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Membrane Array Analysis of Tear Proteins in Ocular Cicatricial Pemphigoid

Matilda F. Chan, MD, PhD, **Robert Sack, PhD**, **David A. Quigley, MS**, **Sonal Sathe, BS**, **Trinka Vijmasi, BS**, **Shimin Li, PhD**, **Douglas Holsclaw, MD**, **Erich C. Strauss, MD**, and **Nancy A. McNamara, OD, PhD**
Francis I. Proctor Research Foundation, University of California, San Francisco Medical School, San Francisco, California (MFC, TV, SL, DH, ECS, NAM), State University of New York, SUNY College of Optometry, New York, New York (RS, SS), and Cancer Research Institute, University of California, San Francisco Medical School, San Francisco, California (DAQ)

Abstract

Purpose—To explore non-invasive, protein-based, membrane array technology as a means to evaluate the global immune and angiogenic profile of tear proteins in patients with active ocular cicatricial pemphigoid (OCP).

Methods—Forty-three proteins consisting of cytokines, angiogenic/growth factors, and immuno-inflammatory modulators were measured by membrane array in tear samples of four control patients and four OCP patients during active disease and after treatment.

Results—Signals for several distinct and consistent molecular entities were upregulated in all four active OCP tear samples relative to controls. In particular, IL-8 and MMP-9 were elevated during active disease and decreased following systemic immunomodulatory therapy.

Conclusions—Protein array analysis may provide a well-tolerated assay to monitor levels of inflammatory markers in the tears of OCP patients in response to therapy.

Keywords

cicatrizing conjunctivitis; ocular cicatricial pemphigoid; inflammation; protein array; tears

Ocular cicatricial pemphigoid (OCP) is characterized by chronic inflammation and subsequent conjunctival and corneal scarring. The clinical manifestations of inflammation in OCP are associated with the expression of various fibrogenic cytokines. Prior studies have examined mRNA and protein expression of individual cytokines in conjunctival biopsy specimens of patients with active disease.[1] The goal of this study was to explore protein-based, membrane array technology as a means of evaluating the global immune and angiogenic profile of tear proteins collected via Schirmer strip from the eyes of patients with active OCP.

MATERIALS AND METHODS

Patients

This prospective study was approved by the Committee for Human Research at the University of California at San Francisco and followed the tenets of the Declaration of

Corresponding author: Nancy A. McNamara, F. I. Proctor Foundation, Dept of Ophthalmology, UCSF, 513 Parnassus Ave, Box 0412, San Francisco, CA 94143-0412, nancy.mcnamara@ucsf.edu.

Helsinki. Details of the study were explained and informed consent was obtained. Two groups of patients were recruited prospectively:

1. Consecutive patients presenting to the Proctor Foundation with a new diagnosis of cicatrizing conjunctivitis or subepithelial scarring and without a history of systemic or topical immunosuppressive therapy within one month of presentation. Only patients with a positive biopsy showing linear junctional deposition of IgG and complement component C3 on direct immunofluorescence were included.
2. Control patients free of ocular disease as evaluated by history and biomicroscopy. Patients with a history of contact lens wear were excluded.

Tear Collection

Tears were collected by Schirmer strip (EagleVision, Memphis, TN) without anesthesia from both eyes of four OCP patients and four age-matched patients with negative ocular and systemic disease histories. Strips were left in place for 5 minutes then immediately stored at −80 °C until processing.

