Ocular Surface Extracellular DNA and Nuclease Activity Imbalance: A New Paradigm for Inflammation in Dry Eye Disease

Snehal Sonawane,¹ Vishakha Khanolkar,¹ Abed Namavari,¹ Shweta Chaudhary,¹ Sonal Gandhi,¹ Sapna Tibrewal,¹ Sarmad H. Jassim,¹ Brittany Shaheen,¹ Joelle Hallak,¹ John H. Horner,² Martin Newcomb,² Joy Sarkar,¹ and Sandeep Jain¹

PURPOSE. We determined whether nucleases are deficient in the tear fluid of dry eye disease (DED) patients, and whether this causes extracellular DNA (eDNA) and neutrophil extracellular trap (NET) accumulation in the precorneal tear film, thus causing ocular surface inflammation.

METHODS. Exfoliated cells adhered to Schirmer test strips were collected on glass slides, and immunofluorescence confocal microscopy was used to evaluate neutrophils, eDNA, NETs, and their molecular components. Similar experiments were performed with mucoid films collected from the inferior conjunctival fornix or bulbar conjunctiva. We used quantitative PCR to evaluate eDNA signaling pathways and inflammatory cytokine expression. We also determined the amount of ocular surface eDNA and evaluated tear fluid nuclease activity.

RESULTS. eDNA, NETs, and neutrophils were present on the ocular surface in DED patients and abundant in mucoid films. NETs consisted of eDNA, histones, cathelicidin, and neutrophil elastase. Tear fluid nuclease activity was decreased significantly in DED patients, whereas the amount of eDNA on the ocular surface was increased significantly. Expression of genes downstream of eDNA signaling, such as *TLR9*, *MyD88*, and type I interferon, as well as the inflammatory cytokines interleukin-6 and tumor necrosis factor- α , was significantly increased in DED patients.

CONCLUSIONS. Extracellular DNA production and clearance mechanisms are dysregulated in DED. Nuclease deficiency in tear fluid allows eDNA and NETs to accumulate in precorneal tear film, and results in ocular surface inflammation. These findings point to novel therapeutic interventions in severe DED based on clearance of eDNA, NETs, and other molecular components from the ocular surface. (*Invest Ophthalmol Vis Sci.* 2012;53:8253–8263) DOI:10.1167/iov.12-10430

From the ¹Corneal Neurobiology Laboratory, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, College of Medicine, Chicago, Illinois; and the ²Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois.

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Corresponding author: Sandeep Jain, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, 1855 W. Taylor Street, Chicago, IL 60612; jains@uic.edu.

Dry eye disease (DED) is a multifactorial disorder of tears and the ocular surface that affects millions of people worldwide, and negatively influences their quality of life.¹ Although DED pathogenesis is not understood fully, inflammation has a prominent role in DED symptom development and amplification.² The current paradigm suggests that ocular surface inflammation is triggered by surface epithelium stress caused by tear hyperosmolarity. Inflammation is sustained by activated antigen-presenting cells (APCs) and T-cells via the afferent and efferent limbs of the adaptive immune system.^{3,4}

The immunopathologic events that sustain the systemic adaptive immune response in DED have been characterized.^{2–4} However, the mechanisms that activate the adaptive immune response are understood poorly. Ocular surface epithelial stress is a key initial event, and a major source of innate cytokines and chemokines that can damage epithelial cells and activate APCs. Tear hyperosmolarity is recognized as an important stressor. However, tear replacement to decrease osmolarity provides limited therapeutic benefit. Therefore, additional stressors may activate DED ocular surface inflammation, and link the innate and adaptive immune mechanisms.

The ocular surface epithelium undergoes continuous, dynamic turnover,^{5,6} which is increased in DED patients.⁷ Superficial corneal cells are shed into the precorneal tear film.^{8,9} The corneal epithelial cell shedding process, or desquamation, is regulated by apoptotic mechanisms.¹⁰ Dead and dying cells release extracellular DNA (eDNA), a type of damage-associated molecular pattern molecule that can stimulate the innate immune system and link it to adaptive immune system.^{11,12} eDNA strands have been reported in corneal filaments, which frequently are present on the corneas of patients with severe DED.¹³ Desquamated cells in the precorneal tear film are a potential source of eDNA. Nucleases are present in the tear fluid, such as lipocalin and DNase, that can hydrolyze and clear eDNA from the ocular surface.^{14–17} Tear lipocalin, an endonuclease, accounts for over 75% of the DNA catalytic activity in tears;¹⁴ however, its DNA hydrolyzing efficiency is three orders of magnitude lower than that of DNase I.¹⁷ Additionally, tear fluid contains several neutrophil extracellular trap (NET) components. Neutrophils undergo a low level of recruitment on the ocular surface,¹⁸ and numerous neutrophils are present in the tear film during ocular surface inflammation.¹⁹ Neutrophil elastase and histone proteins also have been reported in tear fluid.²⁰ Taken together, these reports document the presence of eDNA, histones, neutrophils, neutrophil elastase, and nucleases in tear fluid, and suggest mechanisms exist for the continual production and clearance of eDNA in the precorneal tear film.

