI 6000B; Leica Microsystems, Wetzlar, Germany). Negative controls included the omission of primary antibodies.

Cell proliferation assays

To evaluate proliferation, the non-toxic fluorescence dye alamarBlue® (AbD Serotec, Oxford, UK) was used. The alamarBlue® assay was performed at days 3 and 7. Matrices were incubated in medium with 10% alamarBlue® for 6 hours. After incubation, that medium was recovered, and its fluorescence was measured at 560 nm excitation and 590 nm emission wavelengths, using the SpectraMax M5 fluorescence plate reader (Molecular Devices). Four independent experiments were performed.

Scanning Electron Microscopy

Fibrin-matrices were evaluated by scanning electron microscopy (SEM). Matrices were cut and fixed with 2.5% glutaraldehyde, dehydrated in a graded ethanol series, and dried in a critical point drier. Samples were then viewed in a scanning electron microscope ESEM QUANTA 200 FEG (FEI Company, Hillsboro, OR, USA).

Statistical Analysis

Statistical analysis was done using the Statistical Package for the Social Sciences software (SPSS 15.0, SPSS Inc., Chicago, IL, USA). Data were calculated as means ± standard error of the mean (SEM). To compare means a one-way analysis of variance (ANOVA) was done after assuring equality of variance (Levene's test). After that, pairwise comparisons (Tukey test) were performed. In the

absence of variance equality, a robust test was performed (Welch test), and the Games-Howell test was used for pairwise comparisons. Differences were considered to be significant when $p \leq 0.05$.

RESULTS

Fibrin-Based Matrices Supported Conjunctival Cell Growth

Fibrin matrices supported human conjunctival cell growth. Both fibroblasts and epithelial cells can be maintained in the scaffolds for at least 21 days, as observed in the hematoxylin/eosin staining (**Figure 1**). Conjunctival epithelial cells stratified on the surface of the scaffolds. The number of layers increased over time, ranging from 1-2 layers at day 3 to 5-6 layers at day 14.

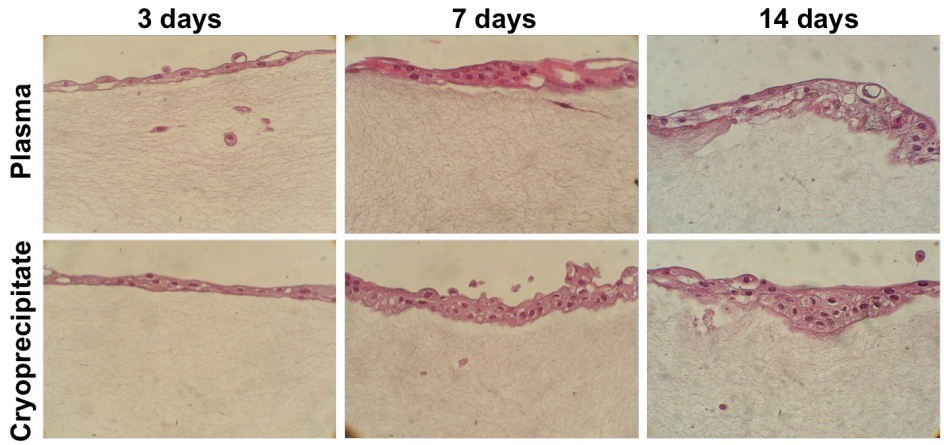

Figure 1. Micrographs showing conjunctival constructs stained with hematoxylin/eosin. The number of epithelial cell layer increased over time. Magnification: X20.

Epithelial Cells Maintained Epithelial Phenotype when Cultured on Fibrin Scaffolds

A problem that often occurs when culturing epithelial cells is epithelial-mesenchymal transition (EMT). To assure that the epithelial cells cultured in the matrices were not suffering EMT, the conjunctival epithelial marker CK19 was analyzed at different days. Cultured epithelial cells on both plasma and cryoprecipitate matrices expressed CK19 at all times (**Figure 2**).

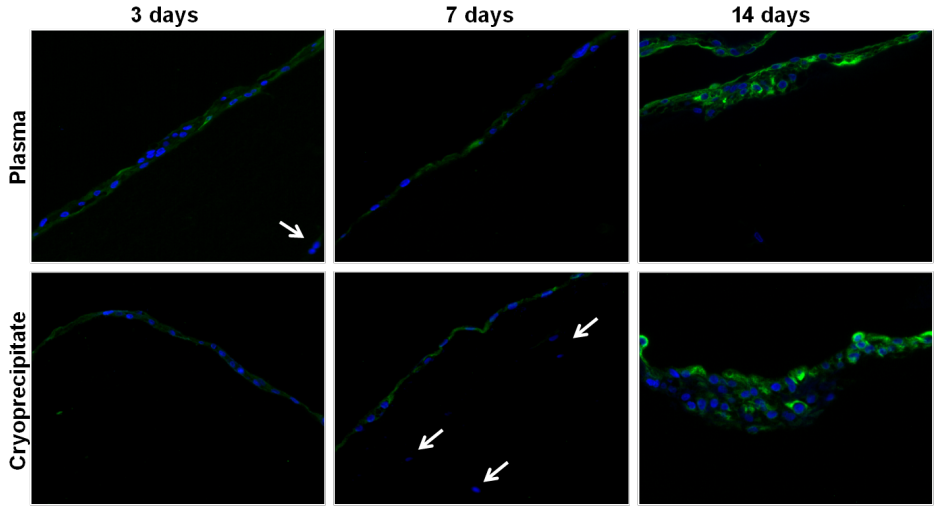

Figure 2. Representative microphotographs of CK19 staining. Epithelial cells maintained CK19 staining up to day 14. Fibroblasts that do not express CK19 are indicated by arrows. Magnification: X20

In addition, the presence of mucus-secretory cells was analyzed using the HPA lectin. HPA-stained cells were observed at days 3 and 7, but almost none at day 14 (**Figure 3**). This indicates that goblet-like cells are present in the scaffolds at least up to seven days.

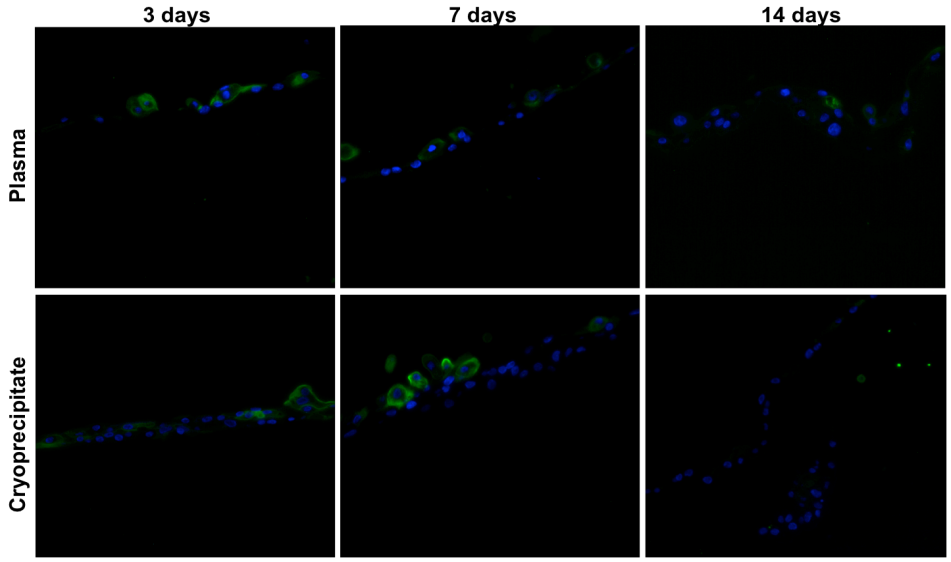

Figure 3. Representative microphotographs of HPA lectin staining. Cells in the matrices produce mucins at days 3 and 7, but they do not produce them at day 14. Magnification: X20.

