Pathogenesis of Dry Eye in Graft Versus Host Disease (GVHD): Role of Ocular Mucins and Conjunctival Fibrosis

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PATHOGENESIS OF DRY EYE IN GRAFT VERSUS HOST DISEASE (GVHD): ROLE OF OCULAR MUCINS AND CONJUNCTIVAL FIBROSIS

A Dissertation by

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ABSTRACT

PATHOGENESIS OF DRY EYE IN GRAFT VERSUS HOST DISEASE (GVHD): ROLE OF OCULAR MUCINS AND CONJUNCTIVAL FIBROSIS

by Kiumars Shamloo

Allogenic hematopoietic stem cell transplantation is a procedure that offers a possible cure for hematologic cancers and other hematologic disorders. Unfortunately, despite the increasing survival rate of patients, the quality of their life is adversely affected by the allogeneic bone marrow transplantation’s major side effect i.e Graft vs. Host Disease (GVHD). GVHD is a complex, multi-organ disease resulting from an immunological attack by donor engrafted immune cells to host organs, including the eye surface. Mostly based on the time of disease onset after transplantation, GVHD is divided into the acute and chronic phase. The eyes may be involved in both acute and chronic GVHD, although ocular involvement is more common in the chronic GVHD with a more severe presentation. As high as 40%-60% of patients with chronic GVHD suffer from debilitating ocular surface damage manifesting as severe dry eye and cicatricial conjunctivitis. Several pathological mechanisms likely contribute to the etiology of ocular GVHD-associated dry eye disease. Despite the high frequency of ocular surface involvement in patients experiencing GVHD, little is known about the underlying pathogenesis responsible for ocular GVHD-associated dry eye. In our first manuscript, using a mouse model of allogeneic transplantation, we demonstrated that ocular GVHD causes a decrease in tear film volume and corneal keratopathy. These ocular surfaces changes are accompanied by a significant decrease in the area and thickness of corneal glycocalyx, a decrease in ocular surface mucins, MUC4 and MUC5AC and loss of
conjunctival goblet cells. Our data showed that topical treatment by mucin secretagogue, rebamipide, partially attenuates GVHD-associated damage to ocular surface. Hyperosmolar tears are a consistent feature of dry eye disease. Our data demonstrated that hyperosmolar stress increases the gene expression of NFAT5, a tonicity-related transcription factor and pro-inflammatory cytokines (IL1, IL6, TNFα, IFN-γ) in human corneal and conjunctival epithelial cells. Next, we demonstrated that these proinflammatory cytokines differentially modulate the expression of MUC 1, and MUC 4 in ocular surface epithelial cells, but they do not cause any notable change in glycocalyx or apoptotic cell death in stratified human corneal and conjunctival epithelial cells. Immune-mediated ocular surface damage in GVHD can initiate a wound healing response leading to fibrosis in the conjunctiva, a fibroblast-rich tissue. Conjunctival fibrosis may underlie the noted decrease in goblet cells and ocular surface mucins, thus contributing to GVHD-associated dry eye. Our results demonstrated that ocular GVHD causes a significant increase in expression of α-smooth muscle actin (SMA), a marker of myofibroblasts, in the conjunctiva. Immunostaining detected the presence of large number of myofibroblasts in bulbar orbital conjunctiva of GVHD mice. An increase in the components of renin-angiotensin system component (RAS), angiotensinogen and angiotensin converting enzyme, was also noted in the conjunctiva suggesting that ocular GVHD causes conjunctival fibrosis by myofibroblast formation and activation of conjunctival RAS.
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<tr>
<td>AT1</td>
<td>Angiotensin II type 1</td>
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<tr>
<td>AACP</td>
<td>American Association of Colleges of Pharmacy</td>
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<tr>
<td>ARVO</td>
<td>Association for Research in Vision and Ophthalmology</td>
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<tr>
<td>α-SMA</td>
<td>Alpha-Smooth muscle actin</td>
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<tr>
<td>BSS</td>
<td>Balanced Salt Solution</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>cGy</td>
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<td>CTGF</td>
<td>Connective tissue growth factor</td>
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<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>GVHD</td>
<td>Growth versus host disease</td>
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<td>IFN-γ</td>
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<td>HEPES/F12</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
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<tr>
<td>IL6</td>
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<tr>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>μg/ml</td>
<td>Microgram per milliliter</td>
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<tr>
<td>ml/ml</td>
<td>Milliliter per milliliter</td>
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<tr>
<td>Acronym</td>
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<td>---------</td>
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<td>mM</td>
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<td>NFATx</td>
<td>Nuclear factor of activated T cells x</td>
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<tr>
<td>OCT</td>
<td>Optimal cutting temperature medium</td>
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<tr>
<td>PAS</td>
<td>Periodic acid–Schiff</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>RAS</td>
<td>renin–angiotensin system</td>
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<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
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<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
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CHAPTER 1

Introduction

Over the past few decades, allogeneic hematopoietic stem cell transplantation has emerged as a potentially curative therapy for some hematologic malignancies and hemoglobinopathies\(^1\). Despite the advances in HLA matching and the use of immunosuppressive drugs, graft versus host disease (GVHD) remains a frequent complication of allogeneic hematopoietic stem cell transplantation. GVHD is an immune condition caused by a complex interplay between donor immune cells, host immune organs, and host tissues\(^2-6\). Depending upon the time of onset and clinical manifestations, GVHD is divided into the acute and chronic phases. Acute GVHD primarily affects the liver, skin, and intestine. On the other hand, chronic GVHD causes a high incidence of ocular complications\(^7-9\).

Ocular GVHD

Ocular GVHD most often presents as severe dry eye and ocular surface disease, which can have a profound impact on the quality of life in its survivors\(^6\). It is diagnosed based on eye-related signs and symptoms such as hyperemia, hemorrhagic conjunctivitis, pseudomembrane formation, lagophthalmos, pain, blurry vision, foreign body sensation, burning sensation, serious light sensitivity, acute conjunctivitis and corneal ulceration\(^10\). It happens in more than 60\% of patients with chronic GVHD\(^10\). Without adequate treatment, dry eye disease in patients with GVHD may advance to corneal keratopathy, ulceration and lead to impairment of vision\(^11\).
**Ocular surface mucins and glycocalyx**

Tear film is a thin layer of fluid that covers the eye surface. Normal tear film is critical for keeping the ocular surface hydrated and lubricated, thus preventing desiccation-induced damage to the ocular surface\textsuperscript{12}. The lacrimal functional unit, including eye surface nerves, ocular surface glycocalyx, lacrimal glands, meibomian glands, and normal blinking response, all contribute collectively to a healthy tear film’s secretion and maintenance\textsuperscript{12}. The tear film, primarily made up of a muco-aqueous gel layer that underlies and partially mixes with an overlaying lipid layer, is critical for keeping the eye surface hydrated and lubricated \textsuperscript{12}. Tear film's mucous layer mainly consists of membrane-bound and secreted mucins. Mucins are high molecular weight glycoproteins made up of a protein core with extensive glycan N-acetyl galactosamine side chains\textsuperscript{13}. Heavy glycosylation imparts a negative charge and hydrophilicity to the mucins\textsuperscript{14}. These structural features account for their two vital physiological functions of repelling pathogens and keeping the eye surface hydrated and lubricated. The tear film mucins include the membrane-bound mucins (MUC): MUC1, MUC4, MUC16, soluble MUC7; it also includes the gel-forming secreted mucins MUC19, MUC5AC\textsuperscript{15}. The membrane-bound mucins which are expressed on the apical surface of corneal and conjunctival epithelial cells, are glycosylated and sialylated along with galectin 3 form a continuous layer called glycocalyx, which help to protect cells against mechanical and chemical damage, prevent pathogen penetration into the eye, reduce friction during blinking, and maintain the hydrophilicity of the ocular surface. MUC5AC is mainly secreted by the goblet cells\textsuperscript{16}. How GVHD affects ocular surface mucins and glycocalyx is not known \textsuperscript{15}. 
Besides providing hydration, lubrication and antimicrobial functions, ocular surface membrane-tethered mucins have also been shown to act as signaling molecules. Specific localization of mucins to the apical surfaces of epithelial cells suggests that their signaling functions may be important as sensor mechanisms in response to invasion or damage of epithelia. MUC1 and MUC4 are the two membrane mucins, which have apparently similar structures. Recent studies have also implicated them in cellular signaling. MUC4 has been shown to act as an intramembrane receptor-ligand for the receptor tyrosine kinase ErbB2/HER2/Neu triggering specific phosphorylation of the ErbB2 in the absence of other ErbB ligands\textsuperscript{17}. MUC1, through its highly conserved cytoplasmic tail, has been shown to bind to \(\beta\)-catenin\textsuperscript{17}.

**Hyperosmolar stress and ocular surface epithelial cells**

Osmotic stress is one of the common stresses for organs, such as the kidney, skin, respiratory track, and eye. Osmotic stress can have longstanding detrimental effects that can cause tissue damage in these organs. Increased levels of tear osmolarity is a consistent feature of GVHD dry eye\textsuperscript{18}. Nuclear factor of activated T-cells (NFAT) proteins is a family of transcription factors, which were originally identified to be expressed in most immune-system cells. This family contains five members: NFAT1 (NFATp), NFAT2 (NFATc), NFAT3, 4, (NFATx), and NFAT5 (tonicity response element-binding protein or EBP27) was initially identified as a transcription factor involved in cellular responses to hypertonic stress\textsuperscript{28}. NFAT5 is activated by phosphorylation under a hyperosmotic environment and is amongst the few known tonicity-regulated transcription factors in mammalian cells. NFAT5 transcriptionally regulates the expression of target genes.
responsible for the metabolism of organic osmolytes, including aldose reductase, taurine transporter, betaine/GABA transporter, and sodium/myo-inositol transporter. NFAT5 also induces molecular chaperones, such as heat shock protein 70 and 94. Does osmotic stress modulate NFAT5 expression in corneal and conjunctival epithelial cells is worth testing?

Patients with dry eye have been shown to have elevated levels of proinflammatory cytokines. How tear hyperosmolarity affects the production of proinflammatory cytokines from ocular surface epithelial cells needs to be investigated. Proinflammatory cytokines released by immune cells in GVHD have been shown to exert a damaging effect on various tissues. The promoter region of genes encoding for mucins has response elements for signaling pathways activated by these proinflammatory cytokines, suggesting that these cytokines can modulate the gene expression of mucins. Additionally, these cytokines have been shown to induce the expression of enzymes, such as heparanase and hyaluronidase that may have a detrimental effect on the glycocalyx.

**GVHD associated fibrosis**

GVHD patients suffer from longstanding fibrosis of skin, liver, lungs and gastrointestinal tract resulting in clinical manifestations of the disease such as scleroderma, bronchiolitis obliterans syndrome, and liver damage. Mouse models of chronic GVHD manifest fibrosis of skin, liver, lungs, and gastrointestinal tract. There are anecdotal clinical reports of conjunctival fibrosis in GVHD patients. However, there is no systemic preclinical study in a mouse model that examines the time course and severity of conjunctival fibrosis and its correlation to GVHD associated dry eye.
Myofibroblasts are key feature of fibrosis and can form by trans-differentiation of resident fibroblasts, mesenchymal cells, and circulating fibrocytes. Myofibroblasts are contractile metabolically active cells that synthesis large quantities of extracellular matrix proteins and play a key role in fibrosis. Conjunctiva has resident fibroblasts and donor-origin fibrocytes have also been demonstrated in the eyes of GVHD patients. Thus, conjunctival fibrosis may be likely caused myofibroblast formation and excessive deposition of extracellular matrix.

Conjunctival tissue has been shown to express components of local renin angiotensin system (RAS), including renin, angiotensinogen, angiotensin converting enzyme (ACE) and AT1 receptors. Conjunctival injury such as trabeculectomy has been shown to upregulate RAS. Angiotensin II has been shown to cause transdifferentiation of conjunctival fibroblasts to myofibroblasts. Profibrotic cytokines such as transforming growth factor beta (TGF-β), PDGF, CTGF released by immune cells in GVHD can serve as the initial trigger for fibroblasts transdifferentiation to myofibroblasts. An interaction between RAS and profibrotic cytokines, especially TGF-β and RAS, can serve to amplify and perpetuate conjunctival fibrosis.

Hypothesis

GVHD-associated dry eye is caused by ocular surface glycocalyx damage, mucin layer dysfunction, and conjunctival fibrosis. We tested this hypothesis using the following aims:

Specific Aim 1: To examine the role of glycocalyx and mucins in the etiology of GVHD-associated dry eye. To test this aim
1a: we studied the effect of GVHD on ocular surface glycocalyx, ocular surface membrane-bound mucins, secreted tear film mucins, and conjunctival goblet cells;
1b: we investigated the effect of augmentation of ocular surface mucins as a pharmacological approach to treat GVHD-associated dry eye.