Neutrophils are key players in the host innate immune response and constitute the first line of defense.²¹ They contain several molecules that are highly potent antimicrobials, but because of poor target specificity, these molecules have the potential to cause inflammation and toxicity to the host cells as well. One strategy that neutrophils use for defense against microbes is to release nuclear contents into the extracellular space to form NETs.²² Stimulation of neutrophils with microorganisms is much more efficient at inducing NET formation than stimulation with single bacterial components, such as lipopolysaccharide (LPS), suggesting a requirement for multiple signaling to achieve optimal net production by neutrophils.^{23,24} NET formation seems to be the consequence of neither necrosis nor apoptosis, but rather a new death cell pathway, which has been termed NETosis.²⁵ NETs contain decondensed chromatin, histones, neutrophil elastase, as well as antimicrobial peptides, such as cathelicidin (LL-37).²⁶ Although NET formation is an important innate immune system defense mechanism and beneficial for health, they have been implicated in pathobiology of inflammatory conditions, like systemic lupus erythematosus (SLE), an autoimmune condition that frequently is associated with DED.²⁷ Patients with SLE have antibodies to NET components, and have DNase I inhibitors and antibodies that prevent NET degradation.²⁸ Emerging evidence also suggests that increased expression of cathelicidin peptides, a molecular component of NETs, is associated with inflammatory diseases, such as rosacea, another condition associated frequently with DED.^{29,30}

We hypothesized that in DED, eDNA production, and clearance mechanisms are dysregulated, allowing eDNA and NETs to accumulate in the precorneal tear film, thus causing ocular surface inflammation. We presented experimental data to support our hypothesis. These findings may contribute to novel therapeutic interventions based upon a new paradigm for ocular surface inflammation in DED.

METHODS

Study Population

Symptomatic tear-deficient DED patients and asymptomatic healthy individuals with normal tear production were enrolled, and provided written informed consent according to the Declaration of Helsinki under an Institutional Review Board approved protocol. Patients were included if they complained of any DED symptom (dryness, irritation, grittiness, light sensitivity, or foreign body sensation) and additionally had severe aqueous tear deficiency, defined as Schirmer I value ≤ 5 mm in 5 minutes (without anesthesia). Individuals were included in the control group if they had no ocular symptoms and a Schirmer I value ≥ 12 mm in 5 minutes (without anesthesia).

Conjunctival Exfoliated Cells and Mucoid Films Analyses

The Schirmer I test was performed without topical anesthesia by placing Schirmer test strips (Haag-Streit, Essex, UK) over the lower lid margin, at the lateral and middle third junctions, for 5 minutes. Strip wetting was recorded in millimeters. Because the test strips contacted the palpebral and bulbar conjunctiva, cells from these areas were exfoliated upon strip removal. Cells that adhered to the strips were transferred to silane-coated adhesive slides (Cat. #061-1225; Tekdon Inc., Myakka City, FL). Slides were fixed immediately for 30 minutes in neutral buffered 10% formaldehyde (Sigma-Aldrich, St. Louis, MO) before further analyses. Mucoid films were collected using disposable microcapillary glass tubes (5 μ l volume; Sigma-Aldrich) over the bulbar

conjunctiva or from the inferior conjunctival fornix. The mucoid films were spread on silane-coated slides and processed as described above.

Hematoxylin and Eosin (H&E) Staining. Slides with conjunctival exfoliated cells ($n = 15$) or mucoid films ($n = 10$) were stained with hematoxylin (H-3401; Vector Labs, Burlingame, CA), rinsed in acid, dipped in bluing solution, and counterstained with eosin (Thermo Scientific, Waltham, MA). Slides were examined using an upright Axioscope 100 microscope (Carl Zeiss Meditec GmbH, Hamburg, Germany), imaged using a Zeiss MRc color camera, and analyzed using Zeiss Axiovision.

Immunostaining and Confocal Microscopy. Immunofluorescence staining and confocal microscopy were performed to localize molecular components of NETs and eDNA as described previously.^{31,32} Slides were permeabilized for 5 minutes in 0.025% Triton X-100 and blocked for 2 hours at room temperature with 1% BSA and 10% normal donkey serum in PBS. Slides were incubated overnight at 4°C with the primary antibody diluted in blocking solution (1:200). The slides were washed four times in PBS (15 minutes each) and incubated for 1 hour with the secondary antibody diluted in blocking solution (1:200). Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI; Cat. #H-1200; Vector Labs) was placed over the slides and covered with a glass coverslip. The primary antibodies used were: mouse monoclonal anti-human neutrophil elastase (clone NP57; DAKO, Glostrup, Denmark), goat polyclonal anti-histone H2B (Cat. #SC-8650; Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit polyclonal anti-cathelicidin (Cat. #ab64892; Abcam, Cambridge, MA). The secondary antibodies used were Dylight 594-conjugated anti-mouse IgG for neutrophil elastase, (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA), and FITC 480 anti-goat IgG for histone and cathelicidin (1:200; Jackson ImmunoResearch Laboratories). Specimens were analyzed using a LSM 710 META confocal microscope (Carl Zeiss GmbH). Patient samples were imaged first to optimize the fluorescent signals, and immediately thereafter negative control slides (with the primary antibody omitted) were imaged using the identical settings. The specificity of the primary antibodies antineutrophil elastase,^{33,34} antihistone H2B,³⁵⁻³⁷ and anticathelicidin³⁸ has been validated previously.