Cell Proliferation in the Matrices

Conjunctival cells proliferate in the matrices, as shown by the alamarBlue® assay results (**Figure 4**). Epithelial cells showed better proliferation rates in cryoprecipitate matrices, whereas fibroblasts proliferated better in plasma scaffolds. When both cell types where analyzed together, no statistically significant differences were observed, what would suggest a crosstalk between both cell types to modulate each other proliferative behaviour.

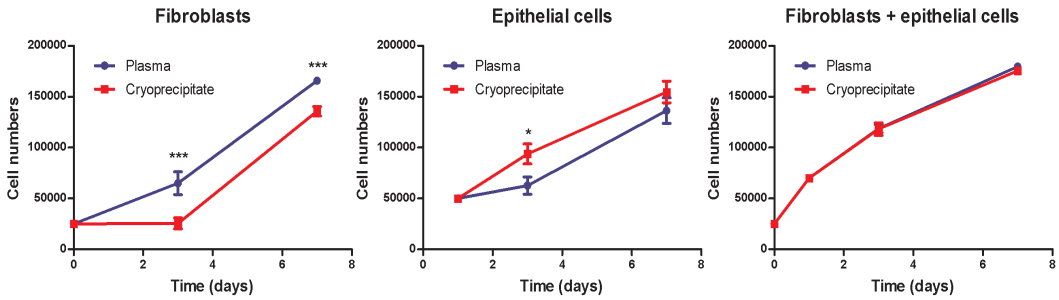

Figure 4. Conjunctival cells proliferate at different rates when seeded in plasma or cryoprecipitate matrices.

In addition, the expression of the proliferation marker Ki67 was analyzed in cell-seeded plasma and cryoprecipitate matrices to determine the proliferative capacity of epithelial cells at 3, 7, and 14 days. Several positive-stained cells were observed in both types of matrices (plasma and cryoprecipitate) at all studied times (**Figure 5**).

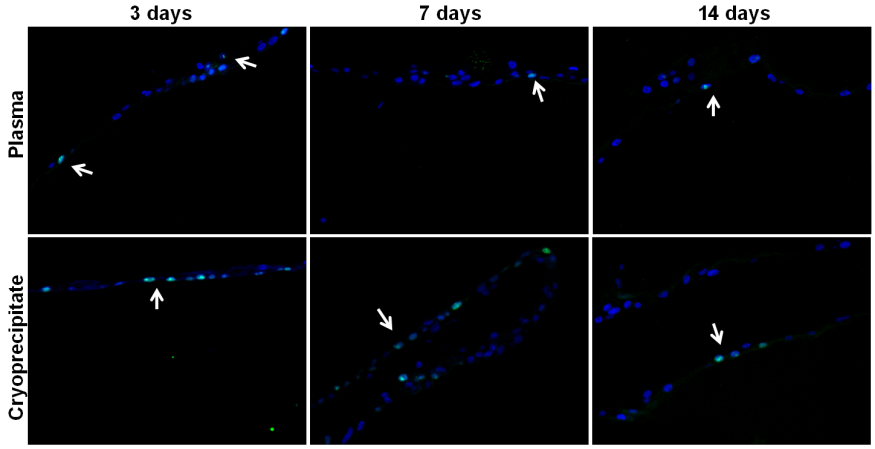

Figure 5. The proliferation marker Ki67 was studied in plasma and cryoprecipitate matrices. The number of cells that stained for Ki67 (arrows) decreased at 14 days. Magnification: X20.

However, significant differences were observed when the percentage (%) of positive cells in each condition was compared (**Figure 6**). The number of positive cells was significantly higher in cryoprecipitate matrices compared to plasma matrices at days 3 and 7 ($p = 0.012$ and $p = 0.023$, respectively). In cryoprecipitate scaffolds, a significant decreased in the percentage of positive cells was observed over time, from 38.97 ± 4.63 % at day 3 to 35.28 ± 7.92 % at day 7 and 10.96 ± 2.48 % at day 14 ($p = 0.015$ for day 3 and $p = 0.0004$ for day 7).

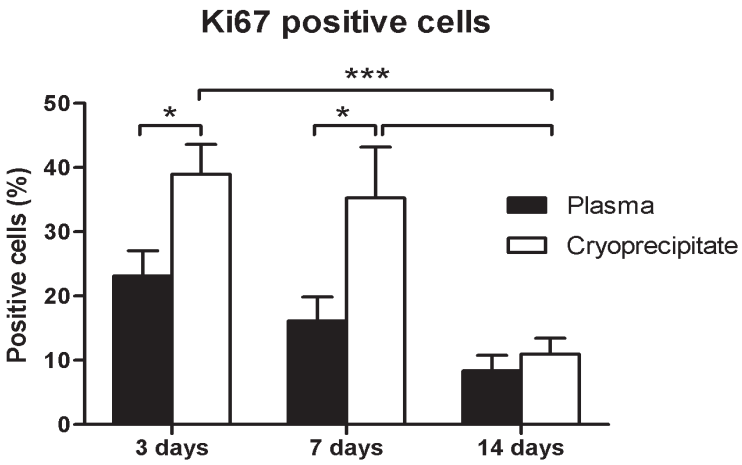

Figure 6. The % of positive cells for the proliferation marker Ki67 was higher in cryoprecipitate scaffolds than in plasma ones at days 3 and 7. That % significantly decreased after 14 days.

Epithelial Cells and Fibroblasts Maintained Cell Morphology in the Fibrin Scaffolds

The morphology of fibroblasts and epithelial cells incorporated to both plasma and cryoprecipitate scaffolds was estudied by SEM. Scaffold structure was similar in both cases, although slightly greater density of fibrils was observed in cryoprecipitate matrices compared to that of plasma matrices (Figure 7).

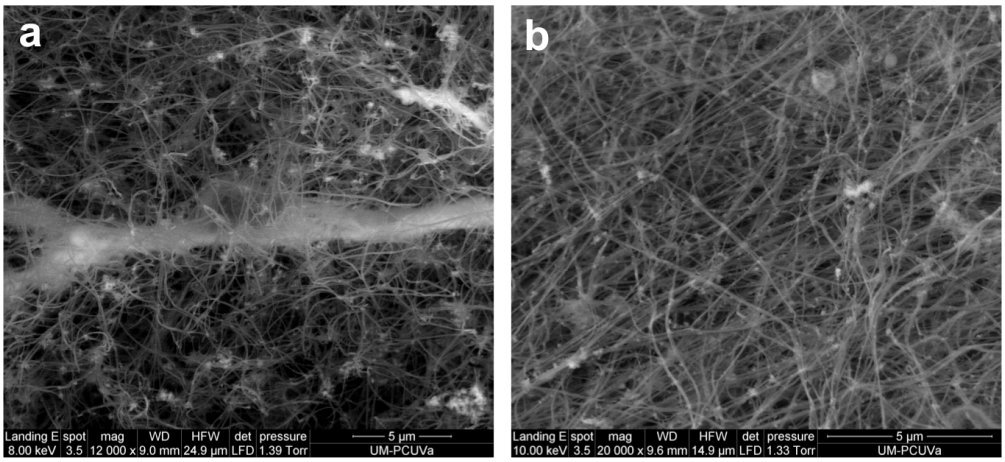

Figure 7. Plasma (a) and cryoprecipitate (b) scaffold structure. Higher densitiy of fibrils was observed in cryoprecipitate matrices (b). Magnification: X12,000 for a, and X20,000 for b.

Fibroblasts were evenly distributed inside the fibrin scaffolds and maintained their elongated shape in plasma (Figure 8a) and cryoprecipitate matrices (Figure 8b). Epithelial cells form layers in which polygonal shaped cells were identified in close contact with each other, in both plasma (Figure 8c) and cryoprecipitate matrices (Figure 8d). Also, microvilli were observed in the surface of epithelial cells cultured on the surface of both plasma and cryoprecipitate scaffolds. A detail of the microvilli is shown in Figure 9.