Specific Aim 2: To examine the effect of hyperosmolar stress on the release of pro-inflammatory cytokines. To test this aim
2a: we studied the effect of hyperosmolar stress on the release of proinflammatory cytokines from ocular surface epithelial cells.
2b: we investigated the effect of proinflammatory cytokines on ocular surface glycocalyx and mucins

Specific Aim 3: To examine the role of conjunctival fibrosis in etiology of GVHD-associated dry eye. To test this aim,
3a: we studied whether GVHD causes conjunctival fibrosis and myofibroblast formation.
3b: we investigated the role of local ocular renin angiotensin system (RAS) in GVHD-mediated conjunctival fibrosis.

Figure 1. Central Hypothesis
Figure 2. Structure of the four types of ocular surface epithelial cells mucins (MUC)
A) secreted, gel forming MUC5AC which produce by goblet cells.
B) membrane bond mucins MUC1, MUC4, MUC16 expressed by the ocular

Figure 3. Ocular surface barrier consisting of glycocalyx and intracellular junctions\textsuperscript{42}. 
References


CHAPTER 2

Graft versus Host Disease-Associated Dry Eye: Role of Ocular Surface Mucins and the Effect of Rebamipide, a Nucin Secretagogue

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Abstract

Purpose: The present study was designed to investigate the role of ocular surface mucins in GVHD-associated dry eye. The ameliorative effect of topical rebamipide, a mucin secretagogue, on GVHD-associated dry eye was also tested.

Methods: A mouse model of allogeneic transplantation was used to induce ocular GVHD with C57BL/6 as donors and B6D2F1 as recipient mice. Phenol red thread method and fluorescein staining were used to quantify tear secretion and corneal keratopathy. At 8 weeks after the allogeneic transplantation, corneas were harvested to perform glyocalyx staining and confocal microscopy. The goblet cell staining was performed using periodic acid Schiff’s staining. Corneal and tear film levels of Mucin1, 4, 16, 19 and 5AC were quantified using ELISA and real-time PCR. Rebamipide was applied topically twice daily to mice eyes and BSS was used as vehicle control.

Results: Allogeneic transplantation resulted in ocular GVHD-associated dry eye characterized by a significant decrease in tear film volume and the onset of corneal keratopathy. Ocular GVHD caused a significant decrease in the area and thickness of corneal glyocalyx. A significant decrease in the goblet cells was also noted. A significant decrease in mucin 4 and mucin 5AC levels was also observed. Topical treatment with rebamipide partially attenuated ocular GVHD-mediated decrease in tear film volume and significantly reduced the severity of corneal keratopathy.

Conclusions: Ocular GVHD has a detrimental impact on ocular surface glyocalyx and mucins. Rebamipide, a mucin secretagogue, partially prevents ocular GVHD-associated decrease in the tear film and reduces the severity of corneal keratopathy.
**Introduction:**

Allogeneic hematopoietic stem cell transplantation is a successful treatment option for hematological malignancies. However, graft versus host disease (GVHD) is a serious complication of hematopoietic stem cell transplantation, and its incidence remains high in spite of the advances in HLA matching. Depending upon the time of onset and clinical manifestations, GVHD is divided into the acute and chronic phase. Acute GVHD primarily affects the liver, skin, and intestine. On the other hand, chronic GVHD has been shown to cause a high incidence of ocular complications. As high as 60-90% of chronic GVHD patients suffer from ocular manifestations. Ocular signs in chronic GVHD patients may be noticeable even before the other systemic symptoms. Dry eye disease is one of the most frequent complications of ocular GVHD. The dry eye disease in ocular GVHD patients is severe, resulting in signs of blurred vision, photophobia, redness, gritty sensation, and pain. These symptoms cause significant visual discomfort and reduce the overall quality of life of GVHD patients. In the absence of timely and appropriate treatment, dry eye disease in GVHD patients may progress to corneal keratopathy, ulceration, and visual impairment.

Lacrimal functional unit including ocular surface nerves, apical surface glycocalyx, lacrimal glands, meibomian glands and a normal blinking response all collectively contribute to the secretion and maintenance of a healthy tear film. Tear film is critical for keeping the ocular surface hydrated and lubricated, thus preventing desiccation-induced damage to the ocular surface. Tear film is comprised of mucous, aqueous and lipid layers. Mucous layer of tear film is primarily comprised of membrane-bound and secreted mucins. Mucins are high molecular weight glycoproteins made
up of a protein core with extensive glycan $N$-acetyl galactosamine side chains. The heavy glycosylation imparts the mucins with hydrophilicity and a high negative charge$^{11-15}$. These structural features account for their two key physiological functions of repelling pathogens and keeping the ocular surface hydrated$^{11-15}$. The mucous layer of tear film contains membrane-bound mucins (MUC): MUC1, MUC4, MUC16, soluble MUC7 and, gel-forming secreted mucins, MUC19, MUC5AC and MUC5B$^{11-15}$. The membrane-bound mucins are expressed on the apical surface of corneal and conjunctival epithelial cells. The gel-forming mucins MUC5AC and MUC5B are primarily secreted by the goblet cells present in the conjunctiva and lacrimal gland respectively. Lacrimal gland also releases soluble MUC7 in the tear film. Tear film mucins retard tear film evaporation keep the eye surface lubricated and entrap allergens and pathogens$^{11-15}$.

Patients with dry eye disease show reduced levels of mucins or an alteration in the degree of their glycosylation$^{16-18}$. A significant aqueous deficit has been observed in the tears of GVHD patients suffering from dry eye$^{19}$. Multiple studies have shown that GVHD causes lacrimal gland fibrosis$^{20-23}$. How GVHD impacts ocular surface mucins is not known. Therefore, the aim of the present study was to investigate the role of ocular surface mucins in GVHD-associated dry eye and to test the ameliorative effect of rebamipide, a mucin secretagogue, on GVHD-associated dry eye.
Methods:

Allogenic bone marrow transplantation

The animal protocol was approved by Institutional Animal Care and Use Committee of Chapman University. All the animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A previously published mouse model of MHC class I mismatch induced ocular GVHD was used\textsuperscript{23}. The B6D2F1 mice (The Jackson Laboratories, Bar Harbor, ME, USA) having a heterozygous MHC haplotype b/d were used as recipients and C57BL/6 mice (The Jackson Laboratories, Bar Harbor, ME, USA) having a homozygous haplotype b/b were used as donors. The bone marrow and spleen cells were harvested from 8 weeks old donor female C57B6 mice. Ten weeks old female B6D2F1 recipient mice were exposed to a total body irradiation of 1100 cGy delivered in two equally divided doses three hours apart (RS 2000 X-ray Biological Irradiator, Rad Source Technologies, Buford, GA, USA). The irradiated B6D2F1 mice were then injected with 2X10\textsuperscript{6} spleen cells and 5X10\textsuperscript{6} bone marrow cells obtained from C57B6 mice by retro-orbital injection. The mice were housed in a sterile cage, fed with diet gel (ClearH2O, Portland, ME, USA) and received sulfatrim (0.672 mg/ml) in their drinking water for the first 14 days. At 8 weeks after the transplantation, animals were euthanized by CO2 administration for the collection of ocular tissue.

The study design included three different group of mice. 1) Control group (No transplant) n= 6 mice included age matched B6D2F1 mice, which did not receive any bone marrow or spleen cell transplantation. 2) Ocular GVHD group n= 12 included B6D2F1 mice that received allogenic bone marrow and spleen cell transplantation.
3) Rebamipide treated group n=6 included B6D2F1 mice that received allogenic bone marrow and spleen cell transplantation and were treated with 2 μl topical ophthalmic drops of 2% rebamipide suspended in BSS in left eye two times daily. The right eye of these mice received 2 μl topical ophthalmic drops of vehicle BSS two times daily.

**Tear quantification:**

Tear secretion was quantified by phenol red thread test before the allogeneic transplantation and at weekly intervals after the transplantation. The phenol red impregnated thread (FCI Ophthalmics, Pembroke, MA, USA) was placed in the lower eyelid of mice on the temporal side for 1 minute. Upon wetting by tears, the phenol red thread changes color from yellow to red due to pH change. After 1 minute, the thread was removed, and the length of the red color on the thread was measured. The length was converted to the volume by using a standard curve plotted by measuring the length of the phenol red thread wetted with a known volume of artificial tears.

**Fluorescein staining:**

Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). A 2 μl sterile solution of 0.5% fluorescein was applied to mouse eye, and imaging was performed under a green fluorescent filter using stereomicroscope equipped with a digital camera. The captured corneal images were divided into hypothetical four quadrants for scoring the keratopathy using a previously published method. Each quadrant was scored as follows: No staining= 0; slightly punctate staining less than 30 spots= 1; punctate staining more than 30 spots, but not diffuse= 2; diffuse staining but no positive plaque= 3; positive fluorescein plaque= 4. The scores of
Each quadrant was added to arrive at a final grade (total maximum possible score=16).

**Glycocalyx staining:**

The eyes were collected from euthanized animals at 8 weeks after the allogeneic transplantation and were fixed by immersing overnight in 4% paraformaldehyde. The corneas were isolated and blocked in 5% BSA for 20 minutes. Glycocalyx staining on the corneas was performed using 1.5 μg/ml solution of Alexa 488 conjugated wheat germ agglutinin lectin (Thermo Fisher Scientific, Hanover Park, IL, USA) for 20 minutes. Wheat germ agglutinin lectin binds to the N-acetylglucosamine and N-acetylneuraminic acid residues present on the ocular surface glycocalyx and have been used in multiple studies, including human patients, to stain corneal glycocalyx\(^{25-27}\). The stained corneas were imaged using a confocal microscope. A total of 4 images were captured from each cornea. The quantification of glycocalyx stained area and thickness was performed using Image J software in a blinded manner.

**Goblet Cell Staining:**

The eyes along with eyelids were harvested from euthanized animals at 8 weeks after the allogeneic transplantation and processed for paraffin embedding. The 7 μm thin paraffin sections were cut, and periodic Acid Schiff's (PAS) staining was performed for goblet cells using a commercially available kit (Polysciences, Inc., Warrington, PA, USA). The stained sections were imaged at 100X magnification using a brightfield microscope (Keyence corporation of America, Itasca, IL, USA).

**ELISA quantification of mucin 1, 4 & 16:**

At 8 weeks after the allogeneic transplantation, animals were euthanized by CO2 administration. The eyeballs were collected, and the corneas were separated. The
CORNEAS WERE HOMOGENIZED IN RIPA BUFFER CONTAINING PROTEASE INHIBITOR (PIERCE, THERMO FISHER SCIENTIFIC, HANOVER PARK, IL, USA). THE TOTAL PROTEIN IN THE CORNEAL HOMOGENATES WAS QUANTIFIED BY BCA METHOD USING A COMMERCIALLY AVAILABLE KIT (PIERCE, THERMO FISHER SCIENTIFIC, HANOVER PARK, IL, USA). THE MUC1, MUC4 AND MUC16 LEVELS WERE QUANTIFIED IN THE CORNEAL PROTEIN LYSESTES USING COMMERCIALLY AVAILABLE ELISA KITS (LSBIO, SEATTLE, WA, USA). THE MUCIN LEVELS WERE NORMALIZED FOR THE MILLIGRAM OF TOTAL PROTEIN IN THE CORNEAL LYSATES.

ELISA QUANTIFICATION OF MUCIN 5AC:

The Muc5ac levels were quantified in the tears collected from mice at 8 weeks after the allogeneic transplantation. For tear collection, mice were lightly anesthetized with isoflurane. A 1 μL solution of 1X PBS containing 0.1% BSA was placed on each eye of the mouse and then collected back by using a Drummond microcapillary tube. The 1 μL collected from each eye was pooled and added to 8 μL of BSS solution. The tears were stored at -80°C for quantification of Muc5ac using a commercially available ELISA kit (LSBio, Seattle, WA, USA).

