Elevated Neutrophil Elastase in Tears of Ocular Graft-Versus-Host Disease Patients

SAMER N. ARAFAT, MARIE-CLAUDE ROBERT, TULIO ABUD, SANDRA SPURR-MICHAUD, FRANCISCO AMPARO, CLAES H. DOHLMAN, REZA DANA, AND ILENE K. GIPSON

• PURPOSE: To investigate the levels of neutrophil elastase (NE), matrix metalloproteinases (MMPs), and myeloperoxidase (MPO) in tear washes of patients with ocular graft-vs-host disease (oGVHD).
• DESIGN: Case-control study.
• METHODS: Based on established criteria, oGVHD patients (n = 14; 28 eyes) and age-/sex-matched healthy controls (n = 14; 28 eyes) were enrolled. Tear washes were collected and analyzed for NE using a single-analyte enzyme-linked immunosorbent assay (ELISA). MMPs (1, 2, 3, 7, 8, 9, 12), MPO, and tissue inhibitor of matrix metalloproteinase (TIMP)-1 were analyzed using multianalyte bead-based ELISA assays. Total MMP activity was measured using a fluorimetric assay. Correlation studies were performed between NE, MMP-8, MMP-9, and MPO within study groups.
• RESULTS: NE, MMP-8, MMP-9, and MPO levels were elevated in oGVHD tears when compared with controls ($P < .0001$). NE was the most elevated analyte. MMP activity was higher and TIMP-1 levels were lower in oGVHD than in control ($P < .0001$). In oGVHD, NE significantly correlated with MMP-8 (r = 0.92), MMP-9 (r = 0.90), and MPO (r = 0.79) ($P < .0001$). MMP-8 correlated with MMP-9 (r = 0.96, $P < .0001$), and MPO (r = 0.60, $P = .001$). MMP-9 correlated with MPO (r = 0.55, $P = .002$). In controls, NE, MMP-9, and MPO significantly correlated with each other ($P < .0001$).
• CONCLUSIONS: The marked increase in NE in oGVHD tears that correlated strongly with elevated MMP-8, MMP-9, and MPO suggests a common neutrophilic source and provides evidence of neutrophil activity on the ocular surface of oGVHD patients. (Am J Ophthalmol 2017;176:46–52. © 2017 Elsevier Inc. All rights reserved.)

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From Schepens Eye Research Institute-Massachusetts Eye and Ear (S.N.A., M.-C.R., T.A., S.S.-M., F.A., R.D., I.K.G.), and Massachusetts Eye and Ear Infirmary (S.N.A., M.-C.R., F.A., C.H.D., R.D.), Harvard Medical School, Boston, Massachusetts; and Department of Ophthalmology and Visual Sciences, Paulista School of Medicine, Federal University of Sao Paulo-UNIFESP, Sao Paulo, Brazil (T.A.).

Inquiries to Ilene K. Gipson, Schepens Eye Research Institute-Massachusetts Eye and Ear, Harvard Medical School, 20 Staniford St, Boston, MA 02114; e-mail: ilene_gipson@meei.harvard.edu

GRAFT-VS-HOST DISEASE (GVHD) IS A SYSTEMIC complication and major cause of morbidity and mortality in patients after allogeneic hematopoietic stem cell transplantation (allo-HSCT) for neoplastic and nonneoplastic hematologic disorders.[1,2] It is a complex, multiorgan, multifactorial, immune-mediated disease that involves an interplay between the donor and recipient adaptive and innate immune systems and affects many organs, primarily the skin, gastrointestinal system, liver, lungs, oral mucosa, and eyes.[1,2] Ocular involvement in GVHD, also known as ocular graft-vs-host disease (oGVHD), occurs in 40%–60% of allo-HSCT recipients and can be seen in up to 90% of patients with chronic GVHD.[2,3] Ocular GVHD can involve virtually all components of the eye, particularly the cornea, conjunctiva, and lacrimal and meibomian glands, and predominantly manifests as dry eye syndrome/keratoconjunctivitis sicca (KCS).[4–7] The pathologic mechanism of ocular surface manifestations in GVHD remains unclear.

The clinical evaluation of oGVHD severity relies primarily on the assessment of signs and symptoms of dry eye. This includes, but is not limited to, tear breakup time (TBUT), Schirmer I test, corneal fluorescein staining (CFS), meibomian gland evaluation (MGE), and the Ocular Surface Disease Index (OSDI) questionnaire. Ocular complications that contribute to symptoms and signs of dry eye include lacrimal and meibomian gland dysfunction, lid scarring, pseudomembranous conjunctivitis and subsequent cicatricial changes that all lead to impaired quality of life.[2,3,8] In addition, persistent inflammation and ocular surface damage in oGVHD can lead to stem cell deficiency and subsequent corneal epitheliopathy, a condition that increases the risk for corneal ulceration and perforation.[9,10]

Neutrophils are the primary effector cells of acute inflammation that are recruited to the cornea and ocular surface in response to tissue injury, stress, and infection to provide constant protection and maintain tissue integrity and hemostasis.[11] However, in nonresolving inflammation, abundant and activated neutrophils may play a role in tissue damage and pathology and have been suggested to be associated with a number of ocular surface diseases by their presence in cornea and conjunctiva and their inflammatory enzymes in tears.[12–15]

Neutrophil elastase (NE) is an important multifunctional enzyme predominantly released by neutrophils. NE