Lacrimal Gland DNase I Immunostaining. Lacrimal gland sections (6–8 μ m) were obtained from archived noninflammatory and nonmalignant lacrimal biopsies ($n = 5$). Sections were deparaffinized at 56°C for 30 minutes, followed by a graded alcohol series treatment. Sections were processed for staining as described above. The primary antibody used was rabbit polyclonal anti-DNase I (1:50, Cat. #HPA010703; Sigma Prestige, St. Louis, MO). The secondary antibody was Dylight 594-conjugated anti-rabbit IgG. Negative controls were: primary antibody omitted, rabbit polyclonal IgG isotype control (Cat. #ab27427; Abcam), and peptide pre-incubation with primary antibody (Peptide for Cat. #HPA010703; Atlas Antibodies AB, Stockholm, Sweden). Imaging and analyses were performed as described above.

Laser Capture Microdissection of eDNA Strands

Schirmer test strip impressions were taken on membrane slides (Cat. #11505158; Leica, Solms, Germany) as described above. Slides were fixed in 10% formaldehyde for 30 minutes and washed in 1× PBS. The slides were stained with DAPI, washed briefly in PBS, and dried for 30 minutes at 37°C. DAPI-stained eDNA strands were visualized, dissected, and captured using a PALM laser capture microdissection microscope (Carl Zeiss, Thornwood, NY). Captured strands were collected in an adhesive cap, and DNA was extracted using DNAzol (Cat. #1-0503-027; Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PCR was performed using the GoTaq PCR kit (Cat. #M7122; Promega, Madison, WI) per the manufacturer's protocol. The human *GAPDH* gene (Cat. #PPH00150F; SA Biosciences, Frederick, MD) was amplified using gene-specific primers. PCR products were electrophoresed and visualized on a 2% ethidium bromide-stained agarose gel.

Quantification of Ocular Surface eDNA

We used two strategies to calculate the amount of eDNA on the ocular surface. First, we determined the total length of eDNA strands in exfoliated material derived from the Schirmer test strip impressions on glass slides. Second, we extracted eDNA from the Schirmer test strip using DNase I and determined the fluorescence intensity using PicoGreen DNA fluorescent dye.

eDNA Length. Slides with Schirmer strip impressions were fixed and stained with DAPI. Five random 20× objective fields were imaged using an inverted microscope (Axio Observer; Carl Zeiss) and analyzed using Neurolucida software (MBF Bioscience, Williston, VT). The eDNA fibers were traced and lengths were calculated using Neuroexplorer (MBF Bioscience) as described previously for corneal nerves.^{31,32} We compared the average total eDNA length in dry eye patients ($n = 10$ patients with non-Sjögren dry eyes, including 4 with graft-versus-host disease [GVHD]) to that in normal controls ($n = 10$).

PicoGreen Assays. PicoGreen assays were performed as described previously.²⁵ The folded end of the Schirmer strip that contacted the conjunctiva was collected in an Eppendorf tube and 200 μ L of 100 U/mL DNase I (Cat. #EN0521; Fermentas Life Sciences, Hanover, MD) was added. After 20 minutes, nuclease activity was stopped with 0.5 mM of EDTA and the Schirmer strip end was removed. PicoGreen DNA fluorescent dye (Cat. #P7589; Invitrogen Detection Technologies) was added, and fluorescence intensity was determined using a microplate reader (Synergy H1; BioTek, Winooski, VT). Values were averaged and compared between DED patients ($n = 10$) and normal controls ($n = 10$).

Exfoliated Conjunctival Cell Gene Expression

RNA was extracted from exfoliated conjunctival cells on Schirmer test strips from DED patients ($n = 17$, 3 with Sjögren disease and 14 with non-Sjögren dry eyes, including 6 with GVHD, 2 with ocular cicatricial pemphigoid [OCP] and 1 with neurotrophic DED) and normal controls ($n = 16$). The folded ends of Schirmer test strips were placed directly in TRIzol (Invitrogen) for RNA extraction, which was performed according to the manufacturer's protocol. Reverse transcription was performed with 1000 ng total RNA using the RT² First Strand cDNA Synthesis Kit (SA Biosciences). The resulting cDNA was pre-amplified using the RT² Nano PreAMP Kit according to the manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed with SYBR using a 7900HT ABI real-time instrument. eDNA signaling gene expression and inflammatory cytokines were analyzed by real-time qPCR as previously described.^{39–41} All primers and reagents were purchased from SA Biosciences unless specified otherwise. The primers used were toll-like receptor 9 (TLR 9; Cat. #PPH01809A), interferon- α (INFA; Cat. #PPH01321A), MyD88 (Cat. #PPH00911A), interferon- β (INFB; Cat. #PPH00384E), interleukin-6 (IL-6; Cat. #PPH00560B), tumor necrosis factor- α (TNF- α ; Cat. #PPH00341E), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cat. #PPH00150F). Samples were assayed in duplicate in a total volume of 25 μ L using the following cycling conditions: 10 minutes at 95°C, 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. A human genomic DNA contamination control was used to confirm that amplification reagents were not contaminated with genomic DNA. For data analyses, the cycle threshold (CT) of each gene for DED patients was normalized to the corresponding value for normal subjects and used to calculate fold change using the $2^{-\Delta\Delta CT}$ method.

Nuclease Activity and DNase I in Tear Fluid

Tears were collected in disposable microcapillary glass tubes using a slit-lamp biomicroscope. The tubes were placed in the lower conjunctival fornix, and tears were collected by capillary action. Tears were transferred to DNase-free Eppendorf tubes for analyses.