Membrane Array Analysis of Tears

Assays were carried out using components of the membrane array kit purchased from RayBiotech Inc., Norcross, GA. Protein extracted from strips combined from both eyes was applied to membrane arrays specific for 43 angiogenic modulators. Schirmer strips were extracted in 400 μl phosphate buffered saline (PBS) with vortexing at room temperature for one hour. Following centrifugation, extract supernatants were adjusted to 1 ml per 10mm of strip in blocking buffer. Each array contained duplicate dots of capture antibodies that were specific for each protein as well as positive and negative controls. The 20 proteins included on Array 1 and the 23 proteins included on Array 2 are summarized in Table 1. Membrane arrays were blocked in 2 ml of 5% biotin-free casein colloidal buffer (RDI, Flanders, NJ) in PBS at pH 7.4. All incubations were carried out with constant rocking at room temperature. After two hours, the blocking solution was discarded and 1 ml of strip extract was added to each membrane. After two hours, the membranes were washed six times for five minutes each with 2 ml of PBS and 0.05% Tween-20 followed by six washes of five minutes each in PBS without detergent. The membranes were then incubated with the supplied cocktail of biotinylated-secondary antibodies diluted to one-half of the recommended concentration in 1 ml of biotin-free casein colloidal buffer for 2 hours at room temperature. The washing sequence was repeated and the membranes were incubated with 1 ml of the supplied horseradish peroxidase (HRP)-conjugated streptavidin diluted 1:20,000 in the casein blocking solution. After one half-hour, the washing sequence was repeated. Matched sets of tear samples and control arrays were developed and imaged in tandem. Negative controls consisted of membranes developed in the absence of tears. Membranes were incubated with 1 ml of ECL Advance Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ) and imaged using a Bio-Rad ChemiDoc XRS image station equipped with an enhanced sensitivity −45°C cooled-backed 12–bit CCD.[2] Relative protein levels were quantified by densitometry and analyzed in R using the Statistical Analysis of Microarrays (SAM) package.[3] Fold-changes were calculated for each protein as the mean post-treatment/pre-treatment value for each patient.

Statistical Analysis

Statistical analysis in this study was designed to assess whether the differences in protein levels between pre- and post-treatment were statistically significant. The Statistical Analysis of Microarrays (SAM) method performs a modified t-test to determine whether two sample populations of measurements are significantly different. Significance of the SAM result is determined by permutation analysis that accounts for the number of comparisons performed

and typically reported as a q-value, which indicates the expected percentage of false (Type One) discoveries which would be made if the analyst rejects the null hypothesis for all results with that degree of statistical significance.

RESULTS

During a 26-month period, four patients were identified that met our criteria of a new diagnosis of OCP as defined by a positive biopsy showing linear junctional deposition of IgG and complement component C3 on direct immunofluorescence. A summary of each OCP patient's ocular exam findings, associated systemic disease history and course of immuno-modulatory therapy (IMT) is provided in Table 2. Three patients (OCP1, OCP3, OCP4) had bulbar, intrapalpebral, conjunctival biopsies and one patient (OCP2) had a skin biopsy for pathologic analysis. All four patients showed linear junctional deposition of IgG and complement component C3 on direct immunofluorescence consistent with a diagnosis of ocular cicatricial pemphigoid. Tear samples were collected prior to examination and biopsy. Control patients ranged in age from 57 years to 77 years, had negative histories for prior ocular or systemic disease, and showed no signs of conjunctival or other ocular inflammation on biomicroscopic examination.

Figure 1 shows representative membrane arrays comparing normal tears to patient OCP3's tears collected before (pre) and following 4- and 8-weeks of IMT. Control membranes run in tandem with each tear sample to elicit background signal are shown (No tear control). The two post-treatment measurements for OCP3 had a correlation coefficient of 0.97 (Pearson correlation, 95% confidence interval 0.95 to 0.99), demonstrating reproducibility and stability of the post-treatment assay. Compared to normal controls, OCP patients showed moderately increased levels of angiogenin, IL-6, IL-8, MMP-9, TIMP-1 and TIMP-2 (Figure 2). IL-8 and MMP-9 were significantly elevated in three out of four OCP patients (OCP1, OCP2, OCP3) with active disease (Pre-Treatment), with the mean fold-increases reaching 4.6 and 4.2, respectively, (False Discovery Rate q-value < 0.001). IL-8 and MMP-9 levels were reduced following IMT (Post-treatment), except in the case of OCP2, where paraneoplastic syndrome associated with non-small cell lung carcinoma required an alternative treatment regimen consisting of radiation, cisplatin, etoposide, prednisone and Mycophenolate mofetil.

DISCUSSION

Previous studies have focused specifically on mRNA expression and protein analysis of individual cytokines in OCP conjunctival biopsy specimens. Because tear proteomics has been shown to reflect the dynamics of ocular surface processes occurring in various ocular diseases, including vernal keratoconjunctivitis (VKC), atopic keratoconjunctivitis (AKC), or Sjögren's syndrome (SS), [4–8] we decided to study the tears of patients with OCP.

Using membrane array technology, we identified specific tear proteins present in a single OCP tear sample and how these proteins change in response to IMT. Over a 26-month period, we identified four patients with active and currently untreated OCP in our tertiary referral clinic. IL-8 and MMP-9 were significantly elevated in three out of the four patients and decreased with the resolution of active, clinical inflammation in as little as one month of IMT. An increase in IL-8 and MMP-9 in active OCP may result from the activation of infiltrating neutrophils by IL-8, which is known to induce the release of MMP-9. The reduction in levels of IL-8 and MMP-9 to near baseline in patients OCP1 and OCP3 following systemic IMT correlated with resolution of active inflammation by clinical examination.