GENE EXPRESSION QUANTIFICATION OF MUCIN 1, 4, 16 & 19:

Corneas were harvested from animals at 8 weeks after transplantation as described above. The mRNA was extracted from the corneas using the RNeasy Mini kit (RNeasy kit; Qiagen Inc., Valencia, CA, USA). The mRNA was immediately reverse transcribed to cDNA using a commercially available kit (Superscript III First-strand synthesis, Thermo Fisher Scientific, Hanover Park, IL, USA) for cDNA synthesis. The cDNA was used to quantify Muc1, Muc4, Muc16, and Muc19 gene expressions using real-time PCR. A 20 μl reaction mixture containing 2 μl of cDNA, 2 μl of forward primer (200 nM),
2 μl of reverse primer (200 nM), and 10 μl of 2X SYBR green supermix was run at a universal cycle (95°C for 10 min, 40 cycles at 95°C for 15 s, and 55°C for 60 s) in a thermocycler (Biorad CFX thermocycler, Bio-Rad Laboratories). β-actin was used as the housekeeping gene. The relative change in gene expression was calculated using ΔΔCt method.

**Statistical Analysis:**

The data is presented as mean + S.E.M. One-way ANOVA followed by Dunnet’s and Duncan’s test was used to analyze time dependent changes in tear film volume for Figure 1 and corneal keratopathy score for Figure 7B, respectively. The data presented in Figures 2-6 for comparing control and allogeneic transplantation groups was analyzed using unpaired t test. Two-way ANOVA was used for data analysis of tear film volume presented in Figure 7 A.

**Results:**

**Effect of ocular GVHD on tear film volume and corneal keratopathy:**

The present study used MHC mismatched allogeneic transplantation mouse model that has been shown to develop ocular GVHD. Our results further confirm that this mouse model of allogeneic transplant results in significant manifestations of dry eye due to ocular GVHD as is evident from a decrease in tear film volume and appearance of corneal keratopathy. Fig. 1 shows a baseline mean tear film volume of 300 nanoliters in the mice prior to the allogeneic transplantation. After the bone marrow and spleen cell transplantation, a statistically significant 3-fold decrease in tear film volume was noted starting at 3 weeks, and this decrease persisted till the tested time point of 8 weeks. The observed 2 weeks delay in the onset of tear film decrease is anticipated because
immune-mediated damage to the lacrimal functional unit is expected to precede prior to a decrease in tear film volume becomes apparent.

Fig. 2 shows a representative fluorescein-stained image of a mouse cornea before (Fig. 2A) and at 8 weeks after the allogeneic bone marrow and spleen cell transplantation (Fig. 2B). As can be seen in fig. 2B, the corneas of mice that underwent allogeneic transplant showed significant punctate and plaque staining. The scoring of fluorescein-stained corneal images was performed in a blinded manner using a previously described method\textsuperscript{24}. The corneas of mice that received allogeneic transplantation had a mean fluorescein staining score of 8 (Fig. 2C), suggesting that ocular GVHD caused a moderate to severe degree of corneal keratopathy.

**Effect of ocular GVHD on corneal glycocalyx and goblet cells:**

Corneal epithelial cells express 3 different types of membrane-tethered mucins on their apical surface. These mucins together with galectin 3 form a continuous network of glycocalyx. Wheat germ agglutinin lectin binds to the sialic acid residues present on these mucins and has been previously used to stain the corneal glycocalyx\textsuperscript{27}. Fig. 3 shows the top and orthogonal projection confocal images of the mouse corneas stained for glycocalyx using Alexa 488 conjugated wheat germ agglutinin. The top view of confocal z stack images show a dense and uniformly distributed glycocalyx staining in the corneas of control mice that did not receive any transplantation (Fig. 3A). On the other hand, corneal glycocalyx was sparse and patchy in the corneas obtained from mice at 8 weeks after they received allogeneic bone marrow and spleen cell transplantation (Figure 3B). The glycocalyx-stained area was quantified as percentage of the total corneal area using binary image analysis of 16
images each of corneas obtained control mice and the mice that received allogeneic transplantation (4 images captured from different parts of each cornea and n=4 mice). As is evident from binary quantification data presented in Fig. 3C, a significant decrease of 37% in glycocalyx stained area was observed in the mice corneas that received allogeneic transplantation compared to the control mice without any transplantation. Fig. 3D and 3E show the orthogonal projection confocal Z stack images of glycocalyx-stained corneas. A significant decrease in the glycocalyx thickness was observed in the corneas obtained from mice that received allogeneic transplantation (Fig. 3D) compared to control corneas obtained from mice without any transplantation (Fig. 3E). Glycocalyx thickness was also quantified in 16 images each obtained from corneas of control mice (n=4) and from the corneas of mice that received allogeneic transplantation (n=4). Fig. 3F shows a mean decrease of 33% in the corneal glycocalyx thickness in the mice that received allogeneic transplantation compared to control mice.

Fig. 4 shows a representative image of periodic acid Schiff’s-stained goblet cells in the eyelids of control mice and mice that underwent allogenic bone marrow and spleen cell transplant. It is apparent from the staining that allogenic bone marrow and spleen cell transplantation mediated ocular GVHD caused a notable decrease in the number of goblet cells. It can also be noted that the morphology and mucin content of goblet cells has also been altered by the ocular GVHD in mice that received allogenic bone marrow and spleen cell transplant as compared to the control mice that did not receive any transplantation.
**Effect of ocular GVHD on mucins:**

We further investigated the effect of allogeneic bone marrow and spleen cell transplantation associated with ocular GVHD on membrane bound Muc1, 4 and 16 mucins using corneal homogenates and on secreted Muc5ac in tear film. A slight reduction in Muc1 (Fig. 5A) levels was noted in the corneal homogenates obtained from mice at 8 weeks after allogeneic bone marrow and spleen cell transplantation as compared to the levels in the control corneal homogenates obtained from mice that did not receive any transplantation. A statistically significant decrease in corneal homogenate levels of Muc4 (Fig. 5B) and tear film levels of Muc5ac (Fig. 5D) was also observed in the mice that underwent allogeneic transplant as compared to the control mice. On the other hand, a slight increase in Muc16 was observed (Fig. 5C) in the corneal homogenates of transplanted mice compared to control mice.

To test the effect of ocular GVHD on mucin gene expression, mRNA levels were quantified in the corneas of control mice and in the cornea obtained from mice at 8 weeks after the allogeneic transplant. A significant increase in Muc1 gene expression was observed in the corneas obtained from mice that received allogeneic transplantation compared to control mice that did not receive any transplantation (Fig. 6A). However, no significant change was noted in the mRNA levels of Muc4 (Fig. 6B), Muc16 (Fig. 6C) and Muc19 (Fig. 6D) between the control mice without the transplantation and mice that received allogeneic bone marrow and spleen cell transplantation.
Effect of topical rebamipide on ocular GVHD-mediated changes in tear film and corneal keratopathy:

Lastly, we tested the effect of rebamipide, a mucin secretagogue, on ocular GVHD mediated decrease in tear film volume and cornel keratopathy. As can be seen from the Fig. 7 A, twice daily topical ophthalmic application of rebamipide attenuated ocular GVHD-mediated decrease in tear film volume. The results were statistically significant at week 3 and 4 compared to the GVHD mice who received allogeneic transplantation but did not receive any eye drops. The BSS was used as a vehicle for compounding rebamipide. Therefore, we also tested the effect of topical ophthalmic application of BSS as vehicle, but BSS application in GVHD mice had no notable effect on the tear film volume compared to untreated (no eye drops) control GVHD mice. Further, rebamipide application also significantly mitigated ocular GVHD-mediated corneal keratopathy (Fig. 7B). Rebamipide treated GVHD mice showed a mean corneal keratopathy score of 3 compared to a score of 8 for the untreated (no eye drops) GVHD mice (Fig. 7B). It is interesting to note though that keeping the ocular surface hydrated by BSS vehicle application also partly attenuated corneal keratopathy. BSS treated GVHD mice showed a mean corneal keratopathy score of 5 compared to a score of 8 for the untreated (no eye drops) GVHD mice (Fig. 7B).

Discussion:

The apical surface of the corneal and conjunctival epithelium is covered with glycocalyx, a thin layer of glycoproteins largely composed of membrane-tethered mucins and galectin-3. The glycocalyx forms a boundary between the ocular surface epithelium and...
and the tear film. Glycocalyx serves to protect the cells against mechanical and chemical damage. An intact glycocalyx is also essential to reduce the friction during blinking and to keep the ocular surface hydrated\textsuperscript{28-30}. We used fluorescent wheat germ agglutinin labeling and whole cornea mount 3D confocal microscopy to visualize glycocalyx on the corneas of GVHD mice. Wheat germ agglutinin binds to \textit{N}-acetyl-glucosamines and sialic acid side chains of the membrane-tethered mucins and has been used to specifically label, visualize and quantify glycocalyx in the cornea and vascular endothelium\textsuperscript{25-27}. Our data demonstrate a significant decrease in the area and thickness of ocular surface glycocalyx in mice that received allogeneic bone marrow and spleen cell transplantation suggesting that GVHD has a detrimental effect on the ocular surface glycocalyx.

Membrane-tethered mucins are an integral component of glycocalyx. Thus, we further examined the effect of GVHD on corneal epithelial membrane-tethered mucins. Our results demonstrate that GVHD caused a significant decrease in protein levels of membrane-tethered Muc4 but did not cause any notable change in protein levels of membrane-tethered Muc1 or Muc16. Interestingly, a significant increase in Muc1 gene expression was observed in the corneas of GVHD mice, which can possibly be a compensatory response to partially circumvent the GVHD-mediated damage to the ocular surface glycocalyx. Besides membrane-tethered mucins, tear film also contains soluble mucins. Goblet cells are the primary source of large gel forming mucin Muc5AC which is secreted into the tear film\textsuperscript{31,32}. The results of the present study demonstrate that GVHD has a detrimental effect on goblet cells because a decrease in the number of goblet cells was observed in the tissue sections obtained from mice suffering from
GVHD due to allogeneic transplantation. The histology data also consistently demonstrated partially empty goblet cells, suggesting the possibility that the surviving goblet cells in the GVHD mice were releasing Muc5AC in the tear film possibly in an effort to salvage the ongoing damage to the ocular surface. Our observations are further supported by the ELISA quantification data showing a significant decrease in the tear film levels of Muc5AC in GVHD mice. Alterations in ocular surface mucins and glycocalyx has been previously reported in non-autoimmune dry eye and dry eye due to Sjogren’s disease. To the best of our knowledge, this is the first study to demonstrate that GVHD causes a damage to the ocular surface glycocalyx and alters ocular surface mucins.

In this study, we used an allogeneic MHC heterozygous mismatch hematopoietic transplant mouse model to induce ocular GVHD. Our data demonstrate that this mouse model develops ocular GVHD-associated dry eye as demonstrated by a significant decrease in tear film and the corneal keratopathy. Previous studies have shown the development of ocular GVHD in this mouse model and support the results of the present study. Using this mouse model, Hassan et al have demonstrated that GVHD has a detrimental effect on the lacrimal gland. Studies using MHC matched allogeneic hematopoietic transplant mouse has also shown lacrimal gland damage in ocular GVHD. However, our data is the first one to demonstrate that besides lacrimal gland, GVHD also causes damage to the ocular surface glycocalyx.

Rebamipide, an amino acid analog of 2 (1H)-quinolinone, has long been used for the treatment of gastric ulcers. The ophthalmic formulation of rebamipide has recently been launched for the treatment of dry eye in Japan.
shown to stimulate gastric mucosal prostaglandin production, increase gastric mucus synthesis, and scavenge reactive oxygen radicals\textsuperscript{40-42}. Recent studies have shown that rebamipide increases MUC1, MUC4 and MUC16 synthesis in stratified cultures of human corneal epithelial cells\textsuperscript{43,44}. In vivo administration of rebamipide has been demonstrated to have an ameliorative effect in mouse model of Sjogren’s syndrome, superoxide dismutase knockout mice and rabbit model of dry eye\textsuperscript{46-47}. Given the beneficial effects of rebamipide on mucous layer and dry eye, we tested the effect of topical administration of rebamipide in GVHD-associated dry eye in mouse model. Our data demonstrate that topical rebamipide administration provided significant protection against GVHD associated dry eye as indicated by the sustenance of tear film and a notable decrease in corneal keratopathy score. Interestingly, topical administration of BSS vehicle alone also had some ameliorative effect suggesting that keeping the ocular surface hydrated can partially rescue GVHD-associated corneal keratopathy.

In summary, our results demonstrate that allogeneic transplantation-associated ocular GVHD can have a significant detrimental effect on ocular surface glycocalyx and ocular surface mucins. Further, modulation of ocular surface mucins by rebamipide, a mucin secretagogue, can partially prevent ocular GVHD-associated decrease in tear film and reduced the severity of corneal keratopathy.

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Figure 1. Tear film volume in mice before (baseline) and at various time points after allogeneic bone marrow & spleen cell transplantation. A significant (* p<0.05 compared to baseline) decrease in tear film volume was observed at 3 weeks after allogeneic transplantation and it remained significantly low for the tested duration of 8 weeks.