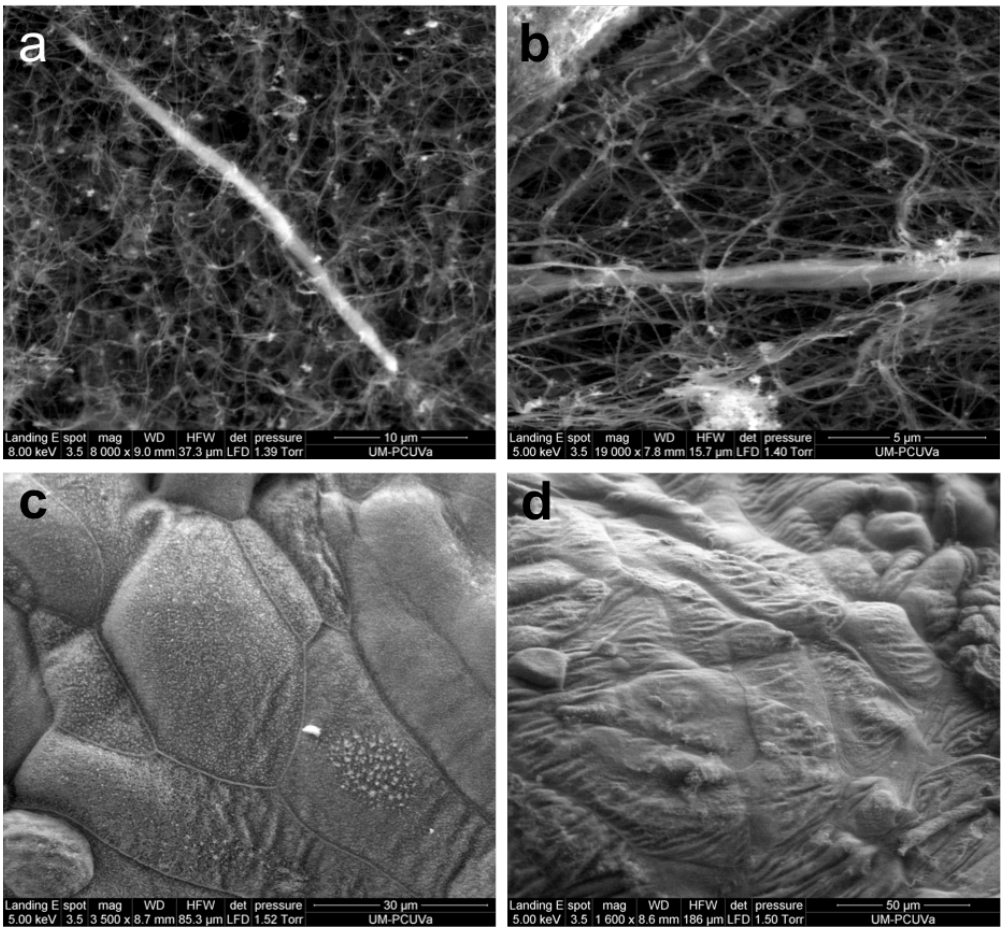

Figure 8. SEM micrographs showing fibroblasts (a, b) and epithelial cells (c, d) in plasma (a, c) and cryoprecipitate (b, d) conjunctival constructs. Fibroblasts are integrated in the scaffold, whereas epithelial cells are covering all the matrix surface. Magnification: X8,000 for a, X19,000 for b, X3,500 for c, X1,600 for d.

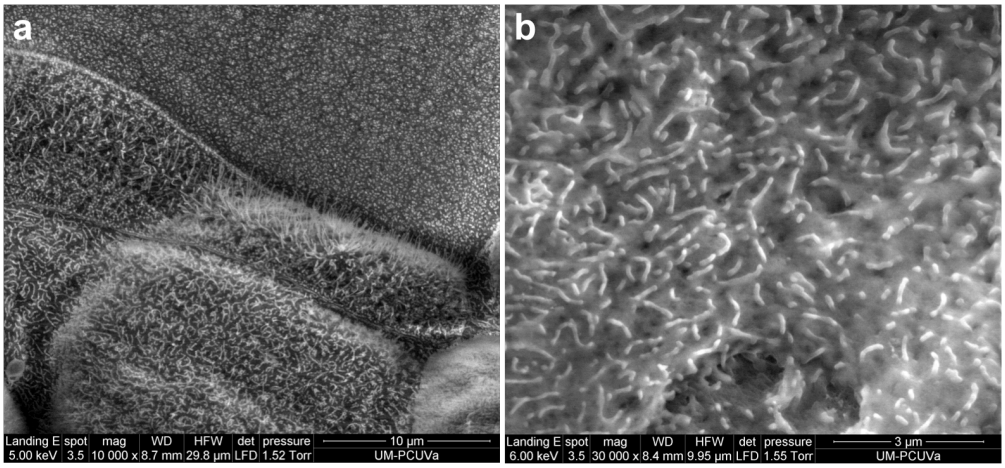

Figure 9. SEM microphotographs from the surface of the three-dimensional model. Epithelial cells were covered by microvilli (a). A detail of the microvilli at higher magnification (b). Magnification: X10,000 for a, and X30,000 for b.

DISCUSSION

In this study we have developed a new three-dimensional model of human conjunctiva using for that purpose a biocompatible scaffold made of fibrin and primary epithelial and stromal cells.

Conjunctival epithelial cells stratified on the surface of fibrin scaffolds. This is essential to mimic a normal conjunctival epithelium. Other authors have also described conjunctival primary cell stratification *in vitro* (Chung et al., 2007), but their model was limited to the epithelium.

The epithelial cells maintained CK19 staining up to 14 days, showing that the cells in the matrices keep their epithelial phenotype. This is important because EMT often occurs in cell culture. When this happens, cells are no longer actual epithelial cells and turn to a fibroblastic phenotype (Costa et al., 2015). Therefore, results obtained from cultures showing EMT are not completely reliable.

An important function of the conjunctiva is to secrete mucins that lubricate and protect the ocular surface. The specialized cells that secrete MUC5AC in the ocular surface are conjunctival goblet cells. These cells can be identified with HPA lectin that stained high molecular weight glycoconjugates contained in the goblet cell secretory products. In our constructs, we found some cells that stained with HPA at

days 3 and 7, in both plasma and cryoprecipitate matrices. However, no HPA staining was found at 14 days. There could be two explanations to this finding. It could happen that at 14 days goblet cells had secreted all their contents. Then, even if goblet cells are present in the culture, they cannot be identified with HPA lectin staining. The other possibility is that goblet cells that were present at 7 days died before 14 days. Goblet cells are difficult to culture, and for this reason it is plausible that they are not able to grow in the three-dimensional culture for a long time.

Although fibrin has been widely used as a scaffold in tissue engineering, to the best of our knowledge this is the first time that two types of fibrin matrices, derived from plasma or cryoprecipitate, are compared. Differences found between both matrices reveal how different is the behavior of stromal and epithelial cell types in contact with them. While epithelial cells grew better in cryoprecipitate matrices, fibroblasts did it better in plasma ones. The reasons of this differential performance can be different for each cell type.

Fibroblasts were incorporated inside the fibrin scaffolds. Then, the space that the fibrin fibrils let inside can be the main reason why fibroblasts proliferate better in plasma matrices. On the contrary, epithelial cells showed the best results in cryoprecipitate matrices. Cryoprecipitate contains more growth factors than plasma. Epithelial cells need many different nutrients to grow *in vitro*. This could be the reason why epithelial cells exhibited higher proliferation rates in cryoprecipitate matrices.