Nuclease Activity in Normal Tear Fluid. Tear film nuclease activity was quantitated in DED patients ($n = 5$) and normal controls (n

$= 5$) using a DNA digestion assay (DNase detection kit; MO-BIO Laboratories, Carlsbad, CA) per the manufacturer's instructions. Tear samples and DNA standards (1-kb DNA ladder) were electrophoresed on a 2% ethidium bromide-stained agarose gel and photographed using a UV-transilluminator. DNase activity was evaluated by comparing the band intensity and pattern of the DNA standard to that of the tear samples. If nucleases were present in tear fluid, there was smearing and decreased band intensity in the sample lanes. The test can quantitate only DNase activity up to 0.05 Kunitz units and this level represents the upper limit of readout. DNase activity above 0.05 Kunitz units, no matter how high, will degrade the DNA ladder in all lanes and DNase activity still will be read as 0.05 Kunitz units.

Nuclease Activity in Tear Fluid. We used a fluorescence resonance energy transfer (FRET)-based assay to compare total nuclease activity in tear fluid from DED patients ($n = 17$ patients with non-Sjögren dry eyes, including 6 with GVHD and 1 with OCP) and normal controls ($n = 15$). FRET nuclease assays were performed as described by Kiedrowski et al.⁴² The detailed protocol was provided by Alexander R. Horswill, PhD, University of Iowa, Iowa City, IA. The FRET substrate, a "PrimeTime" qPCR probe, was purchased from Integrated DNA Technologies (Coralville, IA). It consists of a short (15 mer) single-stranded oligonucleotide that is modified at the 5' end with a Cy3 fluorophore and at the 3' end with Black Hole Quencher 2 (BHQ2). The sequence of the oligonucleotide substrate is 5' CCC CGG ATC CAC CCC 3'. When the oligonucleotide is intact, the Cy3 and BHQ2 are close enough to quench fluorescence. Upon oligonucleotide cleavage, Cy3 fluorescence is proportional to the amount of cleavage and can be used to quantify nuclease activity. We observed considerable variation in yield of HPLC purified FRET substrate in the three orders (21.1 and 35.8 nmol for the 250 nmol synthesis order, and 124.5 nmol for the 1 μ mol synthesis order); therefore, we have not pooled the data and have reported tear fluid nuclease activity analysis performed with 1 μ mol order. The variation in yield comes from the HPLC purification process and is related to the FRET substrate purity. Tear samples were collected as described above. The samples were incubated on ice until the assay was performed. The assay was performed within 3 hours of sample collection. Tear samples (5 μ L) were added to a microtiter plate. FRET substrate (2.5 nmol in 50 μ L buffer solution) was added to wells containing the tear samples. The fluorescence emitted (RFU) and the rate of fluorescence change were measured using the microplate reader (Synergy H1; BioTek) at 37°C, excitation 552 nm, and emission 580 nm. Plates were agitated for 5 seconds before the readings and readings were taken at 3-second intervals for 30 minutes. From the time of mixing of FRET substrate and tears, there was a 20-second lag before the first fluorescence measurements were obtained. This was the time that is needed for mixing the samples, drawing of the microtiter plate in the microplate reader, and start of readout. Data were corrected for background and a 20-second lag in recording, and analyzed using SigmaPlot software (SigmaPlot, San Jose, CA) to determine the relative amount of fluorescence formed in the burst and rates of slow cleavage reaction. Fluorescence measurements were averaged over 30 minutes and compared.

DNase I Quantification in Tear Fluid. We used a commercially available human DNase I Elisa Kit (Cat. #E01D0214; Life Sciences Advanced Tech, St. Petersburg, FL) to determine the amount of DNase I in tear fluid. We collected 25 μ L of tear fluid ($n = 5$) and 0.5 mL of saliva ($n = 5$) from healthy subjects. Saliva was centrifuged at 15,000 rpm for 5 minutes at 4°C, and 25 μ L of the supernatant were analyzed. The standard or samples (25 μ L) were added to the appropriate well in the antibody precoated microtiter plate, and the assay was performed per the manufacturer's instructions. The sensitivity of this assay was 0.1 ng/mL.

Statistical Analyses

Mean values and SEs of the mean were computed for DED patients and normal subjects, and analyzed using Student's *t*-tests. Microsoft Excel

FIGURE 1. eDNA and neutrophil NETs in exfoliated material derived from Schirmer test strip impressions. (A1, A2) Impression cytology method using Schirmer test strips (A1, arrowhead) and silane coated glass slides (A2). (B) H&E staining showing exfoliated surface cells. (C) Wide-field fluorescent microscope image after DAPI stain of conjunctival impression material reveals short and sparse eDNA strands (arrowhead) in normal subjects (C1) and numerous long eDNA strands (C2) in DED patients (arrowhead). (D) Confocal immunofluorescence image after DAPI staining demonstrates numerous strands (arrowhead) and neutrophils with multilobed nucleus. (E) Confocal immunofluorescence staining image shows that histones (E1, green), neutrophil elastase (E2, red), and eDNA (E3, blue) are the molecular components of NETs (E4, overlay). Arrowhead points to the same area in the same section and indicates a NET strand. Inset in (E2) shows neutrophils with DAPI-stained multilobed nucleus. Scale bars: (B, C1, C2, D) 50 µm; (E1) 10 µm; (E2, inset) 10 µm. Scale bar in (E1) applies to (E1-E4).

office statistics software packages were used for analyses and graphs. $P \leq 0.05$ was considered statistically significant.