Figure 2. Representative fluorescein-stained images of mouse corneas before (A) and at 8 weeks (B) after allogeneic bone marrow & spleen cell transplantation.
Quantification of fluorescein staining (C) showed significant (* p<0.05 compared to before transplantation) corneal keratopathy at 8 weeks after allogeneic transplantation.

**Figure 3.** Representative confocal Z stacks images of top (A & B) and orthogonal (D & E) view of mouse corneas stained for glycocalyx (green) using wheat germ agglutinin. Nuclei are stained blue. Panel A and B is top view of corneas obtained from control mice that did not receive any transplantation and mice at 8 weeks after allogeneic bone marrow & spleen cell transplantation. Quantification of percent-stained area (Panel C) shows a significant decrease (*p<0.05 compared to control mice that received no transplantation) in the glycocalyx in mice corneas at 8 weeks after allogeneic transplantation. Panel D & E is orthogonal view of corneas obtained from control mice that did not receive any transplantation and mice at 8 weeks after allogeneic transplantation. Quantification (Panel F) shows a significant decrease (*p<0.05
compared to control mice that received no transplantation) in the glycocalyx thickness in mouse corneas at 8 weeks after allogeneic transplantation. Area and thickness quantifications were calculated using 16 different images, each of control mice (n=4) and mice that received allogeneic transplantation (n=4).

**Figure 4.** Representative images showing periodic acid Schiff’s-stained goblet cells in the tissue sections obtained from control mice (no transplant) and mice at 8 weeks after allogeneic bone marrow & spleen cell transplantation
Figure 5. ELISA quantification of mucin 1, mucin 4, mucin 16, and mucin 5AC in the corneal homogenates and tears obtained from control mice (no transplant) and mice at 8 weeks after allogeneic bone marrow and spleen cell transplantation. A decrease in mucin 4 (* p <0.05) and mucin 5AC (* p <0.05) was observed compared to the levels in control mice that received no allogeneic transplantation.
Figure 6. Gene expression quantification of mucin 1, mucin 4, mucin 16, and mucin 19 in the corneal homogenates obtained from control mice (no transplant) and mice at 8 weeks after allogeneic bone marrow and spleen cell transplantation. A significant increase in mucin1 gene expression (* p <0.05) was observed compared to the levels in control mice that received no allogeneic transplantation.
Figure 7. Effect of rebamipide ophthalmic drops on allogeneic bone marrow and spleen cell transplantation-mediated decrease in tear film volume (A) and corneal keratopathy score (B). Rebamipide attenuated allogeneic transplantation mediated decrease in tear film volume, and the results were statistically significant (* p<0.05) at 3 and 4 weeks after the allogeneic transplantation as compared to mice that also received the allogeneic transplantation but were either treated with BSS vehicle or did not receive any eye drops. Rebamipide treatment also significantly decreased the corneal keratopathy score (* p<0.05 compared to no eye drops-treated mice; τ p<0.05 compared to BSS vehicle). Animals that received BSS vehicle alone also showed significantly (* p<0.05) lower corneal keratopathy score compared to mice that received no eye drops.
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CHAPTER 3

EFFECT OF OSMOTIC STRESS ON NFAT5 AND PROINFLAMMATORY CYTOKINES GENE EXPRESSION IN OCULAR SURFACE EPITHELIAL CELLS

Abstract

An increase in tear osmolarity is a cardinal feature of dry eye disease. Osmotic stress has been shown to cause an increase in the expression of proinflammatory cytokines. The ocular surface is covered by conjunctival and corneal epithelial cells, which may show a different biological response to osmotic stress. Therefore, we investigated the effect of hyperosmolar stress on gene expression of NFAT5, a tonicity-regulated transcription factor and cytokines IL1, IL6, TNFα, and IFN-γ in human conjunctival and corneal epithelial cells.

Methods: Telomerase immortalized human corneal and conjunctival epithelial cells were exposed to hyperosmolar cell culture media (430 mOsm) for 12 and 24 hours. The mRNA was isolated from these cells and was reverse transcribed to cDNA. The cDNA was used for the quantification of gene expression by real-time PCR.

Results: Hyperosmotic stress caused a 15.8- and 2.2-fold increase in NFAT5 gene expression in corneal and conjunctival epithelial cells. Hyperosmotic stress also caused 1.5- and 2-fold increase in the gene expression of IL1 in the human conjunctival and corneal epithelial cells, respectively. Hyperosmotic stress also caused a 3- 4 fold increase in IL6 expression in conjunctival cells, whereas the increase in IL6 gene expression was much more robust in human corneal epithelial cells. Similarly, hyperosmotic stress caused a 2-3 fold increase in TNFα in human conjunctival cells, whereas a >10-fold increase in TNFα expression was observed in the human corneal
epithelial cells. Lastly, a 3-4 fold increase in IFN-γ was observed in corneal epithelial cells compared to a 1.8 fold increase noted in conjunctival epithelial cells.

Conclusion: Our data demonstrated that human conjunctival and corneal epithelial cells show a differential response to the hyperosmotic stress-mediated change in the gene expression of NFAT 5 and proinflammatory cytokines. Human corneal epithelial cells are more sensitive to hyperosmotic stress.

Introduction

Tear Film and Ocular Surface Society defines dry eye disease is a multifactorial disorder of the ocular surface characterized by impairment and loss of tear homeostasis, ocular discomfort or pain, and neurosensory abnormalities\(^1\)-\(^3\). Healthy tear fluid is iso-osmolar, but dry eye disease can cause an increase in tear osmolarity\(^4\)-\(^5\). An increase in tear osmolarity has also been observed in the tears of GVHD patients. NFAT5 is activated by phosphorylation under a hyperosmotic environment and is amongst the few known tonicity-regulated transcription factors in mammalian cells\(^6\). NFAT5 transcriptionally regulates the expression of target genes responsible for organic osmolytes' metabolism, including aldose reductase, taurine transporter, betaine/GABA transporter, and sodium/myo-inositol transporter. Osmotic stress also activates stress-related kinases, and these kinases are responsible for transcription of the proinflammatory cytokines, thus forming a link between osmolarity and gene expression of these cytokines\(^6\).\(^7\). Therefore, we tested the effect of hyperosmolar stress on the changes in gene expression of NFAT5, IL-1, IL-6, TNF- α, and IFN-γ in human corneal and conjunctival epithelial cells\(^8\)-\(^12\).
Methods

Culture of stratified human corneal and conjunctival epithelial cells

Telomerase-transformed human corneal and conjunctival epithelial cell lines were used for this study. Human corneal epithelial cells were propagated in keratinocyte growth medium supplemented with bovine pituitary extract (0.004 ml/ml), human epidermal growth factor (0.125ng/ml), human insulin (5µg/ml), hydrocortisone (0.33µg/ml), epinephrine (0.39 µg/ml), transferrin (10 µg/ml) and calcium chloride (0.15 mM) (PromoCell GmbH, Heidelberg, Germany). To induce stratification, the cells were plated on a collagen-coated 3.0 µm PTFE transwell membrane inserts (Corning Inc., Corning, NY, USA), and the media was switched to a supplemented keratinocyte growth medium containing a higher concentration of CaCl$_2$ (1.15 mM). This media was added both on the inserts and the bottom well until the cells reached 100% confluence. Once the cells reached 100% confluence, the media was added to the bottom of the well only, leaving the membrane inserts containing the cells exposed to air for one week to achieve stratification into a multilayered epithelium.

The human conjunctival epithelial cells were propagated in keratinocyte serum-free medium supplemented with 25 µg/mL bovine pituitary extract, 0.2 ng/mL epidermal growth factor, and 0.4 mM CaCl$_2$ (Gibco-Invitrogen Corp., Rockville, MD, USA). Once the cells reached 50% confluence, the media was switched to a 1:1 mixture of supplemented keratinocyte serum-free medium and low-calcium DMEM without HEPES/F12 media (Gibco-Invitrogen Corp., Rockville, MD, USA). When the cells reached 100% confluence, the media was replaced with DMEM/F12 media.
supplemented with 1mM CaCl₂, 10% Calf serum, and 10 ng/mL EGF for about 5 to 7 days to induce stratification.

**Osmotic stress treatment**

Human corneal and conjunctival epithelial cells were exposed to hyperosmolar cell culture media (430 mOsm) for 12 hours and 24 hours. The hyperosmolar media was prepared by adding sodium chloride to the isotonic cell culture media. The control cells were cultured in the isotonic media (300 mOsm). Each experiment was conducted in triplicate.

**Isolation of mRNA and preparation of cDNA**

The total RNA from the control and hyperosmolar exposed corneal and conjunctival epithelial cells were isolated using a commercially available kit (Qiagen’s RNeasy Mini Kit, Valencia, CA, USA) as per manufacturer’s instructions. The RNA was immediately reverse transcribed to cDNA using a commercially available kit (SuperScript® III First-Stand, Invitrogen, Carlsbad, CA, USA).

**Cytokine gene expression quantification**

The gene expression of cytokines was quantified using Real-Time PCR. A 20 µl reaction mixture containing 2 µl of cDNA and 18 µl of SYBR Green Master Mix was run at a universal cycle (95°C for 10 min, 40 cycles at 95°C for 15 seconds, and 55°C for 60 seconds) using Real-Time thermocycler (Biorad CFX thermal cycler, Bio-Rad Laboratories, Hercules, California, USA). β-actin was used as the housekeeping gene. The relative change in gene expression was calculated using the ΔΔCt method.
Results and Discussion

Exposure of human corneal and conjunctival epithelial cells to hyperosmotic stress caused an average of 15.8- and 2.2-fold increase in NFAT5 gene expression (Figure 1). For IL-1, there was a 2-fold increase in the gene expression corneal epithelial cells noted at 24-hour after exposure to hyper-osmolar media. On the other hand, a 1.5-fold increase in gene expression of IL-1 was noted at 12-hour after hyperosmolar media exposure in conjunctival epithelial cells (Figure 2). Next, for IL-6, there was >150-fold, and 3.5-fold increase in gene expression noted at 24-hour after hyperosmolar media exposure in corneal and conjunctival epithelial cells, respectively (Figure 3). For TNF-α, there was an 8-fold increase in the 12-hour treated corneal epithelial cells, a greater than 10-fold increase in the 24-hour treated corneal epithelial cells, and a 2-fold increase in gene expression in 24-hour treated conjunctival epithelial cells (Figure 4). Lastly, a 3-4 fold increase in IFN-γ was observed in corneal epithelial cells compared to a 1.8 fold increase noted in conjunctival epithelial cells. Based on this data, it can be deduced that hyperosmolar stress increases the gene expression of NFAT5 and proinflammatory cytokines in human corneal and conjunctival epithelial cells, and it can be concluded that human corneal epithelial cells are more sensitive to hyperosmotic stress.
**Figure 1.** Effect of hyperosmolar stress on gene expression of NFAT5 in human corneal and conjunctival epithelial cells. * p< 0.05 versus cells exposed to iso-osmolar media.

**Figure 2.** Effect of hyperosmolar stress on gene expression of IL-1 in human corneal and conjunctival epithelial cells. * p< 0.05 versus cells exposed to iso-osmolar media.
**Figure 3.** Effect of hyperosmolar stress on gene expression IL-6 in human corneal and conjunctival epithelial cells. *p* < 0.05 versus cells exposed to iso-osmolar media

**Figure 4.** Effect of hyperosmolar stress on gene expression of TNF-α in human corneal and conjunctival epithelial cells. *p* < 0.05 versus cells exposed to iso-osmolar media
Figure 5. Effect of hyperosmolar stress on gene expression of IFN-γ in human corneal and conjunctival epithelial cells. * p< 0.05 versus cells exposed to iso-osmolar media.
References:


CHAPTER 4

EFFECT OF PROINFLAMMATORY CYTOKINES ON OCULAR SURFACE MUCINS AND GLYCOCALYX

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Abstract:
Purpose: Proinflammatory cytokines are elevated in tears of patients with dry eye. Apical surface of ocular surface epithelial cells is covered with membrane-tethered mucins (MUC) that, together with galactin-3, form glycocalyx network, which plays a critical role in ocular health. The present study tested the effect of proinflammatory cytokines IL-6, TNF-α, and IFN-γ on membrane-tethered mucins gene expression, glycocalyx, and viability of ocular surface epithelial cells.
Methods: Stratified cultures of human corneal and conjunctival epithelial cells were exposed to five different concentrations of IL-6, TNF-α, and IFN-γ for 24 hours. The mRNA was isolated, and reverse-transcribed to cDNA for gene expression quantification using real time PCR. The glycocalyx was stained using Alexa 488-conjugated wheat germ agglutinin lectin and images using confocal microscopy. Apoptotic and necrotic cell death was quantified using flow cytometry.
Results: IL-6, TNF-α, and IFN-γ treatment resulted in a significant increase in the MUC1 and MUC4 gene expression in stratified human corneal epithelial cells but did not cause a significant change MUC16 expression. Unlike corneal epithelial cells, these cytokines caused no significant changes in MUC1, MUC4, and MUC16 expression in stratified human conjunctival epithelial cells. Further, IL-6, TNF-α, and IFN-γ did not cause a notable change in glycocalyx or apoptotic cell death in stratified human corneal and conjunctival epithelial cells, but IL-6 resulted in an increase in necrotic cell death in stratified human corneal epithelial cells while TNF-α caused a significant increase in necrotic cell death of stratified human conjunctival epithelial cells.
Conclusions: Our results have shown that IL-6, TNF-α, and IFN-γ have differential effects on human corneal and conjunctival epithelial cell mucin gene expression, resulting in an increase in MUC1 and MUC4 gene expression only in stratified human corneal epithelial cells. Furthermore, these cytokines did not cause significant changes in stratified human corneal and conjunctival epithelial cell glycocalyx.