The morphological study performed by SEM revealed some interesting data. First, we could see that the fibroblasts maintained their elongated shape and interacted with the fibrin. The epithelial cells completely covered the matrix surface. We could clearly identify microvilli in the epithelial cells, what means that there is some polarization in the same way that happens in the native tissue. Cell polarity is essential for the function of epithelial tissues (Roignot et al., 2013), including the conjuntiva. Alteration in conjunctival epithelia microvilli was observed in several diseases, such as Sjögren's syndrome and graft versus host disease, among other pathologies (Mancel et al., 1993; Tatematsu et al., 2012).

In summary, we have developed a three-dimensional model of human conjunctival tissue that can be used within a week. In that period, epithelial cells seeded on the surface of the scaffolds proliferated and stratified in different layers, showing polarization and presence of goblet cell-like cells that can secrete mucins.

In addition, fibroblasts are maintained inside the scaffolds. Thus, we have a functional three-dimensional model that can be used to perform *in vitro* studies.

ACKNOWLEDGMENTS

This research was supported by FEDER-CICYT Grant MAT2013-47501-C2-1-R and FPI Scholarship Program BES-2011-046381 (Ministry of Economy and Competitiveness), and by Regional JCyL Grant VA132A11-2. Additional financial support was provided by CIBER-BBN.

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Study 8

Simulation of inflammatory diseases in a three-dimensional model of human conjunctiva

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Manuscript in preparation

ESTUDIO 8

Una vez desarrollado un modelo tridimensional de conjuntiva humana normal, el siguiente y último objetivo era lograr que dicho modelo sirviera para representar distintas enfermedades inflamatorias de la superficie ocular, así como para llevar a cabo estudios relacionados con el ácido hialurónico.

En este estudio se somete al modelo tridimensional a distintas condiciones para intentar simular parcialmente el síndrome de ojo seco y las alergias oculares. También se lleva a cabo un estudio de los receptores de ácido hialurónico.

STUDY 8

After developing the three-dimensional model of human conjunctiva, only the last objective of this thesis remained unconcluded. To address this objective, a last study was planned to test whether the three-dimensional construct developed is useful to study ocular surface inflammation.

In this last study, an in vitro simulation of an inflammatory microenvironment was done. The three-dimensional model of human conjunctiva is exposed to different conditions to simulate some of the main changes observed in ocular surface diseases, such as dry eye or allergic conjunctivitis. In addition, we analyze changes in hyaluronan receptor expression.

Simulation of Inflammatory Diseases in the Three-Dimensional Model of Human Conjunctiva

ABSTRACT

In this study, a proof of concept for the use of a three-dimensional model of human conjunctiva to study ocular surface inflammation is presented. The three-dimensional model was made with primary cells and fibrin matrices derived from cryoprecipitate. In this study, four different experimental conditions were analyzed: control, air-lifted culture, desiccation, and IL-13 treatment. Desiccation condition was used to mimic dry eye disease, and IL-13 treatment was intended to mimic allergy. Matrices were kept in culture for 7 days and after that they were processed and analyzed. Matrices were stained with hematoxylin/eosin to observe changes in morphology. Ki67 proliferation marker, high molecular weight glycoconjugates identified by HPA lectin, and hyaluronan receptors CD44 and RHAMM expression was analyzed by immunofluoresence. MUC5AC, IL-6, and sCD44 secretion was evaluated by ELISA. An increase in epithelial stratification was observed in air-lifted matrices compared to control. In addition, increased numbers of cells stained with HPA lectin and upregulated MUC5AC secretion was observed in that condition. Increased MUC5AC secretion was also found in constructs exposed to desiccating conditions or IL-13. Some Ki67 positive cells were observed in all conditions, althought the greater numbers were found in air-lifted condition. The inflammatory status of cells was evaluated by IL-6 levels, and those levels were found elevated in air-lifted and desiccating conditions, but not in IL-13 treated constructs. Finally, CD44 and RHAMM hyaluronan receptors were elevated in IL-13 matrices, and decreased in desiccating conditions. These results are in agreement with previous data obtained from patients with inflammatory diseases, such as atopic keratoconjunctivitis or rosacea. In conclusion, it is possible to use the three-dimensional model of human conjunctiva to study ocular surface inflammatory diseases.

INTRODUCTION

Inflammatory ocular surface diseases are very prevalent among global population. Patients demand more eficacious new treatments for their diseases and, at the same time, governments and pharmaceutical companies are worried about the cost of the research needed to develop new drugs (Bielory and Syed, 2013).

The conjunctiva is involved in the majority of inflammatory ocular surface diseases, playing an active role in the pathophisiology of those conditions. The conjunctiva has a conjunctival-associated lymphoid tissue (CALT) that is a key player in inflammation (Knop and Knop, 2010). But also, the most abundant cells in the conjunctiva, epithelial cells, including goblet cells, and fibroblasts, participate in these processes (Fukuda et al., 2006; Cavet et al., 2013; Dartt and Masli, 2014).

Current *in vitro* models to study conjunctival inflammation are usually composed of just one cell type that is cultured as a cell monolayer. These models do not recapitulate the complexity of the whole tissue, and interactions between different cells, and between cells and the extracellular matrix can not be studied.

In a previous study from our group (García-Posadas et al., manuscript in preparation), we engineered a three-dimensional model of normal human conjunctiva using fibrin-based scaffolds. Two different constructs were introduced, one using a plasma matrix and the other one using a cryoprecipitate matrix as scaffold. Epithelial cells showed better proliferation rates in cryoprecipitate scaffolds, and for that reason cryoprecipitate matrices were chosen to develop a three-dimensional model of inflammed conjunctiva.

There are several factors that induce inflammation in conjunctival tissue in the different diseases. In dry eye disease, desiccation is one condition that triggers the inflammatory response (Tost et al., 2012). In allergy, the presence of large amounts of some Th2-type cytokines, such as interleukin 13 (IL-13) (Leonardi et al., 2006), is the cause of some of the signs observed in allergic patients, such as mucus hypersecretion. For that reason, desiccation and IL-13 treatment were used as experimental conditions to try to mimic some of the features observed in conjunctival diseases and to develop a three-dimensional model of inflammed human conjunctiva.

MATERIALS AND METHODS

Conjunctival Cell Culture

Conjunctival cells were cultured as previously described (García-Posadas et al., 2013) from conjunctival tissues obtained from cadaveric donors (n = 8). Corneoscleral buttons were obtained with informed research consent from Barraquer Eye Bank of Barcelona (Spain). This study was in strict accordance with the tenets of the Declaration of Helsinki and Spanish Regulations concerning the use of human tissues for biomedical research, and had the approval of Institutional Review Board of the University of Valladolid.

Fibroblasts were grown inside the fibrin-based scaffolds. The cells were incorporated to them while they were prepared, adding 100,000 cells/ml to the cryoprecipitate. Epithelial cells were seeded on the surface of the scaffolds 24 hour after they were prepared, at a cell density of 100,000 cells/cm^2.

Fibrin Scaffold Preparation

Fibrin scaffolds were prepared from cryoprecipitate obtained from Centro de Hemoterapia y Hemodonación from Castille and Leon (CHEMCYL, Valladolid, Spain). Scaffolds were produced by mixing 333 µl/ml cryoprecipitate with 40 µl/ml tranexamic acid (Rottapharm, Valencia, Spain) and 40 µl/ml calcium chloride (Braun, Barcelona, Spain), all diluted in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) culture medium (Invitrogen, Inchinnan, UK).