RESULTS

Study Population

DED patients ($n = 37$, 73 eyes) had an average aqueous tear production of 2.76 ± 0.35 mm. Normal individuals ($n = 18$, 36 eyes) had an average aqueous tear production of 19.1 ± 1.25 mm, which was significantly greater than DED patients ($P < 0.001$). Patients had non-Sjögren dry eyes (NSS, $n = 33$), which included etiologies, such as idiopathic, post-LASIK, neurotrophic, GVHD, and OCP, and Sjögren disease (SS, $n = 4$).

eDNA and NETs are Present on the Ocular Surface

We performed H&E and immunofluorescence staining on conjunctival cells from patients with severe DED. Cells were derived from test strip impressions on a glass slide after performing the Schirmer I test (Figs. 1A1, 1A2). H&E staining showed exfoliated conjunctival cells present singly or in groups. The cells were round or oval shaped, with an eosinophilic cytoplasm and uniform round basophilic nuclei (Fig. 1B). DAPI nuclear staining revealed a few sparse eDNA strands in normal subjects (Fig. 1C1) and numerous long eDNA strands in DED patients (Fig. 1C2). Confocal microscopy

revealed neutrophils were present among the eDNA strands (Fig. 1D). Histone (Fig. 1E1) and neutrophil elastase (Fig. 1E2) colocalized with DAPI stained eDNA strands, confirming that these were NETs (Fig. 1E4). To rule out the possibility that eDNA could be an artifact of impression cytology with Schirmer strips, we performed similar analyses on exfoliated buccal mucosa cells obtained using identical methodology and did not observe eDNA strands (data not shown).

We also performed H&E and immunofluorescence staining on mucoid films present on the bulbar conjunctiva/cornea (Fig. 2A1) or in the inferior fornix (Fig. 2A2). Mucoid films were present in 7 patients (5 GVHD and 2 idiopathic NSS). Mucoid films appeared as a frothy white mucoid collection that sometimes dispersed with blinking. We used a microcapillary tube to lift these mucoid films for analyses. In some instances, turbid white fluid was drawn in the microcapillary tube. However, the analyses still revealed similar findings. H&E showed numerous neutrophils and exfoliated cells within the mucoid films (Fig. 2B1). DAPI staining showed eDNA (Fig. 2B2) and numerous neutrophil elastase positive neutrophils (Fig. 2B3). Neutrophil elastase (Fig. 2C1) and histone (Fig. 2C2) colocalized with DAPI-stained eDNA strands, confirming that these were NETs (Fig. 2C4).

To confirm that the DAPI-stained strands contained DNA, we captured DAPI strands from membrane slides using a laser capture microdissection microscope (Figs. 2D1, D2). The

FIGURE 2. eDNA and NETs in mucoid films. (A) Clinical photographs of eyes of patients with severe tear-deficient DED. Arrowhead indicates a mucoid film over the cornea and bulbar conjunctiva (A1), and inferior fornix (A2). Inset shows magnified view of mucoid films. (B) Cytological examination of the mucoid films. (B1) H&E staining shows the surface epithelial cells and numerous neutrophils. (B2) DAPI staining shows the presence of eDNA strands (arrowhead) and multilobed nucleus of neutrophils. (B3) Neutrophil elastase immunostaining (red) confirms the presence of neutrophils. (C) Confocal immunofluorescent staining image shows that neutrophil elastase (C1, red), histones (C2, green), and eDNA (C3, blue) are molecular components of NETs (C4, overlay). Arrowhead indicates a NET strand. (D) Laser capture microdissection (LCM) was performed to capture DAPI-stained strands to confirm the presence of DNA in them. (D1) Arrowhead indicates an eDNA strand. (D2) Asterisk occupies the area of the strand post LCM. (D3) GAPDH PCR product in eDNA strand lane confirms the presence of DNA material. Scale bars: (B1, B2, B3) 20 µm; (C, D1, D2) 50 µm. Scale bar in (C3) applies to (C1-C4).

extracellular DAPI-stained strands contain DNA as shown by GAPDH gene product PCR amplification (Fig. 2D3).

We investigated whether cathelicidin was present in NETs. Cathelicidin was present within mucoid films (Fig. 3A1), and colocalized with neutrophil elastase and DAPI-stained nuclear material (Fig. 3A4). Cathelicidin also was present within neutrophils (Fig. 3B4). Cathelicidin, nuclear material, and neutrophil elastase extruded from the neutrophil to form NETs (Fig. 3C1-3C4).

Amount of eDNA on the Ocular Surface

DED patients had significantly lower aqueous tear production compared to normal subjects (Fig. 4A). eDNA strand length was significantly greater ($P = 0.002$) in DED patients (15.0 ± 4.2 mm) compared to normal subjects (1.58 ± 0.47 mm, Fig. 4B). The amount of eDNA on the ocular surface, as measured by PicoGreen assay, also was significantly greater ($P = 0.006$) in DED patients ($20,137.2 \pm 1507.3$ RFU) compared to controls ($13,055.5 \pm 1787.2$ RFU, Fig. 4B).

eDNA Signaling Pathway Gene Expression

We performed qPCR on exfoliated conjunctival cells to determine the fold change in the expression of genes downstream of eDNA signaling (Fig. 4D). The expression of

TLR9, MyD88, and IFN-type I (IFNA and IFNB), as well as the inflammatory genes *IL-6* and *TNF-α*, was increased significantly in conjunctival cells from DED patients. The fold increase in gene expression in DED patients observed was: TLR9 (5.57 ± 1.6 , $P = 0.003$), MyD88 (4.20 ± 0.6 , $P < 0.0001$), IFNA (3.09 ± 0.5 , $P = 0.0003$), IFNB (4.18 ± 0.6 , $P < 0.0001$), *TNF-α* (20.6 ± 10.0 , $P = 0.03$), and *IL-6* (17.3 ± 3.1 , $P < 0.0001$).