Keywords: Mucins, Glycocalyx, Ocular Surface, Proinflammatory Cytokines

Introduction

The apical surface of human corneal and conjunctival epithelial cells is covered with a layer of glycocalyx that forms a boundary between these cells and the tear film [1-3]. Glycocalyx is a network made up of membrane-tethered mucins, their side chains, and galectin-3 [1-7]. Glycocalyx and mucins are critical for ocular health as they serve many functions, including repelling pathogens, keeping the ocular surface hydrated, protecting the cells against mechanical and chemical damage, as well as reducing friction during blinking [1-7].

Dry eye or keratoconjunctivitis sicca is a common ocular condition with a high global prevalence. The disease is characterized by loss of tear film homeostasis, tear hyperosmolarity, ocular surface inflammation, and neurosensory abnormalities [8,9]. While multiple factors contribute to the pathogenesis of dry eye, the immune-mediated inflammatory response plays a central role in its onset and progression [10-14]. Hyperosmolar stress has been shown to trigger the release of proinflammatory cytokines IL-1, IL-6, TNF-α from ocular surface epithelial cells [15,16]. Hyperosmolar stress due to increased tear osmolarity in dry eye disease and the associated release of cytokines can contribute to the early stage of ocular surface inflammation. Levels of IL-6
and TNF-α have been reported to be elevated in the tears of patients suffering from dry eye [17]. These proinflammatory cytokines along with chemokines, are also responsible for activation, maturation, and migration of antigen-presenting cells in the early stages of dry eye disease [10-14]. Subsequently, activated antigen-presenting cells recruit T cells which have been shown to secret IL-17 and IFN-γ [10-14]. T cell-derived cytokines contribute to the amplification stage of inflammation in dry eye disease [10-14].

The promoter region of genes encoding for mucins have response elements for signaling pathways activated by proinflammatory cytokines, including IL-6, TNF-α, and IFN-γ, suggesting that these cytokines can modulate the gene expression of mucins [18-21]. Additionally, these cytokines have been shown to induce the expression of enzymes such as heparanase and hyaluronidase that may have a detrimental effect on the glycocalyx [22,23]. Immunological and conjunctiva impression cytology studies have shown that the levels of mucins or the degree of their glycosylation is notably altered in both the cornea as well as conjunctiva of patients suffering from Non Sjogren and Sjogren syndrome-related dry eye disease [1,2,24]. Therefore, the present study is designed to investigate the effect of two early-stage (IL-6, TNF-α) and one amplification stage (IFN-γ) proinflammatory cytokines on membrane-tethered mucins (MUC) MUC1, MUC4, MUC16, ocular surface glycocalyx, and cell viability of human stratified corneal and human stratified conjunctival epithelial cells. Since patients suffering from dry eye have elevated tear levels of these cytokines in the picogram range, the present study tested the effect of these cytokines on ocular surface mucins and glycocalyx at the pathologically relevant picogram concentrations.
Methods

Culture of stratified human corneal and conjunctival epithelial cells

Telomerase-transformed human corneal and conjunctival epithelial cell lines were used for this study. Human corneal epithelial cells were propagated in keratinocyte growth medium supplemented with bovine pituitary extract (0.004 ml/ml), human epidermal growth factor (0.125 ng/ml), human insulin (5 µg/ml), hydrocortisone (0.33 µg/ml), epinephrine (0.39 µg/ml), transferrin (10 µg/ml) and calcium chloride (0.15 mM) (PromoCell GmbH, Heidelberg, Germany). To induce stratification, the cells were plated on a collagen-coated 3.0 µm PTFE transwell membrane inserts (Corning Inc., Corning, NY, USA) and the media was switched to supplemented keratinocyte growth medium containing a higher concentration of CaCl$_2$ (1.15 mM). This media was added both on the inserts as well as the bottom well until the cells reached 100% confluence. Once the cells reached 100% confluence, the media was added to the bottom of the well only, leaving the membrane inserts containing the cells exposed to air for one week to achieve stratification into a multilayered epithelium [25].

The human conjunctival epithelial cells were propagated in keratinocyte serum-free medium supplemented with 25 µg/mL bovine pituitary extract, 0.2 ng/mL epidermal growth factor, and 0.4 mM CaCl$_2$ (Gibco-Invitrogen Corp., Rockville, MD, USA). Once the cells reached 50% confluence, the media was switched to a 1:1 mixture of supplemented keratinocyte serum-free medium and low-calcium DMEM without HEPES/F12 media (Gibco-Invitrogen Corp., Rockville, MD, USA). When the cells reached 100% confluence, the media was replaced with DMEM/F12 media.
supplemented with 1mM CaCl$_2$, 10% Calf serum, and 10 ng/mL EGF for about 5 to 7 days to induce stratification [26,27].

**Cytokines treatment**

The cytokines were purchased from PeproTech (Cranbury, NJ, USA). To test the effects of proinflammatory cytokines on mucin expression, glycocalyx, and epithelial viability, the stratified cultures of human corneal and conjunctival epithelial cells were separately exposed to IL-6, TNF-α, and IFN-γ at five different concentrations (30, 60, 125, 250, 500 pg/mL) for 24 hours. Each experiment was conducted in quadruplicates.

**Isolation of mRNA and preparation of cDNA**

The total RNA from the control and cytokine exposed stratified corneal and conjunctival epithelial cells was isolated using a commercially available kit (Qiagen’s RNeasy Mini Kit, Valencia, CA, USA) as per the manufacturer’s instructions. The RNA was immediately reverse transcribed to cDNA using a commercially available kit (SuperScript® III First-Stand, Invitrogen, Carlsbad, CA, USA).

**Mucins gene expression quantification**

The gene expression of MUC1, MUC4, and MUC16 was quantified using Real-Time PCR. A 20 µl reaction mixture containing 2 µl of cDNA and 18 µl of SYBR Green Master Mix was run at a universal cycle (95°C for 10 min, 40 cycles at 95°C for 15 seconds and 55°C for 60 seconds) using Real-Time thermocycler (Biorad CFX thermal cycler, Bio-Rad Laboratories, Hercules, California, USA). β-actin was used as the housekeeping gene. The relative change in gene expression was calculated using the $\Delta\Delta$Ct method.
**Glycocalyx staining**

The stratified cultures of control and cytokine-exposed human corneal and conjunctival epithelial cells were fixed in 4% paraformaldehyde. The cells were blocked in 5% BSA for 20 minutes. Glycocalyx was stained using 1.5 μg/mL solution of Alexa 488-conjugated wheat germ agglutinin lectin (Thermo Fisher Scientific, Hanover Park, IL, USA) for 20 minutes. Wheat germ agglutinin lectin binds to the N-acetylglucosamine and N-acetylneuraminic acid residues present on the ocular surface glycocalyx and has been used in multiple studies, including human patients to stain corneal glycocalyx [28,29]. The stained glycocalyx was imaged using a confocal microscope (Nikon Inc. Mellville, NY). Four images were captured from the randomly selected areas of each cultured well (total n=16 for each cytokine since the experiment was in quadruplicates). The quantification of glycocalyx stained area was performed using Image J software in a blinded manner.

**Flow cytometry quantification of apoptotic and necrotic cell death**

The stratified cultures of control and cytokine exposed human corneal and conjunctival epithelial cells were incubated in a solution containing 1:2000 propidium iodide and 1:300 FITC-conjugated annexin V (Cayman Chemical, Ann Harbor, MI, USA). The populations of stained cells were analyzed using BD FACSverse flow cytometer (BD Sciences, San Jose, CA, USA). The data was analyzed using FlowJo software.
Statistics

The data are presented as mean ± Standard error of mean. Statistical analysis was performed using GraphPad Prism software (GraphPad Prism, Version 8, San Diego, CA, USA). The data were analyzed using One-Way ANOVA, followed by Dunnett’s post-hoc test. A p value of <0.05 was considered statistically significant.

Results

Effect of IL-6, TNF-α, and IFN-γ on membrane-tethered mucins gene expression in stratified human corneal and conjunctival epithelial cells

To test the effect of IL-6, TNF-α, and IFN-γ on ocular surface mucins gene expression, the stratified human corneal and conjunctival epithelial cells were exposed to five concentrations of these cytokines. Fig. 1 shows the effect of IL-6 on membrane-tethered mucins in stratified human corneal and conjunctival epithelial cells. IL-6 treatment caused a significant increase in the gene expression of MUC1 and MUC4 in the human corneal epithelial cells but did not cause a notable change in the gene expression of MUC16 (Fig. 1A). IL-6 showed an inverted bell-shaped dose-response curve with a less pronounced effect at 30 pg/ml followed by a dose-dependent significant (p<0.05 compared to control cells not exposed to IL-6) increase at 60, 125 and 250 pg/ml then a subsequent decrease in effect at 500 pg/ml for both MUC1 and MUC4 gene expression. In contrast to the corneal epithelial cells, IL-6 did not significantly change the MUC1, MUC4, and MUC16 gene expression in stratified human conjunctival epithelial cells (Fig. 1B).

Fig. 2A shows that TNF-α treatment caused significant (p<0.05 compared to control cells not exposed to TNF-α) increase in the gene expression of MUC1 and
MUC4 in stratified human corneal epithelial cells but did not cause any notable change in MUC16. It is worth noting that the various tested concentrations of TNF-α caused a similar increase in the gene expression of MUC1 and MUC4 suggesting that the effect of TNF-α may not be dose-dependent but a likely response of all or none pattern. On the other hand, TNF-α did not cause any notable change in MUC1, MUC4, and MUC16 expression in the stratified human conjunctival epithelial cells (Fig. 2B).

Consistent with the effects observed with IL-6 and TNF-α, IFN-γ exposure also caused a significant (p<0.05 compared to control cells not exposed to IFN-γ) increase in the gene expression of MUC1 and MUC4 in the stratified human corneal epithelial cells but did not cause any change in MUC16 gene expression (Fig. 3A). Just like TNF-α, various doses of IFN-γ also caused a similar increase in the gene expression of MUC1 and MUC4, suggesting that the effect of IFN-γ are not dose-dependent but follow all or none response. On the contrary, Fig. 3B shows that IFN-γ did not cause any significant change in the gene expression of MUC1, MUC4, and MUC 16 in stratified human conjunctival epithelial cells.

**Effect of IL-6, TNF-α and IFN-γ on stratified human corneal and conjunctival epithelial cell glycocalyx**

The integrity of glycocalyx is important for the barrier, lubrication, and hydration functions of ocular surface epithelial cells [1,2]. Glycocalyx, along with mucins, contains side-chain structural and chemical components to form a network [1-7]. Proinflammatory cytokines have been shown to increase enzymes that can digest these components and damage glycocalyx [22,23]. Therefore, we next tested the effect of these proinflammatory cytokines on human corneal and conjunctival epithelial cell glycocalyx. Wheat germ agglutinin lectin binds to the sialic acid residues and has been previously
used to stain the glycocalyx [28,29]. Fig. 4 shows the top projection of 3-D confocal images of glycocalyx (stained green with wheat germ agglutinin) in stratified human corneal epithelial cells. As shown in Fig. 4, treatment of corneal epithelial cells with IL-6, TNF-α, and IFN-γ (250 pg/ml) did not cause any detrimental effect on corneal epithelial cells glycocalyx. Digital quantification of randomly captured images revealed an average stained area of 92 ± 6% in control corneal epithelial cells, 93 ± 8% in IL-6 treated corneal epithelial cells, 90 ± 10 % in TNF-α treated corneal epithelial cells, and 92 ± 9% in IFN-γ treated stratified human corneal epithelial cells.