Experimental Conditions

Four different experimental conditions were used in this study, as seen in Figure 1. Constructs cultured for 7 days in standard submerged conditions were used as controls. In addition, other constructs were cultured submerged for 3 days and then, 3 days in air-lifted conditions. To simulate some of the features found in dry eye patients, a desiccating condition was also used. To simulate desiccation, constructs were placed without medium in the culture hood for two hours. The existing flow in the hood desiccates the constructs. Finally, the last experimental condition that was analyzed in this study consisted in IL-13 treatment. Constructs were cultured in submerged conditions for 6 days, and in the seventh day they were incubated with 20 ng/ml IL-13 (PeproTech, London, UK) for 24 hours.

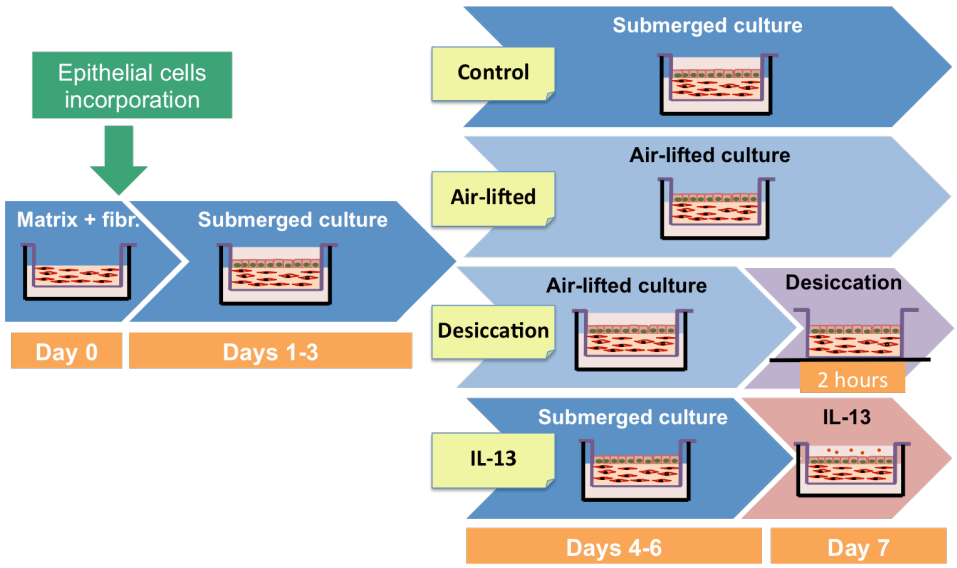

Figure 1. Schematic showing the protocol used in the different experimental conditions.

Histological Processing

At day 8, constructs exposed to each experimental condition were rinsed in PBS and fixed with 4% formaldehyde. Fixed matrices were processed and embedded in paraffin. Five µm sections were cut and collected in poly-L-lysine treated slides. Sections were then stained with hematoxylin and eosin (H/E) or kept at -80ºC until further use for immunoflourescent staining.

Immunofluorescent Staining

Paraffin slides were deparaffinized in xylene, and rehydrated in decreasing concentrations of ethanol. After antigen retrieval with 0.01% trypsin, sections were blocked in PBS with 4% donkey serum (Sigma-Aldrich, St. Louis, MO, USA) and 0.03% Triton X-100. Primary antibodies against Ki67 (Abcam, Cambridge, UK), CD44 (Calbiochem-Merck, Darmstad, Germany), and RHAMM (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied to the slide. After washing 3 times with PBS, AlexaFluor 488 secondary antibody (Invitrogen) was applied for 1 h at room temperature. For HPA lectin staining 1:500 dilution of HPA was used for 30 min at room temperature. Nuclei were counterstained with Hoechst dye (Sigma-Aldrich) in all the slides. The preparations were viewed under an epifluorescence microscope (Leica DMI 6000B; Leica Microsystems, Wetzlar, Germany). Negative controls included the omission of primary antibodies.

Secreted Protein Determination by Enzyme-Linked Immunosorbent Assay

The levels of secreted proteins MUC5AC, IL-6, and sCD44 were measured by enzyme-linked immunosorbent assay (ELISA) technique in culture supernatants recovered from the constructs exposed to the four experimental conditions. Commercial kits used are listed in Table 1. The assays were performed according to the manufacturer's protocols.

Table 1. Commercial ELISA kits used in the study

Protein	Manufacturer	Detection range
MUC5AC	Shanghai Yehua Biological Technology Co., Ltd., Shanghai, China	5-1000 ng/ml
IL-6	Diaclone, Besançon, France	6.25-200 pg/ml
sCD44	eBioscience, Vienna, Austria	62.5-4000 pg/ml

Data Presentation and Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM). Student's *t*-test was performed to analyze data and $p \leq 0.05$ was considered statistically significant.

RESULTS

Conjunctival Constructs Exposed to Experimental Conditions Experienced Structural Changes

Conjunctival constructs were fixed at day 7, processed, and stained with hematoxylin/eosin to analyze cell distribution and potential structural changes (Figure 2). When constructs were cultured in air-lifted conditions, higher stratification was observed. However, when air-lifted cultures were additionally exposed to 2 hours of desiccation, the number of epithelial layers was reduced.

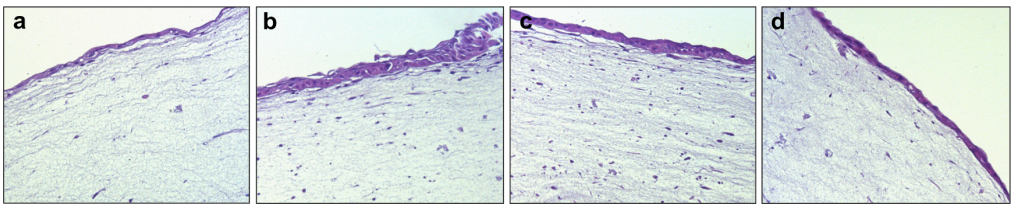

Figure 2. Hematoxylin/eosin section of matrices in control (a), air-lifted (b), desiccation (c) or IL-13 (d) conditions. Magnification: X20.

Conjunctival constructs thickness in the four experimental conditions was measured in 6 independent experiments, and mean values were compared. Control constructs had 558,12 ± 49.42 μm thickness, whereas constructs treated with IL-13 were significantly thicker (871.75 ± 46.37 μm, p = 0.0008) than control constructs (Figure 3). Constructs under the desiccating condition were slightly thinner (503.22 ± 35.05 μm) than control constructs, although difference was not statistically significant.

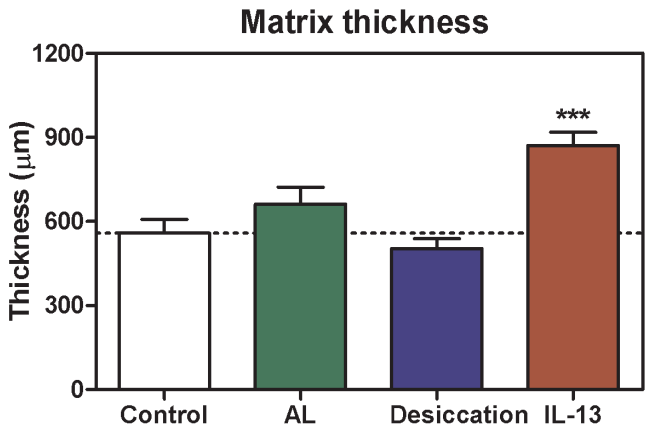

Figure 3. Matrix thickness in the different experimental conditions. Mean values from 6 independent experiments. ***p ≤ 0.005.

Mucous Secretion of Cells in Conjunctival Constructs Changes in the Experimental Conditions

The presence of mucus was evaluated in the constructs by two different techniques. First, constructs exposed to the four experimental conditions were stained with the HPA lectin (Figure 4). High molecular weight glycoconjugates identified by HPA lectin were observed in all the experimental conditions. Increased numbers of stained cells were observed in air-lifted condition (Figure 4b), whereas a decrease was found in IL-13 treated constructs (Figure 4d).