Nucleases and DNase I are Present in Tear Fluid

We used DNase I specific antibodies to immunostain lacrimal gland sections. DNase I localized within the epithelial cells lining the lacrimal gland acini (Fig. 5A). We performed ELISA on tear samples to determine the amount of DNase I in tear and saliva (Fig. 5B1). DNase I concentration in tear was 3.14 ± 0.49 ng/mL and in saliva it was 4.21 ± 1.14 ng/mL.

We quantitated nuclease activity in the tear using a DNase detection kit assay (Fig. 5B2). The nuclease activity in tears of normal subjects and DED patients was greater than or equal to 0.05 Kunitz units. We were unable to use this assay to quantitate and compare tear fluid nuclease activity of DED patients and normal subjects because 0.05 Kunitz units is the upper limit of readout. Therefore, we performed a FRET-based nuclease activity assay to compare total nuclease activity between DED patients and normal subjects (Fig. 5C1). The normals showed a

FIGURE 3. Confocal immunofluorescent staining image showing cathelicidin in NETs and neutrophils (**A1-A4**) Cathelicidin (green) is present in mucoid films (**A1**, arrow) and eDNA strands (**A1**, arrowhead). Cathelicidin colocalizes with neutrophil elastase (**A2**, red) and DAPI nuclear stain (**A3**, blue). (**B1-B4**) Cathelicidin (green) is present in neutrophils (**B1**, arrowhead) and colocalizes with neutrophil elastase (**B2**, red) and DAPI (**B3**, blue). (**C1-C4**) Cathelicidin (**C1**, arrowhead) and DAPI-stained nuclear material (**C3**, arrowhead) are extruded from a neutrophil to form NETs. Scale bars: 10 μ m. Scale bar in (**A1**) applies to (**A1-A4**), in (**B1**) applies to (**B1-B4**), and in (**C1**) applies to (**C1-C4**).

large burst and larger growth in signal as compared to DED patients. DED patients showed either no burst with small growth in signal or a larger burst with no growth in signal. The slope and burst signal was consistently greater in normal than DED patients. Average fluorescence was significantly lower in the DED patients (8557 ± 124 RFU, $P < 0.05$) compared to controls ($22,934 \pm 250$ RFU, Fig. 5C2). To characterize the fast (prompt) and slow reactions, we performed experiments using the FRET substrate and known concentration of tear film nucleases (recombinant human DNase I or recombinant human lipocalin-1). The important observation was that most of the kinetic runs clearly were biphasic (supplementary data, available at <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10430/-/DCSupplemental>).

DISCUSSION

Our study yielded four important findings. First, eDNA and NETs are present in excessive amounts on the ocular surface of patients with severe, tear-deficient DED. Immunolocalization experiments revealed that the molecular components of NETs include histones, cathelicidin, and neutrophil elastase. Next,

using a DNA digestion assay, we determined that tear fluid nuclease activity was >0.05 Kunitz units. Using a FRET-based assay, we found that tear fluid nuclease activity was reduced in DED patients. We determined that DNase I is a DNA hydrolyzing nuclease present in lacrimal glands and a normal concentration in tear fluid is 3.14 ng/mL, similar to that in serum and saliva. We also determined that mucoid films are present on the ocular surface or inferior conjunctival fornix in some patients with severe DED (particularly with GVHD), and are rich in neutrophils, eDNA, and NETs. Finally, exfoliated ocular surface cells from DED patients had increased expression of inflammatory cytokines and eDNA signaling pathway genes.

Taken together, these findings suggested that in healthy eyes, eDNA is produced in the precorneal tear film and cleared by tear fluid nucleases. In patients with severe DED, tear fluid nuclease deficiency allows eDNA, neutrophils, and NETs to accumulate in the precorneal tear film and cause ocular surface inflammation. The practical implication of our findings is the suggestion of new therapeutic interventions based on clearing eDNA, NETs, and their molecular components from the ocular surface, as well as inhibiting eDNA signaling pathway gene expression. Since our study was performed on symptomatic

FIGURE 4. Amount of ocular surface eDNA and eDNA signaling pathway gene expression in DED patients and controls. **(A)** The average aqueous tear production, measured using Schirmer I test, was significantly lower in DED patients. **(B)** eDNA length was measured after impression of Schirmer strip on a glass slide. The eDNA length was significantly greater in DED patients. **(C)** eDNA amount on the Schirmer strips was measured using PicoGreen assay. DED patients have significantly greater eDNA amounts. **(D)** Genes in the eDNA signal transduction pathway were significantly overexpressed in exfoliated conjunctival cells from DED patients. **(E)** Inflammatory gene expression also was significantly increased in DED patients. * $P < 0.05$.

patients with severe tear-deficient DED, additional studies will be needed to determine whether ocular surface eDNA, NETs, and tear film nuclease activity are dysregulated in dry eye patients with milder disease.