Fig. 5 shows the top projection of 3-D confocal images of glycocalyx (stained green with wheat germ agglutinin) in stratified human conjunctival epithelial cells. As was seen with the corneal epithelial cells, treatment with IL-6, TNF-α, and IFN-γ (250 pg/ml) did not cause any detrimental effect on conjunctival epithelial cells glycocalyx. Digital quantification of randomly captured images revealed an average stained area of 80 ± 11% in control corneal epithelial cells, 79 ± 8% in IL-6 treated corneal epithelial cells, 81 ± 10 % in TNF-α treated corneal epithelial cells, and 77 ± 9% in IFN-γ treated conjunctival epithelial cells.

**Effect of IL-6, TNF-α and IFN-γ on stratified human corneal and conjunctival epithelial cell necrosis and apoptosis**

Loss of epithelial cell viability can compromise the ocular surface health and can perpetuate the inflammation. Therefore, we next tested the effect of IL-6, TNF-α, and IFN-γ on human corneal and conjunctival epithelial cell necrosis and apoptosis. None of the cytokines caused any significant increase in apoptotic cell death of human corneal epithelial cells (Fig. 6A). Interestingly, IL-6 treatment (250 pg/ml) caused a significant (p<0.05) increase in necrotic cell death (Fig. 6A). Like the effect observed in human
corneal epithelial cells, none of the tested cytokines caused any increase in apoptotic cell death of human conjunctival epithelial cells (Fig. 6B). In contrast to human corneal epithelial cells, TNF-α instead of IL-6 caused a significant (p<0.05) increase in the necrotic cell death of human conjunctival epithelial cells.

Discussion

Cytokines play an important role in the initiation and perpetuation of dry eye disease [10-14,30,31]. Exposing ocular surface epithelial cells to hyperosmolar stress causes the release of proinflammatory cytokines [15,16]. The level of these proinflammatory cytokines is also significantly increased in the tears of dry eye patients and the conjunctiva of dry eye patients with Sjogren’s syndrome [17,31,32]. Further, an inverse correlation has been demonstrated between the levels of these cytokines and the fluorescein staining and goblet cell density [32].

The apical surface of corneal and conjunctival epithelial cells is covered with glycocalyx [1-7]. Membrane-tethered mucins are important components of glycocalyx [1-7]. Multiple studies have shown that proinflammatory cytokines can modulate the expression of membrane-tethered mucins in oral, nasal, and respiratory mucosal epithelium [33-36]. The results of this study demonstrate that IL-6, TNF-α, and IFN-γ increase the expression of MUC1 and MUC4 in the corneal epithelial cells at the picogram levels. Interestingly, our data demonstrate that these cytokines do not affect the MUC1 and MUC4 expression in conjunctival epithelial cells. Although both corneal and conjunctival epithelial cells are reported to express the receptors for these cytokines [37-39], the differential effect of these cytokines on MUC1 and MUC4 gene expression in corneal and conjunctival epithelial cells may be due to differences in the
receptor density, presence of decoy molecules, differential activation of signaling pathways, or different response of mucin promoters in the two epithelial cell types. Multiple studies have demonstrated that MUC1 has anti-inflammatory properties due to the negative regulation of Toll-like receptors [40,41]. Studies have also demonstrated that MUC1 can act as a microbial scavenger to limit Campylobacter jejuni and Helicobacter pylori infection in the gastrointestinal tract in a mouse model and limit S. pneumoniae infection in epithelial cells [42-44]. Therefore, the cytokine-mediated increase in gene expression of corneal epithelial MUC1 and MUC4 could possibly represent a physiological response to enhance the ocular surface defense against microbial stress. Interestingly, none of the three tested cytokines caused any notable change in the gene expression of MUC16 in both corneal as well as conjunctival epithelial cells, suggesting that the picogram dose of these cytokines is not sufficient to modulate MUC16 gene expression.

Cytokines have also been shown to induce the expression of enzymes such as heparanase and hyaluronidase, which can damage the mucin side chains, thus compromising the integrity of glycocalyx [22,23]. TNF-α has been shown to induce endothelial glycocalyx shedding resulting in increased vascular permeability and aberrant vasodilation [45]. However, the results of the present study demonstrate that IL-6, TNF-α, and IFN-γ do not have any detrimental effect on corneal and conjunctival epithelial cell glycocalyx. Lastly, none of the cytokines caused an increase in apoptotic cell death, but an increase in necrotic cell death was noted with TNF-α in conjunctival epithelial cells and with IL-6 treatment in corneal epithelial cells.
In summary, the results of the present study demonstrate that IL-6, TNF-α, and IFN-γ have differential effects on corneal and conjunctival epithelial cells mucins gene expression and cause an increase in MUC1 and MUC4 gene expression only in corneal epithelial cells. Furthermore, these cytokines did not cause any notable change in the corneal and conjunctival glycocalyx and apoptotic cell death.

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Fig. 1. Effect of IL-6 on MUC1, MUC4, MUC16 gene expression in stratified cultures of human corneal (A) and conjunctival (B) epithelial cells. The cells were exposed to IL6 for 24 hours.

* p < 0.05 compared to control cells (C) that were not exposed to the cytokine
Fig. 2. Effect of TNF-α on MUC1, MUC4, MUC16 gene expression in stratified cultures of human corneal (A) and conjunctival (B) epithelial cells. The cells were exposed to TNF-α for 24 hours.

* p < 0.05 compared to control cells (C) that were not exposed to the cytokine
Fig. 3. Effect of IFN-γ on MUC1, MUC4, MUC16 gene expression in stratified cultures of human corneal (A) and conjunctival (B) epithelial cells. The cells were exposed to IFN-γ for 24 hours.

* p < 0.05 compared to control cells (C) that were not exposed to the cytokine
**Fig. 4.** Representative confocal Z-stack images showing glycocalyx staining in stratified cultures of human corneal epithelial cells exposed to IL-6, TNF-α, and IFN-γ (250 pg/ml) for 24 hours. Nuclei are stained blue with DAPI and glycocalyx is stained green with Alexa-488 conjugated wheat germ agglutinin lectin.
Fig. 5. Representative confocal Z-stack images showing glycocalyx staining in stratified cultures of human conjunctival epithelial cells exposed to IL-6, TNF-α, and IFN-γ (250 pg/ml) for 24 hours. Nuclei are stained blue with DAPI and glycocalyx is stained green with Alexa-488 conjugated wheat germ agglutinin lectin.
Fig. 6. Flow cytometry quantification of apoptosis and necrosis in stratified human corneal (A) and conjunctival (B) epithelial cells treatment with IL-6, TNF-α, and IFN-γ (250 pg/ml) for 24 hours. The cells were stained with annexin V/propidium iodide dual staining for flow cytometry quantification.

* p < 0.05 as compared with the control cells that were not exposed to any cytokine.
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CHAPTER 5

Renin-angiotensin system activation and myofibroblast formation in Graft versus Host Disease (GVHD)-associated conjunctival fibrosis

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Abstract

Purpose: The present study was designed to investigate the role of myofibroblast transdifferentiation and conjunctival renin angiotensin system (RAS) in the pathogenesis of graft versus host (GVHD)-associated conjunctival fibrosis.

Methods: A mouse model of major histocompatibility-matched allogeneic transplantation was used to induce GVHD with male B10.D2 mice as donors and BALB/c mice as recipients. Male BALB/c to female BALB/c syngeneic transplant was used as control. Chromosome Y staining in the spleen cells obtained from female recipient mice was used to confirm engraftment. Phenol red thread test and fluorescein staining were used to quantify tears and corneal keratopathy, respectively. The eye tissue was harvested at 4 and 8 weeks after the transplant for immunostaining of smooth muscle actin, (α-SMA), a myofibroblast marker, angiotensinogen and angiotensin converting enzyme (ACE). Conjunctiva was harvested for RNA isolation, cDNA preparation and gene quantification of α-SMA, angiotensinogen and ACE using real time PCR.

Results: More than 80% spleen cells in recipient mice were chromosome Y positive, thus conforming to successful engraftment. A significant decrease in tear secretion and a marked increase in corneal keratopathy score after allogeneic transplant indicated the onset of ocular GVHD in these mice. A significant increase in α-SMA gene expression and the presence of a large number of α-SMA positive cells was noted in the bulbar orbital conjunctiva of mice after allogeneic transplant. Allogenic transplant also caused a significant increase in the gene expression and protein expression angiotensinogen and ACE in the subconjunctival eyelid area.
Conclusion: Results of the present study demonstrate that GVHD-associated conjunctival fibrosis is accompanied by myofibroblast formation and activation of local tissue RAS.

Introduction

Allogeneic bone marrow transplantation is a standard treatment for many hematologic malignancies, hemoglobinopathies, and immunodeficiency diseases. However, graft versus host disease (GVHD) remains a challenging complication of allogeneic bone marrow transplantation. It is an immune-mediated condition that causes damage to many organs, including eyes, with 60%–90% of GVHD patients showing ocular complications (Kim, 2005; Nassiri et al, 2013). Eyes get affected in both acute and chronic GVHD, although ocular involvement is more common in patients with chronic GVHD. Acute GVHD can cause photophobia, hyperemia, conjunctivitis, lagophthalmos, and corneal ulceration (Franklin et al, 1986; Nassar et al, 2013; Nassiri et al, 2013; Qiu et al, 2018), whereas chronic GVHD is characterized by severe ocular surface damage resulting in dry eye, keratinization, epithelial thinning, squamous metaplasia, corneal ulceration and meibomian gland atrophy (Inamoto et al, 2019; Nassiri et al, 2013).

Conjunctiva is also significantly affected in chronic GVHD patients with manifestations such as pseudomembranous conjunctivitis, symblepharon and fornix shortening (Nassar et al, 2013; Nassiri et al, 2013). Additionally, GVHD patients show a white scar under their bulbar conjunctiva suggestive of subepithelial conjunctival fibrosis (Kheirkhah et al, 2018; Kusne et al., 2017). Injury to conjunctival tissue caused by the chronic inflammatory response due to GVHD can initiate a wound healing response.
Excessive wound healing response may lead to aberrant extracellular matrix synthesis, thus resulting in conjunctival fibrosis. Myofibroblasts are contractile metabolically active cells that synthesis large quantities of extracellular matrix proteins and play a key role in fibrosis (Hinz et al, 2007; Klingberg et al, 2013; Wynn and Ramalingam, 2012). Myofibroblasts can form by trans-differentiation of a variety of cells such as resident fibroblasts, mesenchymal cells, and circulating fibrocytes (Hinz et al, 2007; Klingberg et al, 2013; Wynn and Ramalingam, 2012). Conjunctiva is a connective tissue containing a significant number of fibroblasts and presence of donor-origin fibrocytes has also been demonstrated in the eyes of GVHD patients (Ogawa et al., 2005).

Conjunctival tissue has been shown to express components of local renin angiotensin system (RAS), including renin, angiotensinogen, angiotensin converting enzyme (ACE) and AT1 receptors (Holappa et al, 2017; Ramirez et al, 1996; Savaskan et al, 2004; Sramek et al, 1992; Wagner et al,1996). Like many other organs, role of angiotensin II, the end effector of RAS pathway, has been implicated in conjunctival fibrosis (Shi et al, 2015; Ye et al., 2020). Components of RAS are upregulated after conjunctival injury (Shi et al, 2015). Angiotensin II has been shown to cause transdifferentiation of tenon’s fibroblasts to myofibroblasts (Ye et al., 2020). Profibrotic cytokines such as transforming growth factor beta (TGF-β), PDGF, CTGF released by immune cells in GVHD can serve as initial trigger for fibroblasts transdifferentiation to myofibroblasts (Dale and Saban, 2015; Ogawa et al., 2018). However, upregulation of RAS and a cross talk between profibrotic cytokines, especially TGF-β and RAS, can serve to amplify and perpetuate conjunctival fibrosis. The present study was designed
to investigate the role of myofibroblast transdifferentiation and conjunctival RAS in the pathogenesis of GVHD-associated conjunctival fibrosis.