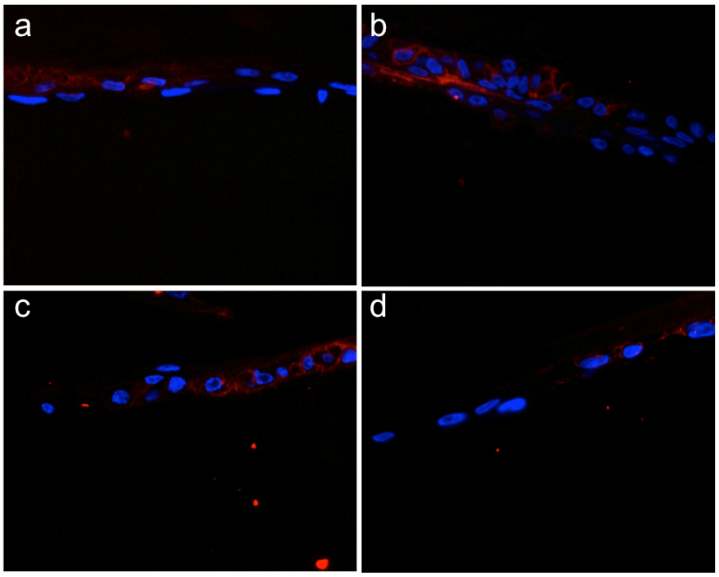

Figure 4. HPA lectin staining (red) was detected in control (a), air-lifted (b), desiccation (c), and IL-13 (d) constructs. Magnification: X40.

In addition, mucin secretion in the constructs was evaluated by MUC5AC ELISA (Figure 5). MUC5AC secreted levels increased from 177.37 ± 3.03 ng/ml in the control matrices to 207.54 ± 11.52 ng/ml in air-lifted culture ($p = 0.020$), to 215.18 ± 10.23 ng/ml in IL-13 desiccating condition ($p = 0.0026$), and to 204.44 ± 10.71 ng/ml in IL-13 treated constructs ($p = 0.0074$).

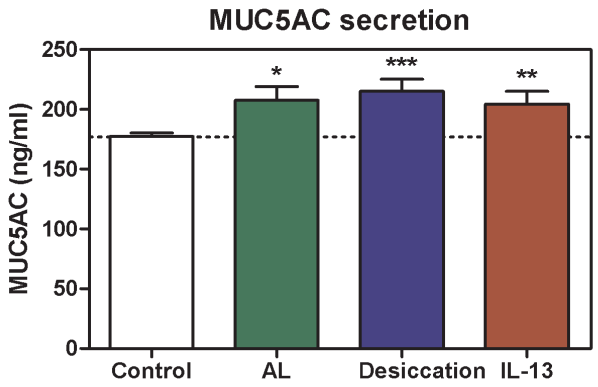

Figure 5. MUC5AC levels secreated by goblet cells present in the constructs was analyzed by ELISA. An increased in secretion in all the experimental conditions was observed compared to control values. Mean values from 6 independent experiments. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.005$.

Cell Proliferation Rate in Conjunctival Constructs Changed under Experimental Conditions

Constructs were stained with an anti-Ki67 antibody to determine the presence of cells with proliferative capacity in the model at day 7. Positive cells were observed in all the experimental conditions (Figure 6), although the greatest numbers were found in the constructs cultured in air-lifted conditions (Figure 6b).

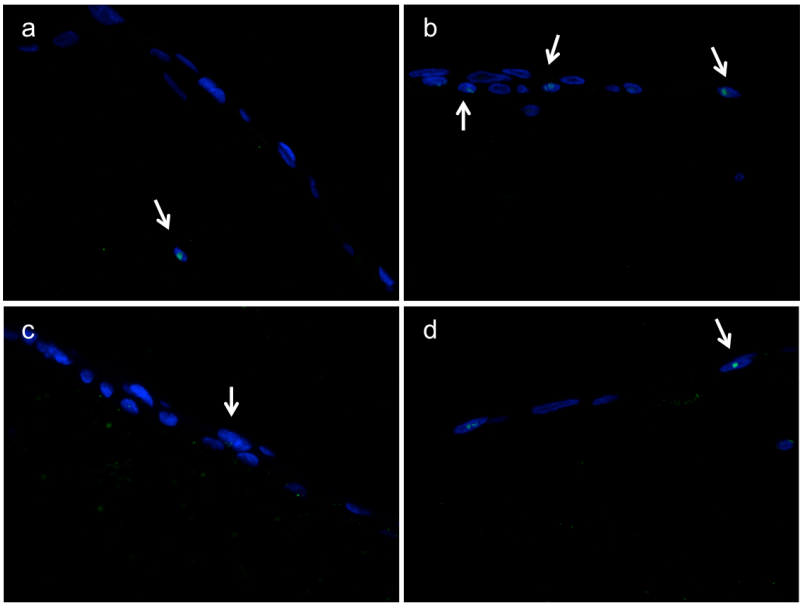

Figure 6. Representative microphotographs showing Ki67 positive cells (arrows) in control (a), air-lifted (b), desiccation (c), and IL-13 (d) conditions. Magnification: X40.

Conjunctival Constructs Exposed to Experimental Conditions Exhibited Inflammatory Responses

The secreted levels of the inflammatory cytokine IL-6 were measured by ELISA to determine whether the different experimental conditions produced an inflammatory response in the cells contained in the three-dimensional model (Figure 7). Cells in the control constructs basaly released 2,230.4 ± 1,076.7 ng/ml IL-6, and that secretion was significantly increased to 9,879.8 ± 2,371.6 ng/ml ($p = 0.0088$) and to 12,023.0 ± 2,766.9 ng/ml ($p = 0.0040$) in air-lifted and desiccating conditions, respectively. Matrices treated with IL-13 secreated 3,553.5 ± 1,702.2 ng/ml that was not significantly different from levels secreated by control matrices.

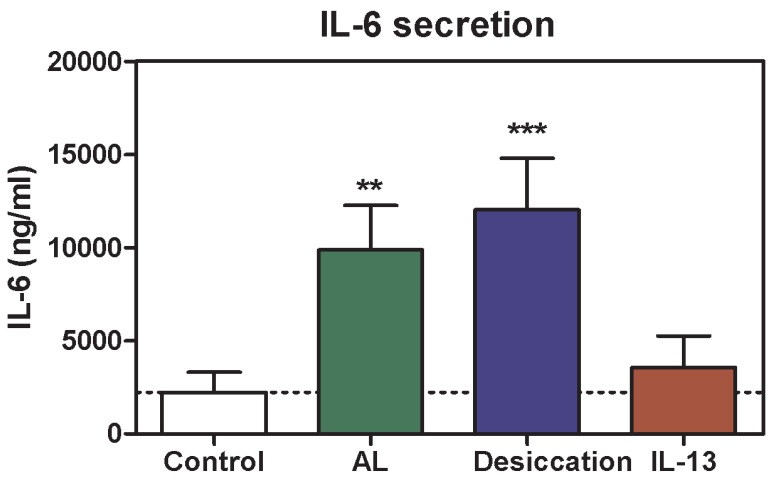

Figure 7. The inflammatory cytokine IL-6 was secreted by conjunctival cells in the fibrin scaffold. In air-lifted culture and desiccation conditions, IL-6 levels were significantly increased. Mean values from 6 independent experiments. **p ≤ 0.01; ***p ≤ 0.005.

Hyaluronan Receptors Expression in the Constructs Changed in the Experimental Conditions

Hyaluronan receptors expression was evaluated in the constructs. CD44 and RHAMM expression was analyzed by immunofluorescence (Figure 8), and sCD44 release was measured by ELISA (Figure 9).