To our knowledge, this is the first report demonstrating the use of novel, tear-based membrane array technology to assess ocular inflammation in patients with active OCP both naïve to specific treatment and following IMT. Relative tear protein levels were reported as we found it particularly difficult to accurately quantify the specific concentration of low abundant tear proteins due to tear specific matrix effects arising, in part, from the variable presence of a highly "sticky" confounding factor, which we have elaborated upon elsewhere. [8] Data obtained using membrane array serves a valuable purpose in that by using dilute blocked samples and incubation wells for membranes with large plastic surfaces, the highly "sticky" tear factor can selectively bind to the plastic and not the membrane and thereby provide a more accurate set of signals. The obtained data is critical for selecting targets for later quantitative assays. For this reason membrane arrays are of particular importance for selecting biomarkers for further more expensive quantitative evaluation.

Acknowledgments

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Membrane 1

	a	b	c	d	e	f	g	h
1	POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
2	POS	POS	NEG	NEG	Angiogenin	EGF	ENA-78	b FGF
3	GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
4	GRO	IFN-γ	IGF-I	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
5	PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
6	PIGF	RANTES	TGF-β1	TIMP-1	TIMP-2	Thrombopoietin	VEGF	VEGF-D
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS
8	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	NEG	POS

Membrane 2

	a	b	c	d	e	f	g	h
1	POS	POS	NEG	NEG	Angiopoietin-1	Angiopoietin-2	Angiostatin	Endostatin
2	POS	POS	NEG	NEG	Angiopoietin-1	Angiopoietin-2	Angiostatin	Endostatin
3	G-CSF	GM-CSF	I-309	IL-10	IL1-α	IL-1β	IL-2	IL-4
4	G-CSF	GM-CSF	I-309	IL-10	IL1-α	IL-1β	IL-2	IL-4
5	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	Tie-2	TNF-α
6	I-TAC	MCP-3	MCP-4	MMP-1	MMP-9	PECAM-1	Tie-2	TNF-α
7	u PAR	VEGF R2	VEGF R3	Blank	Blank	Blank	Blank	POS
8	u PAR	VEGF R2	VEGF R3	Blank	Blank	Blank	Blank	POS

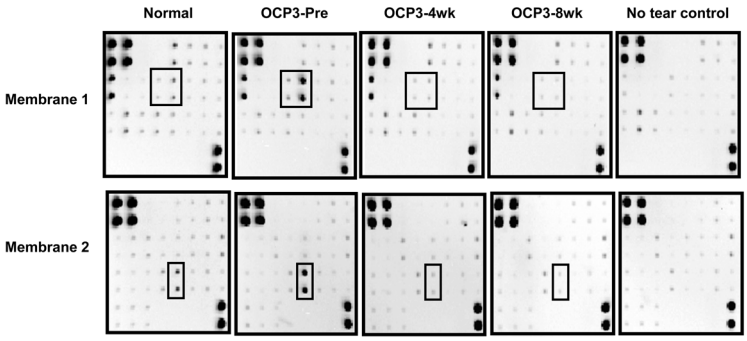

Figure 1.

Duplicate dots of capture antibodies specific for each protein as well as positive (pos) and negative (neg) controls are shown for a representative normal (normal 3) and OCP patient (OCP 3). Low to moderate signals for angiogenin, growth related oncogene (GRO), IL-6, IL-8, TIMP-1 and TIMP-2, MMP1, and MMP9 are detected in normal tears. Tears from the OCP patient were collected at the time of presentation (Pre) and after 4 and 8 weeks of immuno-modulatory therapy (IMT). Boxes denote the decrease in signal for IL-6, IL-8, and MMP-9 with treatment.

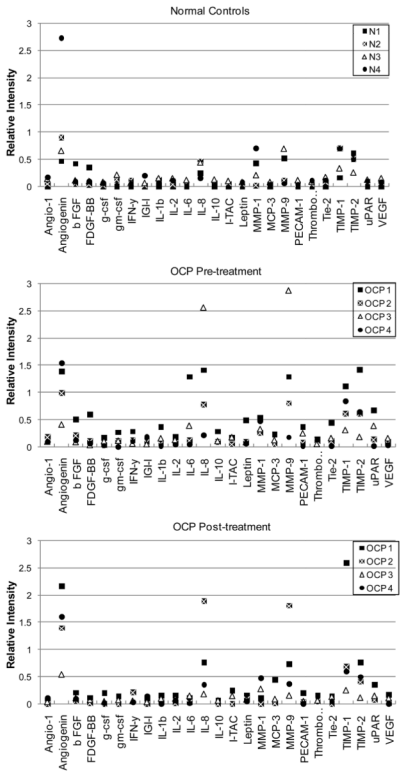

Figure 2.