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is involved in direct intracellular killing of phagocytosed bacteria (in combination with myeloperoxidase [MPO] and reactive oxygen species); extracellular killing via trapping bacteria in neutrophil extracellular traps (NETs) secreted by activated neutrophils; processing of cytokines, chemokines, and growth factors; and processing and activation of cellular receptors including toll-like receptors (TLRs).[16] However, NE is also considered to be one of the most destructive enzymes owing to its ability to degrade almost all components of the extracellular matrix, by activating collagen-degrading enzymes collectively called matrix metalloproteinases (MMPs) and inactivating tissue inhibitors of matrix metalloproteinases (TIMPs)[17] NE was also found to be a potent sheddase of membrane-tethered mucins present on the apical surface of corneal epithelium.[18–20]

The purpose of this study was to compare NE levels in tears of oGVHD patients with age- and sex-matched normal subjects. In addition to NE, MMPs, TIMP-1, MPO, and total MMP activities were also analyzed. Correlations between tear levels of NE, MMPs, and MPO were performed to ascertain their cellular origin on the ocular surface.

METHODS

THIS CASE-CONTROL STUDY INCLUDED 28 EYES OF 14 OGVHD patients (oGVHD group) and 28 eyes of 14 age- and sex-matched normal controls (control group) enrolled in the study. Informed consent was obtained from all participants prior to participation in the study. The study protocol was approved by the Human Studies Committee of the Massachusetts Eye and Ear Infirmary (MEEI), Boston, Massachusetts, and all procedures complied with all the requirements of the Health Insurance Portability and Accountability Act and the tenets of the Declaration of Helsinki. Study participants were recruited and tear washes were performed at MEEI and Schepens Eye Research Institute/Mass Eye and Ear between January 2014 and April 2015.

We analyzed the levels of NE, MMPs, TIMP-1, MPO, and total MMP activity in tear washes. All patients with chronic oGVHD had undergone allo-HSCT, had a previous diagnosis of acute or chronic systemic GVHD, and were diagnosed with chronic oGVHD according to the National Institutes of Health diagnostic criteria.[21] Briefly, all patients presented with new onset of ocular sicca symptoms (dryness, grittiness, pain) after the transplant with an Ocular Surface Disease Index (OSDI) score >23, punctate keratitis (corneal fluorescein staining [CFS] score ≥4, NEI grading system) by slit-lamp examination, and a mean Schirmer test of ≤10 mm in both eyes. All patients in the oGVHD group were in good stable overall health at the time of the study, with no history of immune disease (other than GVHD), ocular or periocular malignancy, corneal epithelial defect, or herpetic keratitis. Normal subjects were in good overall health, with no systemic or ocular surface diseases, allergies, ocular surgeries, ophthalmic drop use, or contact lens wear. Pregnant or lactating women were excluded from the study.

• **TEAR WASH COLLECTION AND PROCESSING:** The tear washes were performed as previously described[22] before any scheduled manipulation (eg, intraocular pressure assessment) or instillation of any diagnostic drops. A total of 60 μL of previously aliquoted sterile physiologic saline was applied into the inferior fornix without topical anesthetics using a sterile micropipette. The subjects were asked to slowly roll their eyes up, down, nasally, and temporally once without blinking. The tear washes were then collected from the inferior fornix by micropipette, transferred into sterile polypropylene tubes, and centrifuged for 30 minutes at 13 500 rpm to remove any cellular debris. The supernatants were stored at −80 C.

Protein concentrations were determined using the Micro BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) according to the manufacturer's instructions and the tears were aliquoted and stored at −80 C for further analysis.

• **TEAR WASH ANALYSIS:** NE levels were measured using the Human Elastase enzyme-linked immunosorbent assay (ELISA) Kit (Hycult Biotech, Uden, The Netherlands) using the manufacturer's instructions and analyzed on a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, California, USA). Enzyme concentrations (ng/mL) were normalized to microgram of protein loaded in the assay (ng/mL/μg protein loaded).

Total (proform and active) levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-12 were measured using the Fluorokine Human MMP MultiAnalyte Profiling Kit (R&D Systems, Minneapolis, Minnesota, USA) and MPO and TIMP-1 levels were measured using the Fluorokine Human Cardiac MultiAnalyte Profiling Kit (R&D Systems). The assays were analyzed using a Bio-Rad Bio-Plex analyzer powered by Luminex 100 xMAP technology (Luminex Corp, Austin, Texas, USA) based on manufacturer's instructions. The final concentrations (ng/mL) were normalized to microgram of protein loaded in the assays (ng/mL/μg protein loaded).

Total MMP activity was measured using a modified protocol of the MMP-9 Fluorometric Drug Discovery Kit (Enzo Life Sciences, Plymouth Meeting, Pennsylvania, USA) as previously described.[23] The tear wash samples were diluted in MMP-1 assay buffer into a SensoPlate black, 96-well, glass-bottom plate (Greiner Bio-One North America, Inc, Monroe, North Carolina, USA). A quenched OmniMMP RED fluorogenic substrate (Enzo Life Sciences) was added into each well. Fluorescence was measured at 540 nm (excitation) and 590 nm (emission) using the Synergy Mx monochromator-based multimode microplate reader (Biotek U.S., Winooski, Vermont, USA) at 1-minute intervals for 45 minutes. The slopes derived from