Materials and Methods

Bone marrow transplantation

The animal protocol was approved by the Institutional Animal Care and Use Committee of Chapman University. All animal experiments were conducted in accordance with the ARVO statements for eye research. Mouse model of major MHC match and minor MHC mismatch was used to induce GVHD. Male B10.D2 mice (The Jackson Laboratories, Bar Harbor, ME, USA) having a homozygous MHC haplotype d/d were used as donors and female BALB/c mice (The Chares River, Wilmington, MA, USA) also having a homozygous haplotype d/d were used as recipients. The control group was syngeneic transplantation, where donors were male BALB/c mice and recipient were female BALB/c mice. The femurs and spleen cells were harvested from 8 weeks old donor mice, as described in our earlier publication (Shamloo et al., 2019). Recipient female BALB/c mice were exposed to 700 cGy irradiation using an X-Ray irradiator (RS 2000 X-ray Biological Irradiator, Rad Source Technologies, Buford, GA, USA). After 6 hours, the irradiated recipient female BALB/c mice were injected with 2X10^6 spleen cells and 1X10^6 bone marrow cells. The mice were fed with diet gel (ClearH2O, Portland, ME, USA), housed in sterile cages, and received sulfatrim (0.672 mg/mL) in their drinking water for two weeks after the transplant. At four and eight weeks after the transplantation, animals were euthanized by CO2 administration for the collection of ocular tissue. The study design included two different groups of mice: 1) Control group included BALB/c female mice (n=9) that received syngeneic bone marrow
and spleen cell transplantation from male BALB/c mice 2) Ocular GVHD group included female BALB/c mice (n =12) that received allogeneic bone marrow and spleen cell transplantation from male B10/D2 mice.

**Monitoring of engraftment**

The presence of chromosome Y in the spleen cells of female recipient mice was used to track engraftment by male donor marrow. A fluorescent paint probe was used to stain chromosome Y by fluorescent in situ hybridization using a commercially available kit (Empire genomics, Williamsville, NY). Mice were also monitored for systemic signs of chronic GVHD, such as loss of body weight and body hair.

**Tear quantification**

Tear secretion was quantified by phenol red thread (FCI Ophthalmics, Pembroke, MA, USA) which is pH sensitive and changes from yellow to red upon wetting by tears. The thread was placed in temporal side of lower eye lid for 1 minute and the length of color change was measured in millimeters and converted to volume using a standard plot. A standard curve was plotted by wetting the thread with known volume of artificial tears and measuring the length of the wetted phenol red thread. Due to the small amount of tear film volume in the mouse eyes, the phenol red thread test typically requires longer time to obtain consistent values, accordingly 1-minute duration for phenol red thread test was used. Tear volume was quantified before the allogeneic and syngeneic transplantation (baseline) and at week 2, 4, 6 and 8 after the transplantation (Shamloo et al, 2019).
Fluorescein staining

Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). A 2-μL sterile solution of 0.5% fluorescein was applied to mouse eye and imaging was performed under cobalt filter with a slit lamp (SL-17, Kowa, USA) using a digital camera. The captured corneal images were divided into four hypothetical quadrants for scoring the keratopathy using a previously published method (Shamloo et al, 2019; Zhang et al, 2014). Each quadrant was scored as follows: no staining = 0; slightly punctate staining less than 30 spots = 1; punctate staining more than 30 spots, but not diffuse = 2; diffuse staining but no positive plaque = 3; positive fluorescein plaque = 4. The scores of each quadrant were added to arrive at a final grade (total maximum possible score =16).

Tissue harvesting and cryosectioning

Eyes along with the eyelids were harvested from euthanized animals. The tissue was fixed in 4% paraformaldehyde overnight followed by immersion in 15% and 30% sucrose, respectively. The tissue was embedded in optimal cutting temperature (OCT) using 2-methylbutane bath cooled over liquid nitrogen. The sagittal sections were cut from frozen tissue at 8-μm thickness using cryostat (CM 1860, Leica, Germany) for performing immunofluorescent staining.

Immunofluorescence staining for α-SMA, angiotensinogen and ACE

Slides containing 8 μm thick sagittal sections of eye and eyelid were rinsed in phosphate buffer saline (PBS) and blocked with PBS solution containing 2% bovine serum albumin for 30 min. The tissue sections were then incubated with primary antibodies for α-SMA (1:1,00 dilution, Invitrogen, Carlsbad, CA), angiotensinogen (1:50
dilution R&D systems, Minneapolis, MN) and ACE (1:25 dilution R&D systems, Minneapolis, MN) for 90 minutes. The slides were washed with PBS three times and incubated with Alexa 488- or Alexa 647-conjugated secondary antibody (1: 500 Dilution, Abcam, Cambridge, MA) for 60 minutes (Sharma et al., 2009; 2012). The nuclei were stained with DAPI. The slides were images using confocal microscope (Nikon, Melville, NY). The number of nuclei showing α-SMA staining and the percent fraction of area showing angiotensinogen and ACE staining was quantified using Image J.

**RNA isolation, cDNA preparation and gene expression quantification**

The whole sheet of bulbar conjunctival tissue was removed by using iris scissor and fine pointed forceps from euthanized animals’ eye at the end of 4 and 8 weeks after transplantation. The mRNA was extracted using the RNeasy Mini kit (RNeasy kit; Qiagen Inc., Valencia, CA). The mRNA was immediately reverse transcribed to complementary DNA (cDNA) using a commercially available kit (Superscript III First-strand synthesis; Thermo Fisher Scientific). The cDNA was used to quantify α-SMA, angiotensinogen and ACE gene expressions using real-time PCR. A 20-μL reaction mixture containing 2 μL of cDNA, 2 μL each of 200 nM of forward primer and reverse primer (Table 1), and 10 μL of 23 SYBR green super mix was run at a universal cycle (95 °C for 10 minutes, 40 cycles at 95 °C for 15 seconds, and 55°C for 60 seconds) in a thermocycler (Biorad CFX thermocycler; Bio-Rad Laboratories, Hercules, CA, USA). β-actin was used as the housekeeping gene. The relative change in gene expression was calculated using ΔΔCt method.
Results

Characterization of GVHD and allogenic transplantation

Mice who received allogeneic transplantation started exhibiting signs of GVHD at around 4 weeks that included significantly lower body weight (Figure 1A), signs of skin scleroderma, and loss of body hair (Figure 1B). Interestingly, a sharp decrease in body weight was observed up to the initial 2 weeks after transplantation in both groups that received either allogeneic or syngeneic transplantation. This early decrease in body weight noted in both the groups can be likely attributed to the irradiation stress (Figure 1A). Both groups regained body weight by week 4. However, the regain of body weight was significantly lower in mice with allogeneic transplants compared to mice that received a syngeneic transplant. Furthermore, after this initial recovery, mice with allogeneic transplant did not show any further gain, and their body weight plateaued, suggesting the onset of systemic GVHD. On the other hand, mice that received syngeneic transplantation continued to show an upward trend with a notable increase in body weight (Figure 1A).

Since the donor mice in our model were male while the recipient mice were female, we used Y chromosome staining to confirm the successful engraftment of donor origin marrow cells. Figure 1C shows fluorescent in-situ hybridization staining for chromosome Y in the spleen cells obtained from recipient female mice at 4 weeks after allogeneic transplant. Quantification of spleen cells from five different animals showed an average of 80% spleen cells showing chromosome Y staining, thus confirming the successful engraftment of recipient mice by cells originating from male donor marrow.
Clinical signs of ocular GVHD

The mice who received allogeneic transplantation showed signs of ocular GVHD that included a decrease in tear film and onset of corneal keratopathy. Figure 2 shows time-dependent changes in tear film volume in mice that received syngeneic and allogeneic transplantation. Both groups had an average tear volume of 153nl and 137nl at the baseline prior to transplant. Just like body weight, both groups showed a decrease in tear film volume at 2 weeks after the transplant, which could be attributed to irradiation stress-related physiological changes. However, mice that received allogeneic transplants continued to show a further decline in tear film volume at weeks 6 and 8 after the initial slight regain at week 4. The tear volume in the mice with allogenic transplant was significantly less compared to mice that received a syngeneic transplant. Figure 3A shows a representative fluorescein-stained image of mice eyes that received syngeneic or allogeneic transplantation. The corneas of mice that received allogeneic transplant showed punctate staining at week 4 and even plaques after week 8. Quantification of corneal keratopathy showed that both groups had a mean score of 2 at the baseline (Figure 3B). Mice that received allogenic transplantation showed a significant increase in the corneal keratopathic score to 6 and 9.3 at 4 weeks and 8 weeks, respectively. The syngeneic mice also showed a slight increase in keratopathy score to 3.6 at week 8, possibly due to aging (Figure 3B).

Presence of conjunctival myofibroblasts in ocular GVHD

The presence of myofibroblasts is a hallmark feature of fibrosis. Myofibroblasts express high levels of intracellular bundles of α-SMA. Therefore, to test whether ocular GVHD causes conjunctival fibrosis due to myofibroblast formation, we used gene
expression to quantify α-SMA and performed α-SMA immunostaining to detect the localization of myofibroblasts. A 2.7-fold (p<0.05) and 2.02-fold increase in α-SMA gene expression was observed in the conjunctival tissue obtained from mice at 4 weeks and 8 weeks after allogeneic transplantation (Figure 4). Furthermore, immunostaining detected the presence of a large number of α-SMA positive myofibroblasts in bulbar orbital conjunctiva of mice at 4 and 8 weeks after an allogeneic transplant (Figure 5A). On the other hand, no myofibroblasts could be detected in the tissue sections obtained from mice that received a syngeneic transplant (Figure 5A). Quantification of the number of nuclei showed an average of 1079 α-SMA positive nuclei at 4 weeks and 978 α-SMA positive nuclei at 8 weeks after allogeneic transplantation compared to around 250 α-SMA positive nuclei in mice that received a syngeneic transplant (Figure 5B).

**Ocular GVHD and conjunctival RAS activation**

To test whether conjunctival fibrosis was accompanied by activation of local RAS, we quantified gene expression of angiotensinogen and ACE. We also detected angiotensinogen and ACE localization by performing immunostaining. A statistically significant 1.4-fold (p<0.05) increase in gene expression of angiotensinogen and a 2-fold (p<0.05) increase in gene expression of ACE was observed in the conjunctival tissue obtained from mice at 4 weeks after allogeneic transplantation. By 8-week time point, this noted increase in angiotensinogen and ACE returned to the baseline (Figure 6).

Immunostaining detected a large area under the bulbar conjunctiva that was intensely stained for angiotensinogen (Figure 7A) and ACE (Figure 8A) at 4 weeks after allogeneic transplant. On the other hand, angiotensinogen and ACE staining was barely
detectable in the eyelids of mice that received syngeneic transplant. Quantification of
the percentage of the stained area as fraction of total eyelid for angiotensinogen
showed only 4% stained area in mice that received syngeneic transplant but 37%
stained area (p<0.05) and 22% stained area (p<0.05) in mice at 4 weeks and 8 weeks
after the allogeneic transplant, respectively (Figure 7B). Similarly, < 0.4% area showed
ACE staining in mice that received syngeneic transplant compared to 2% (p<0.05) and
1.5% area at 4 weeks and 8 weeks in mice that received allogeneic transplantation
(Figure 8B)

Discussion

Fibrosis is one of the key features of chronic GVHD affecting many organs,
including lungs, skin, and GI tract (Ghimire et al., 2017; Kitko et al, 2012; Min, 2011;
Zeiser and Blazar, 2017). Clinical subepithelial fibrosis appearing as a white scar has
been reported in the eyelids of chronic GVHD patients (Kheirkhah et al, 2018; Kusne et
al., 2017). Studies have also shown a correlation between conjunctival fibrosis and
corneal epitheliopathy, suggesting that conjunctival fibrosis likely contributes to dry eye
and ocular surface damage in GVHD (Kheirkhah et al, 2018; Kusne et al., 2017). The
present study used B10D2 to BALB/c major MHC match and minor MHC mismatch
mouse model to investigate whether this mouse model shows features of conjunctival
fibrosis as characterized by the presence of myofibroblasts. This mouse model exhibits
scleroderma-like features, as is also confirmed in the present study, and the model has
been extensively used to study GVHD-associated fibrosis in various organs (Chu and
Gress, 2008; McCormick et al., 1999; Yaguchi et al., 2013). The results of the present
study demonstrate that this model indeed shows features of conjunctival fibrosis as
demonstrated by presence of myofibroblasts. Furthermore, the model recapitulates many other features of ocular GVHD, including dry eye and corneal keratopathy. Interestingly, conjunctival fibrosis was noted at 4 weeks without any further increase in severity at 8 weeks, whereas tear film and keratopathy score continued to decline up to the tested time point of 8 weeks. This trend tentatively suggests that the conjunctival fibrosis sets in early and may contribute to the ocular surface damage in this model as is observed in clinical studies.