Graphical comparison of background-subtracted densitometric values (Y-axis) for tear proteins measured in four normal (N) patients and four OCP patients before and after treatment.

Table 1

Proteins included in Arrays 1 and 2.

Array 1		Array 2	
Protein	**Abbreviation**	**Protein**	**Abbreviation**
Angiogenin	Angiogenin	Angiopoeitin-1	Angiopoeitin-1
Epidermal Growth Factor	EGF	Angiopoeitin-2	Angiopoeitin-2
Epithelial neutrophil-activating protein	78ENA-78	Angiostatin	Angiostatin
Basic Fibroblast Growth Factor	bFGF	Endostatin	Endostatin
Growth Related Oncogene	GRO	Granulocyte-colony Stimulating Factor	G-CSF
Interferon Gamma	IFN-γ	Granulocyte-macrophage Colony Stimulating Factor	GM-CSF
Insulin-like growth factor-1	IGF-1	I-309	I-309
Interleukin 6	IL-6	Interleukin 10	IL-10
Interleukin 8	IL-8	Interleukin 1 Alpha	IL-1α
Leptin	LEPTIN	Interleukin 1 Beta	IL-1β
Monocyte Chemoattractant Protein 1	MCP-1	Interleukin 2	IL-2
Platelet-derived Growth Factor BB	PDGF-BB	Interleukin 4	IL-4
Placenta Growth Factor	PIGF	Interferon-inducible T Cell Alpha Chemoattractant	I-TAC
Regulated upon activation, normal T-cell expressed, and presumably secreted	RANTES	Monocyte Chemoattractant Protein 3	MCP-3
Transforming Growth Factor beta 1	TGF-β1	Monocyte Chemoattractant Protein 4	MCP-4
Tissue Inhibitor of Metalloproteinases-1	TIMP-1	Matrix Metalloproteinase 1	MMP-1
Tissue Inhibitor of Metalloproteinases-2	TIMP-2	Matrix Metalloproteinase 9	MMP-9
Thrombopoeitin	TPO	Platelet Endothelial Cell Adhesion Molecule	PECAM-1
Vascular Endothelial Growth Factor	VEGF	Tyrosine Kinase with Immunoglobulin and Epidermal Growth Factor Homology Domain 2	Tie-2
Transforming Growth Factor beta 1	RANTES	Tumor Necrosis Factor-alpha	TNF-α
		Urokinase Plasminogen Activator Receptor	uPAR
		Vascular Endothelial Growth Factor Receptor 2	VEGF-R2
		Vascular Endothelial Growth Factor Receptor 3	VEGF-R3

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Table 2

Clinical Summary of Ocular Cicatricial Pemphigoid Patients.

Patient No./Sex/Age, y	Clinical Findings	Systemic Disease	IMT	Time on IMT prior to follow- up tear collection (months)	Schirmer's Strip Results (mm) Pre-IMT (OD, OS) and Post-IMT (OD, OS)
OCP1/F/84	Diffuse bulbar conjunctival injection, subepithelial scarring	Oral mucous membrane pemphigoid	Cyclophosphamide	2	16, 16 (Pre) 26, 25 (Post)
OCP2/M/69	Conjunctival bullae, fornix shortening, inferior symblepharon	Non-small cell lung carcinoma	Radiation, Cisplatin, Etoposide, Prednisone, Mycophenolate mofetil	4	5, 8 (Pre) 35, 12 (Post)
OCP3/M/58	Irregular corneal epithelium, fornix shortening, symblepharon	None	Cyclophosphamide, oral prednisone, topical prednisolone	1 and 2	15, 12 (Pre) 20, 1 (1 m Post) 5, 5 (2 m Post)
OCP4/F/77	Diffuse bulbar conjunctival injection, subepithelial scarring, symblepharon	Rheumatoid arthritis, chronic myelogenous leukemia (CML)	Prednisone, Methotrexate	2	8, 6 (Pre) 10, 6 (Post)