Epithelial cells in the constructs expressed the hyaluronan receptor CD44 in all experimental conditions. In the air-lifted condition, where several epithelial layers are found, CD44 was distributed mainly in the basal layers (Figure 8b). However, in IL-13 treated matrices, all epithelial cell layers expressed CD44 (Figure 8d). Fibroblasts did not express CD44 except in the desiccating condition (Figure 8c).

In control constructs RHAMM was expressed by most epithelial cells and, with lower intensitiy, by fibroblasts (Figure 8e). The distribution and intensity was similar in air-lifted matrices (Figure 8f). However, in the desiccating condition no RHAMM expression was observed in neither epithelial cells nor fibroblasts (Figure 8g). Finally, in IL-13 treated matrices (Figure 8h) higher intensity was found in the fibroblasts inside the fibrin scaffold, compared to control constructs.

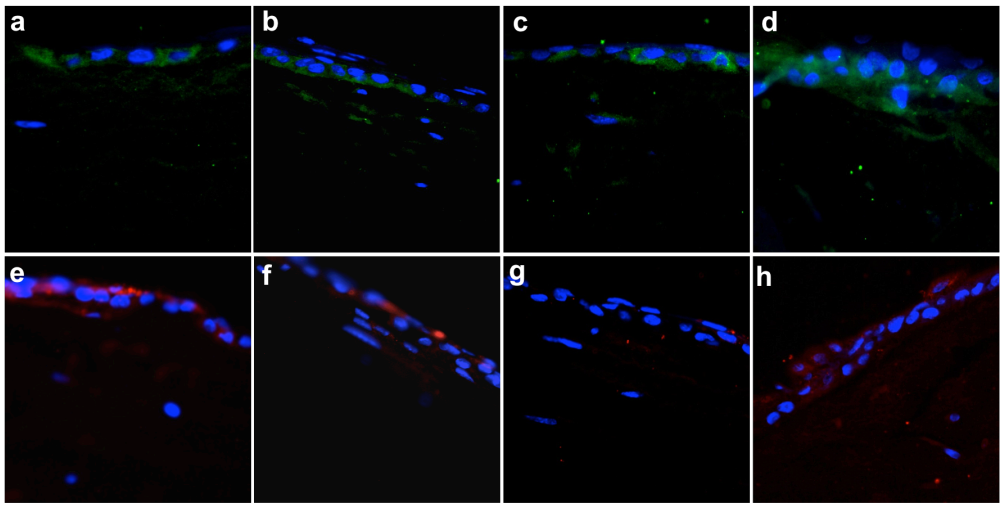

Figure 8. CD44 (green, a-d) and RHAMM (red, e-h) hyaluronan receptors were expressed by epithelial cells in the conjunctival constructs. Magnification: X40.

Conjunctival cells basally secreated 10.30 ± 1.14 ng/ml sCD44 in the control constructs (Figure 9). Significantly higher levels were released in the air-lifted condition (15.65 ± 2.34 ng/ml, p = 0.05), whereas no changes were observed in desiccation or IL-13 conditions.

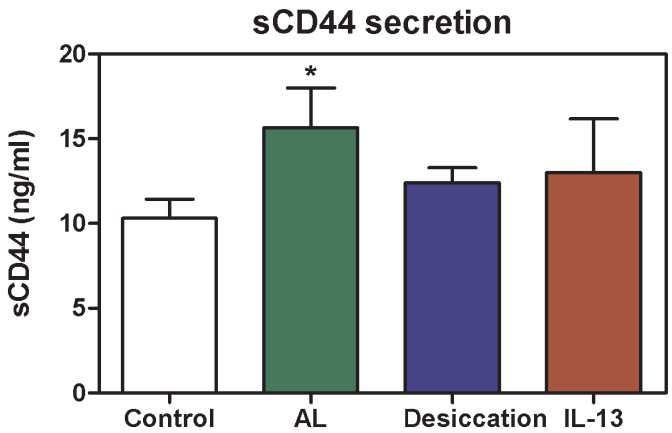

Figure 9. sCD44 was secreted in the three-dimensional model. In the air-lifted condition that secretion was increased compared to the control. Mean values from 5 independent experiments. *p ≤ 0.05.

DISCUSSION

In this study, we presented a proof of concept for the use of a three-dimensional model of human conjunctiva to study ocular surface inflammation. The model was exposed to different experimental conditions such as air-lifted culture, desiccation, or cytokine treatments and after that, morphological and functional changes were observed.

Epithelial cells growing on the surface of the fibrin scaffold showed higher stratification when cultured in air-lifted condition. The ability of epithelial cells to stratiffy when cultured in those conditions was already published. Chung et al. (2007) reported human conjunctival epithelial cell stratification and several authors referred similar results in other mucosal epithelia (de Borja Callejas et al., 2014). Thus, these results corroborated that epithelial cells in our three-dimensional model are able to respond as they were expected to do.

One of the main functions of the conjunctiva that is altered in ocular surface infllammatory diseases is mucin secretion. Changes in mucous secretion were observed in the experimental conditions. When the three-dimensional model of human conjunctiva was characterized, some goblet-like cells were observed. In this study, we found that those goblet-like cells were presented in all the conditions, and that its numbers increased in the air-lifted condition. Again, this response was already reported be several authors in other mucosal epithelium models (Yokoyama et al., 2007; Kesimer et al., 2009). In addition, MUC5AC secretion was measured in the constructs, and the presence of this mucin was found in all conditions. In the conjunctiva, MUC5AC is exclusively secreted by goblet cells. Therefore, our results confirm the presence of goblet cells in the three-dimensional model. Moreover, an increase in MUC5AC secretion was found in air-lifted, desiccation, and IL-13 conditions compared to control constructs. Our results are consistent with those of De Paiva et al., and Contreras-Ruiz et al., that reported elevated MUC5AC secretion in murine goblet cells exposed to IL-13 (De Paiva et al., 2011; Contreras-Ruiz et al., 2014). The important effects of IL-13 on conjunctival goblet cells are the main reason why we choose this cytokine to simulate allergic conditions, instead of using the hallmark allergy cytokine IL-4.

Interleukin 6 has been used in several studies as a marker of inflammation. J. Zhang et al. described overexpression of IL-6 in dry eye patients which correlates with the symptomatic severity of disease (Zhang et al., 2013). In this research we measured IL-6 secreted levels, and found that in the air-lifted and in the desiccating conditions IL-6 levels were significantly increased, what is in accordance with the previously mentioned report. That IL-6 overexpression was indicative of an inflammatory status of the cells in the constructs, what supports the idea of using this model to study ocular surface inflammatory disease. Specifically, IL-6 is found elevated in dry eye disease, and the highest levels of IL-6 in our model were found in the desiccating condition, which try to mimic dry eye inflammation.

Finally, hyaluronan receptors were studied in the constructs exposed to the experimental conditions. Interestingly, we found some changes in the receptors expression. CD44 and RHAMM expression was increased in IL-13 treated matrices. In our previous study with hyaluronan receptors in patients with inflammation, increased mRNA levels were found in immune-atopic diseases, including atopic and vernal keratoconjunctivitis, where IL-13 is elevated (García-Posadas et al., 2014). On the contrary, in the desiccating condition that mimics dry eye, RHAMM is not expressed. This result is also in agreement with the results obtained in patients, where a decrease in RHAMM expression was observed in patients with immune non-atopic diseases (García-Posadas et al., 2014). Finally, sCD44 was secreted in all conditions, and significantly more in air-lifted condition. In our study with patients we detected sCD44 in tears of just 32% of the subjects. We also found that IOBA-NHC cells exposed to IL-13 secreted lower levels of sCD44 than untreated cells. In the current study we did not find significant differences with IL-13 treatment, what remarks once again the differences between cell lines and primary cells.