Extracellular DNA must re-enter a cell and bind its intracellular receptor to stimulate downstream signaling pathways.¹² Cathelicidin binds eDNA and enhances its intracellular entry.⁴³ Cathelicidin localized within neutrophils and NETs on the ocular surface of patients with severe DED, particularly within the mucoid films. Once inside the cell, DNA binds TLR9 to stimulate signaling through MyD88, which initiates a signaling cascade leading to an IFN-type I response.^{44,45} We found significantly increased expression of mRNA for TLR9, MyD88, and IFN-type I in exfoliated conjunctiva cells from DED patients. These data agreed with a previous study that found increased expression of IFN-type I pathway genes, as well as IFN-inducible genes, in salivary gland and conjunctival cells from patients with primary Sjögren's syndrome.⁴⁶ Type I IFNs (IFN- α/β) augment dendritic cell maturation and activate the adaptive immune system. In addition to increased expression of mRNA for TLR9-MyD88 pathway genes, IL-6 and TNF- α gene expression was increased in exfoliated conjunctival cells. The expression of these inflammatory cytokines increases in the corneal and conjunctival epithelium in experimental DED.^{47,48} Thus, based on our findings and those of others, we proposed a mechanism for inflammation in severe DED. Extracellular DNA and NETs in tear fluid bind cathelicidin, and re-enter ocular surface cells to stimulate the TLR9-MyD88 pathway and activate the IFN type I response. In this way, eDNA links the innate and adaptive immune mechanisms.

One limitation of our method of collecting exfoliated cells using Schirmer strips is that cells from bulbar as well as palpebral conjunctiva are collected, and they may not be expressing the same genes as each other. Therefore, with our technique we can comment only on the expression of inflammatory genes on the overall ocular surface. To develop appropriate targets for therapeutic intervention, further investigations are needed to determine the differential expression of these inflammatory genes (bulbar conjunctiva versus palpebral epithelium and conjunctiva versus corneal epithelium), as well as the posttranscriptional and posttranslational modifications that influence final abundance of their protein products.

Neutrophils have an important role in innate immunity. We observed numerous neutrophils on the ocular surface of DED patients, particularly in mucoid films. Furthermore, we found increased TLR9 and MyD88 gene expression in the conjunctival exfoliated material from DED patients. Several lines of evidence suggest that neutrophil recruitment is linked inextricably to TLR9-MyD88 signaling. Increased expression of genes in the TLR9-MyD88 signaling pathway in dead or dying cells is necessary for neutrophil recruitment.^{49–51} Also, topical application of a synthetic bacterial DNA mimic to injured corneal epithelium results in recruitment of neutrophils to the corneas of wild-type, but not TLR9-/- mice.⁵² Therefore, because TLR9 is an eDNA receptor and is expressed in the corneal epithelium, eDNA may stimulate TLR9-MyD88 signaling in desquamated ocular surface cells to recruit neutrophils to the precorneal tear film of DED patients. Once recruited to the ocular surface, neutrophils can form NETs in the precorneal tear film. NETosis denotes a unique neutrophil response, in which nuclear DNA, histones, neutrophil elastase,

FIGURE 5. Nucleases and DNase I are present in tear fluid. (A1–A6) Confocal immunofluorescent microscope image showing DNase I in human lacrimal glands (A1–A3, red). The specificity of the staining was confirmed with peptide competition (A4–A6) and isotype control staining (not shown). (B) Nuclease activity in the tears of normal subjects. DNase I ELISA showed that its concentration in normal tear fluid is 3.14 ng/ml. In the DNase detection kit, normal tears completely degraded DNA (tears lane), Therefore, tear fluid nuclease activity is greater than 0.05 Kunitz units. (C1) Nuclease activity in tears was quantitated using a FRET assay. Plot of corrected fluorescence emission signal versus time for four normal (top four traces) and two DED patients (bottom two traces) is shown. The measured emission values were corrected by subtraction of the background signal from the probe substrate. The legend lists the intercepts in RFU and slopes in RFU/min for each trace; errors are given at the 95% confidence interval. (C2) Graph showing that the nuclease activity in DED patients was significantly lower compared to healthy individuals. * $P < 0.05$. Scale bar in (A1): 20 μ m applies to (A1–A6).

and cathelicidin emerge from the cell as NETs.^{23,25} NETs and their molecular components can injure cells directly. Saffarzadeh et al. reported that NETs, particularly their histone component, can cause direct cytotoxicity to epithelial cells.⁵³ Extracellular histones are major mediators of cell death in sepsis.⁵⁴ Cathelicidin peptide fragments can cause inflammation, erythema, and telangiectasia, particularly in patients with rosacea.⁵⁵ Neutrophil elastase induces epithelial cell apoptosis.⁵⁶ We observed extracellular histones, cathelicidin, and neutrophil elastase within ocular surface NETs. Given the known toxic effects of NETs and their molecular components, it is reasonable to hypothesize that they have a role in producing some of the clinical signs and pathologic features that are characteristic of DED. For example, cathelicidin fragments may have a role in producing conjunctival erythema and eyelid margin telangiectasia, and neutrophil elastase may cause conjunctival epithelial apoptosis. We found that neutrophils and NETs were particularly numerous in mucoid films on the ocular surface and/or in the inferior conjunctival fornix. It is not surprising to find mucins intermixed with NETs because

neutrophil elastase causes membrane-associated mucin release.⁵⁷ Our findings suggested that neutrophil-rich mucoid films on the ocular surface are a clinical sign of active inflammation. This conclusion agrees with previous reports that demonstrate neutrophils on the ocular surface during active corneal inflammation.¹⁹ Thus, we proposed a mechanism for ocular surface disease in patients with severe DED. The presence of excessive amounts of NETs and their molecular components (histone, neutrophil elastase, and cathelicidin) on the ocular surface can cause directly epithelial cell injury, conjunctival erythema, eyelid margin telangiectasia, as well as conjunctival and cell apoptosis.