In summary, we have validated the three-dimensional human conjunctival model for its use to study ocular surface inflammation. In addition, we have shown that by exposing the constructs to desiccation for just two hours it is possible to reproduce some of the features found in dry eye disease, and that treating the conjunctival constructs with IL-13 mimic some of the responses found in allergic diseases. Bearing all this in mind we can conclude that this three-dimensional model is functional, respond to several stimuli and, therefore, it can be use to study ocular inflammatory diseases.

ACKNOWLEDGMENTS

This research was supported by FEDER-CICYT Grant MAT2013-47501-C2-1-R and FPI Scholarship Program BES-2011-046381 (Ministry of Economy and Competitiveness), and by Regional JCyL Grant VA132A11-2. Additional financial support was provided by CIBER-BBN.

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EPÍLOGO/*EPILOGUE*

“Quien mira hacia afuera, sueña. Quien mira hacia adentro, despierta.”

“*Who looks outside, dreams; who looks inside, awakes*”

— Carl Jung

Fortalezas y limitaciones

En esta tesis doctoral se ha desarrollado un nuevo modelo tridimensional de conjuntiva humana que presenta varias ventajas frente a los modelos existentes:

- Está elaborado con células primarias que muestran mayor similitud con el tejido original que las líneas celulares.
- Es un modelo tridimensional con varias capas de células epiteliales, por lo que los resultados obtenidos en él serán presumiblemente más similares a los obtenidos *in vivo* que aquellos obtenidos en modelos celulares en monocapa.
- En este modelo hay tres tipos celulares fundamentales, como son las células epiteliales, las células caliciformes y los fibroblastos conjuntivales. La mayoría de modelos disponibles incluyen solo un tipo celular, por lo que este modelo es más completo.

A pesar de los avances obtenidos con el desarrollo de este nuevo modelo tridimensional, el trabajo presentado en esta tesis tiene varias limitaciones:

- La fuente empleada para obtener las células es tejido conjuntival humano y, en consecuencia, es una fuente limitada.
- Además, la disponibilidad del tejido para investigación no es regular, lo que supone un inconveniente a la hora de planificar experimentos.
- El modelo tridimensional desarrollado no representa al completo la complejidad de la conjuntiva humana, puesto que no tiene vascularización, inervación, ni presencia del CALT.

Strenghts and limitations

In this Doctoral Thesis report we have developed a new three-dimensional model of human conjunctiva. This model has several advantages over the existing ones:

- *It is made with primary cells, more similar to the original tissue than cells lines.*
- *This three-dimensional model with several epithelial cell layers will presumibly allow to obtain more valuable results than those taken from monolayer cultures.*
- *This model comprises three conjunctival cell types: epithelial cells, goblet cells, and fibroblasts. Therefore, it is more complete than most current models, which are just limited to one cell type.*

Despite the progress made with the development of the model, this thesis present several limitations:

- *The source used to get the cells is human conjunctival tissue and, in consequence, is a limited source.*
- *Furthermore, as the tissue availability is uncertain, experiments cannot be scheduled.*
- *The three-dimensional model does not completely recapitulate the complexitiy of the conjunctival tissue, since vascularization, inervation, and CALT are not represented in the model.*

Perspectivas futuras

- Estudiar la interacción entre las células epiteliales y los fibroblastos en las matrices, y la interacción de las células con la propia matriz.
- Perfeccionar los modelos de enfermedad, probando distintos tiempos de desecación y varias concentraciones de IL-13.
- Completar el modelo tridimensional incluyendo, además, células endoteliales que representen la vascularización conjuntival.

Future perspectives

- *To study the interaction between epithelial cells and fibroblasts, and between cells and fibrin-scaffolds.*
- *To improve disease models, testing for that purpose different desiccation times and several IL-13 concentrations.*
- *To complete the three-dimensional model including endothelial cells that could represent conjunctival vascularization.*

Conclusiones generales

1. Se ha demostrado que los receptores CD44 y RHAMM están implicados en la inflamación de la superficie ocular y su expresión varía dependiendo de la patología. Esto debería ser tenido en cuenta a la hora de administrar fármacos o lágrimas artificiales que contengan ácido hialurónico. Además, el estudio comparativo de los receptores de ácido hialurónico en muestras obtenidas de pacientes y en líneas celulares ha corroborado una vez más que hay que ser cautos con los resultados obtenidos en líneas celulares, puesto que estos difieren muy a menudo de los que se obtienen con muestras más directas.

2. Las distintas citoquinas que se encuentran elevadas en determinadas patologías inflamatorias que afectan a la conjuntiva tienen efectos diferenciales sobre la función de las células caliciformes. Se ha demostrado que el IFN-γ es capaz de interaccionar con agonistas colinérgicos para modular esta función, lo que indica que las células caliciformes tienen un papel en la protección de la superficie ocular que va más allá de la simple secreción de mucinas.

3. El tejido conjuntival obtenido de donante cadáver es una fuente adecuada de células para desarrollar cultivos primarios de células epiteliales, células caliciformes y fibroblastos conjuntivales, algo que no estaba optimizado hasta el momento. Esta fuente de material humano puede ayudar a reducir el uso de las líneas celulares que tan a menudo muestran resultados discrepantes con los obtenidos directamente de individuos sanos.

4. Las matrices de fibrina son un buen biomaterial para emplear como andamios en cultivos tridimensionales de conjuntiva humana, puesto que permiten la adhesión y la proliferación de células epiteliales, caliciformes y no caliciformes, y de fibroblastos.

5. Se han establecido unas condiciones estándar con un único medio de cultivo en las que es posible realizar un co-cultivo de fibroblastos, células epiteliales y células caliciformes en una matriz tridimensional de fibrina. En estas condiciones el crecimiento de cada tipo celular alcanza un equilibrio que

permite desarrollar un modelo de conjuntiva humana adecuado para llevar a cabo estudios de hasta dos semanas.

6. Se ha podido elaborar y validar un modelo tridimensional de conjuntiva con una estructura similar a la conjuntiva humana, con un epitelio estratificado y polarizado en el que se encuentran células caliciformes que, además, son capaces de secretar MUC5AC de manera diferencial en respuesta a estímulos clínicamente relevantes, tales como la desecación o la presencia de citoquinas inflamatorias.

General conclusions

1. We demonstrated that CD44 and RHAMM hyaluronan receptors are implicated in ocular surface inflammation. Expression of the hyaluronan receptors changed depending on the etiology of the disease. This should be taken into account when artificial tears or drugs containing HA are prescribed to patients. In addition, comparison of the results based on cell lines and on fresh samples obtained from patients highlighted once again the caution that researchers should exert when extrapolating results obtained from cell lines.

2. Cytokines that are altered in several ocular surface inflammatory diseases, such as dry eye and ocular allergy, had differential effects on goblet cells. We showed that IFN-γ interacts with cholinergic agonists to modulate goblet cell function in terms of proliferation and secretion, indicating that goblet cells have a protective role beyond mucin secretion.

3. Conjunctival tissue obtained from cadaveric donors, a resource that has been neglected for a long time, provided suitable conjunctival epithelial cells and fibroblasts to construct the conjunctival models. This source of human tissue can help to reduce the use of human cell lines, which can drift far from the originating healthy human tissues in terms of marker expression.

4. Fibrin-based matrices were a good biomaterial to develop a three-dimensional scaffold for human conjunctival cells. The properties of these matrices likely facilitated cell adhesion and allowed the proliferation of different cell types.

5. We established standard conditions with a specific culture medium to co-culture conjunctival fibroblasts, epithelial cells, and goblet cells. In these conditions, the performance of each cell type reached an equilibrium that allowed development of an appropriate three-dimensional model suitable to perform different laboratory studies up to two weeks.

6. We designed, constructed, and validated an *in vitro* three-dimensional conjunctival model with a structure similar to that of the native human conjunctival tissue. The model included a stratified and polarized epithelium containing goblet cells that secreted mucins in response to relevant stimuli, such as desiccation or cytokine exposure.