Our experimental data cannot exclude the potential positive effects of NETs, such as defense against pathogens. In addition to their antimicrobial action, confinement of pathogens to a local site of infection might be an important function of NETs.²⁴ In addition, immobilizing neutrophil granule components into NETs may keep potentially noxious proteins and proteases from diffusing away and inducing damage in tissue adjacent to the site of inflammation. NET

formation initially may be a protective response to ward off infection of a severely compromised ocular surface, but which then, in the double-edge sword that often is inflammation, becomes detrimental.

We used two assays to assess tear fluid nuclease activity. The first assay was a commercially available kit that uses the DNA ladder as a template. The second assay observed the ability of nucleases to degrade a short single-stranded oligonucleotide in real time. The nuclease activity in tears from normal subjects and DED patients was >0.05 Kunitz units. We were unable to use a DNase detection kit assay to quantitate and compare tear fluid nuclease activity because 0.05 Kunitz units is the upper limit of readout using this method. Further, in some DED samples, the DNA ladder did not migrate and stuck to the lane wells, presumably due to interaction with tear fluid proteins. Therefore, we performed a FRET-based nuclease activity assay to compare total nuclease activity between the groups. Upon FRET substrate cleavage, fluorescence is proportional to the amount of cleavage and can be used to quantify nuclease activity. The FRET experiments did not show the same baseline at time 0 because there was a prompt (burst) signal formation that was more efficient for normals than in patients with DED. Because there was a 20-second lag between the start of fluorescence measurements and mixing of FRET substrate with tears, we were unable to analyze the prompt process. By the time the first fluorescence measurement was recorded, the prompt response was largely complete. Subsequent growth in signal was recorded over 30 minutes. Overall, our data suggested that there are two important processes at play. One is a fast DNA lysis that we did not follow kinetically but that gives the initial fluorescence signal values far above background. The second is a slow process that resulted in the slow growth in signal seen for most patients. DED patients appeared to be deficient in one or the other process. To characterize the fast (prompt) and slow reactions, we performed experiments using the FRET substrate and known concentrations of recombinant human tear film nucleases (supplementary data, available at <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10430/-/DCSupplemental>). The important observation was that most of the kinetic runs clearly were biphasic (supplementary Fig. S1, available at <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10430/-/DCSupplemental>). There was a "burst" reaction and a slow reaction similar to the data from tears of patients, and the burst reaction was complete in several seconds. There are simple possible explanations for our kinetic data. For example, the fast reaction might result from very fast binding of DNA to the nuclease followed by cleavage of DNA to give the fluorophore liberated from the quencher (i.e., we measured the rate of cleavage reaction). If the product DNA from that reaction binds the nuclease tightly, then the slow reaction could be release of the DNA, which would be followed by another binding and cleavage reaction. Both of these reactions will be dependent only on the nuclease concentration. The above model would fit our data for the normal cases. This model suggested that nuclease concentration is low or absent in DED patients.

Sources of eDNA on the ocular surface and in tear fluid include desquamated surface epithelial cells, inflammatory cells (predominantly neutrophils), and eyelid margin microbes. Nucleases hydrolyze eDNA and enable its clearance from the ocular surface. Lipocalin is a nuclease present in tears and reduced in the tear fluid of patients with evaporative DED.^{14,58} DNase I is another well-known nuclease present in human tears.¹⁵ We localized DNase I protein in the lacrimal gland and used ELISA to determine that its concentration in human tears is 3.14 ng/ml. This value correlates well with the serum DNase I concentration. Deficient tear fluid nuclease activity may lead to decreased eDNA hydrolysis on the ocular surface, allowing

eDNA to accumulate. Interestingly, systemic conditions where DNase I activity is deficient, such as SLE, have a high association with DED.^{59,60} DNase I is present in serum in similar amounts as in tear fluid, and serum tear eye drops are an effective treatment in severe DED patients.^{61,62} Our findings suggested that the therapeutic effect of serum tears may be due partly to the presence of DNase I. These clinical observations, coupled with our findings, should lead to investigations to assess the therapeutic potential of topical DNase I therapy in severe DED patients. Further, determining the hydrolytic products of eDNA may provide a signature that may be unique for each tear nuclease. These follow-up investigations may be important to determine which specific nuclease (lipocalin or DNase) is deficient and which nuclease hydrolyzes eDNA efficiently.

In conclusion, our data substantiated the hypothesis that in DED, eDNA production and clearance mechanisms are dysregulated. Tear fluid nuclease deficiency allows eDNA and NETs to accumulate in the precorneal tear film and drive ocular surface inflammation. Our findings suggested new therapeutic interventions in severe DED, including the use of topical DNase I to hydrolyze eDNA and NETs, as well as molecular strategies to inhibit cathelicidin, neutrophil elastase, and TLR9-MyD88 pathway targets.

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