The repair process during fibrosis typically involves deposition of excessive extracellular matrix, which results in replacement of normal parenchymal tissue with the hypertrophic scar tissue. Myofibroblast formation and proliferation is a critical step in fibrosis (Hinz et al, 2007; Klingberg et al, 2013; Wynn and Ramalingam, 2012). Myofibroblasts are metabolically active cells, which synthesize and secrete large quantities of extracellular matrix during fibrosis (Hinz et al, 2007; Klingberg et al, 2013; Wynn and Ramalingam, 2012). Myofibroblasts also contribute to altered mechanical properties of the tissues because they express high amounts of contractile bundles of cytoskeletal protein, α-SMA. The present study demonstrates a large number of α-SMA positive myofibroblasts in the orbital bulbar conjunctiva, suggesting that GVHD-associated conjunctival fibrosis is accompanied by myofibroblast formation. The noted increase of α-SMA gene expression at week 4 after transplantation followed by a decline at week 8, but a comparable number of α-SMA-stained cells suggest an initial proliferative phase of myofibroblast formation after allogeneic transplantation followed by stabilization phase. The resolution phase of fibrosis is typically characterized by a decline in the myofibroblast number due to apoptosis and other mechanisms.
Interestingly, results of the current study demonstrate that resolution of GVHD-associated conjunctival fibrosis likely requires a longer duration since a significant number of myofibroblasts were still present in the conjunctiva of GVHD mice up to the tested time point of 8 weeks. Furthermore, a shortening of eyelid and fornix was also consistently observed in the eyes of GVHD mice at 8 weeks. Given the detection of large populations of myofibroblasts and their localization in bulbar orbital conjunctiva of GVHD mice, it is highly likely that the tissue contractile changes caused by these myofibroblasts may be contributing to the noted alteration in the eyelid and fornix architect of these GVHD mice.

GVHD is an immune condition mediated by a complex interplay between donor immune cells and host tissue. Immune cells of donor origin have been shown to be present in the recipient conjunctival tissue (Tatematsu et al., 2012). These immune cells can release a variety of profibrotic cytokines such as TGF-β, PDGF, CTGF that can trigger fibroblast activation and their transdifferentiation to myofibroblasts (Mack, 2018 Rockey et al., 2015; Smigiel et al., 2018; Weiskirchen et al., 2019; Wynn, 2008). Further, these immune cells and activated fibroblasts also secrete profibrotic vasoactive peptides such as endothelin and angiotensin that can perpetuate a vicious cycle of excessive wound healing and fibrosis (Mack, 2018; Rockey et al., 2015; Smigiel et al., 2018; Weiskirchen et al., 2019; Wynn, 2008). Activation of RAS and elevated levels of its effector molecule ang II have been shown to play a critical role in mediating fibrosis in liver, skin, lungs, and cardiac tissue (Abdul-Hafez et al., 2018; Ferrario, 2016; Jia et al., 2018; Munshi et al., 2011; Wang et al., 2015). Ocular tissue has been shown to express all components of RAS (Holappa et al, 2017; Savaskan et al, 2004; Sramek et
al, 1992; Ramirez et al, 1996; Wagner et al, 1996). Overaction of RAS has been shown to play a critical role in conjunctival fibrosis after trabeculectomy (Shi et al., 2015; Ye et al., 2020). Results of the present study demonstrate that GVHD-associated myofibroblast formation was accompanied by a significant increase in the gene and protein expression of angiotensinogen and angiotensin converting enzyme in the tissue around conjunctiva. Angiotensin II, directly or through increased levels of TGF-β, can promote fibroblast proliferation and myofibroblast differentiation. Therefore, the noted increase in local angiotensinogen and ACE activity observed in this study is likely to be an important contributor to the initiation and perpetuation of conjunctival fibrosis and myofibroblast formation. Overlapping temporal kinetics between the concomitant increase in angiotensinogen and ACE and an increase in SMA expression, as noted in the present study, further supports this hypothesis.
**Figure 1:** Body weight of mice before (baseline) and at various time points after syngeneic and allogeneic transplantation (A). A significant (* p < 0.05) decrease in body weight was observed in mice who received an allogeneic transplant compared to mice with a syngeneic transplant. Representative photograph (B) showing signs of sclerodermatous skin lesions and hair loss in mice who received an allogeneic transplant. Spleen cells of recipient (female) mice showing Y chromosome staining confirming successful engraftment of donor (male) graft.
Figure 2: Tear film volume in mice before (baseline) and at various time points after syngeneic and allogeneic transplantation. A significant (*) $p < 0.05$ decrease in tear film volume was observed in mice who received an allogeneic transplant compared to mice with a syngeneic transplant.
Figure 3: Representative fluorescein-stained images of corneas at 8 weeks after syngeneic transplant (control) and at 4 weeks, 8 weeks after allogeneic transplant (GVHD). Quantification of fluorescein staining (B) showed significant (* p < 0.05) compared with syngeneic and before transplantation) corneal keratopathy at 4 and 8 weeks after allogeneic transplantation.
**Figure 4:** Gene expression quantification of α-smooth muscle actin in conjunctival homogenates obtained from mice at 8 weeks after syngeneic transplant (control) and at 4 weeks, 8 weeks after allogeneic transplant (GVHD). A significant increase in α-smooth muscle actin gene expression was noted in conjunctival homogenates obtained from mice (*p<0.05) after allogeneic transplant compared to the tissues obtained from mice that received syngeneic transplant.
Figure 5: Representative confocal images (A) showing immunofluorescent staining (green) for α-smooth muscle actin in the eyelid tissue sections obtained from mice at 8 weeks after syngeneic transplant (control) and at 4 weeks, 8 weeks after an allogeneic transplant (GVHD). Nuclei are stained blue with DAPI. Graph (B) shows quantification of α-smooth muscle actin-stained nuclei in images obtained from 5 different slides, each from n=5 mice. A significant increase (* p<0.05) in α-smooth muscle actin-stained nuclei was observed in tissues obtained from mice at 4 weeks and 8 weeks after allogeneic transplant compared to the tissues obtained from mice that received a syngeneic transplant.
Figure 6: Gene expression quantification of angiotensinogen and angiotensin converting enzyme in conjunctival homogenates obtained from mice at 8 weeks after syngeneic transplant (control) and at 4 weeks, 8 weeks after allogeneic transplant (GVHD). A significant increase in angiotensinogen and angiotensin converting enzyme gene expression was noted in conjunctival homogenates obtained from mice (*p<0.05) after allogeneic transplant compared to the tissues obtained from mice that received a syngeneic transplant.
Figure 7: Representative confocal images (A) showing immunofluorescent staining (green) for angiotensinogen in the eyelid tissue sections obtained from mice at 8 weeks after syngeneic transplant (control) and at 4 weeks, 8 weeks after an allogeneic transplant (GVHD). Nuclei are stained blue with DAPI. Graph (B) shows quantification of angiotensinogen-stained area (calculated as a fraction of total eyelid area) using images obtained from 5 different slides each from n=5 mice. A significant increase (* p<0.05) in angiotensinogen-stained area was observed in tissues obtained from mice at 4 weeks and 8 weeks after allogeneic transplant compared to the tissues obtained from mice that received a syngeneic transplant.
**Figure 8:** Representative confocal images (A) showing immunofluorescent staining (red) for angiotensin converting enzyme in the eyelid tissue sections obtained from mice at 8 weeks after syngeneic transplant (control) and at 4 weeks, 8 weeks after allogeneic transplant (GVHD). Nuclei are stained blue with DAPI. Graph (B) shows quantification of angiotensinogen-stained area (calculated as a fraction of total eyelid area) using images obtained from 5 different slides each from n=5 mice. An increase in angiotensin converting enzyme-stained area was observed in tissues obtained from mice at 4 weeks (* p<0.05) and 8 weeks after allogeneic transplant compared to the tissues obtained from mice that received syngeneic transplant.
Table 1: Sequence of forward and reverse primers used for Real-Time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reverse Primer</th>
<th>Forward Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angiotensinogen</td>
<td>5’CAA GTT GAT CTT CCA CCC TGT C3’</td>
<td>5’TCC CAC GCT CTC TGG ATT TA3’</td>
</tr>
<tr>
<td>ACE</td>
<td>5’TTG CTG CCC TCT ATG GTA ATG3’</td>
<td>5’GAC AGG TTC GTG GAA GAG TAT G3’</td>
</tr>
<tr>
<td>SMA</td>
<td>5’GGC AGT AGT CAC GAA GGA ATA G3’</td>
<td>5’CCA TCA TGC GTG ACT T3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’CCA AGA AGG AAG GCT GGA AA3’</td>
<td>5’CTC CCT GGA GAA GAG CTA TGA3’</td>
</tr>
</tbody>
</table>
References:


CHAPTER 6

Discussion, Conclusion and Future Direction

GVHD is a significant complication after stem cell transplantation that happens due to abnormal immune response to the healthy host tissue. It can cause damage to vital organs, including the eye and results in dry eye disease 1-5.

Dry eye is a multifactorial disease of the ocular surface characterized by loss of tear film homeostasis characterized by tear film instability, hyperosmolarity, ocular surface inflammation, and neurosensory abnormalities6. Tear film is composed of three layers. Outer lipid layer which is produced by the meibomian gland, and it covers the tear surface to prevent tear film evaporation. The middle aqueous layer produced by lacrimal gland, lubricates the eye surface and provides oxygen and nutrients to the cornea. Inner mucin layer retains tear to the eye surface, repels microbes and lubricates the eye surface 7. Hemostasis of these three layers maintains the ocular surface's health, and damage to any aspect of these components leads to dry eye disease 6,8.

The pathogenesis of GVHD-associated dry eye is incompletely understood. Previous studies have shown that GVHD causes fibrosis of lacrimal glands, which could potentially lead to reduced aqueous layer, thus resulting in ocular surface dryness 9,10. However, the lipid and mucin layer's role have not been studied in GVHD-associated dry eye disease. Studies in Sjögren and non-Sjögren related dry eye disease demonstrated a decrease in ocular surface mucins and glycocalyx barrier 10,11. Another study among contact lens wearers showed that solutions containing purified gastric mucins could help relief against dry eye 12. However, the effect of GVHD on ocular
surface mucins is not studied yet; therefore, we decided to investigate ocular surface mucins' role in GVHD-associated dry eye disease. Our GVHD mouse model demonstrated clinical signs of dry eye disease, as was evident from the decreased tear film and the presence of keratopathies. In this work, for the first time, we demonstrate that GVHD causes damage to the ocular surface glycocalyx and causes alteration of ocular surface mucins. Our results are in agreement with previous observations of decreased mucins in Sjögren disease-related dry eye disease\textsuperscript{13,14}. In vivo administration of rebamipide has been demonstrated to have a beneficial effect on Sjögren’s syndrome by increasing glycocalyx barrier\textsuperscript{14}. In this study, we tested the effect of rebamipide, a mucin secretagogue, on the GVHD-associated dry eye mouse model, which provided a significant protection against GVHD-associated dry eye by the maintaining of tear film secretion and a notable decrease in corneal keratopathy score. Although in this study, we investigated the effect of GVHD on specific mucins, but we did not investigate rebamipide's effect in the alteration of specific ocular surface mucins. Previous studies on stratified human cultured corneal epithelial cells, which were treated by rebamipide, have demonstrated an increase of mucin\textsuperscript{15}. These findings indicated that rebamipide might repair glycocalyx barrier disruption in dry eye disease as was noted in our data, through MUC16 protein expression.

Multiple possible mechanism can contribute to the decrease in mucins and damage to glycocalyx that we noted in our first study. Conjunctiva forms a large part of ocular surface, contains goblet cells and contributes to both secreted and membrane-tethered ocular surface mucins. Multiorgan fibrosis is a well-known complication in patients with chronic GVHD (cGVHD). While skin, liver, and gastrointestinal involvement
are the most common manifestations of acute GVHD, cGVHD may affect any organ, including lungs, skin, and eyes.\textsuperscript{16-18} Scleroderma-like changes and bronchiolitis obliterans syndrome are two of the most damaging outcomes in these patients, which were reported in 5 to 15 percent of patients with cGVHD.\textsuperscript{19} Ocular involvement in cGVHD is also very common and appears as severe ocular surface damage causing dry eye, keratinization, epithelial thinning, squamous metaplasia, corneal ulceration and meibomian gland atrophy.\textsuperscript{4,5,20} cGVHD has already been shown to cause an inflammatory destruction of the lacrimal glands with fibrosis, which decreases tear production, increases tear film osmolarity, and leads to dry eye disease.\textsuperscript{9,10} Conjunctiva is also significantly affected in chronic GVHD patients with pseudomembranous conjunctivitis, symblepharon, and fornix shortening manifestations.\textsuperscript{5,21} Clinical reports recently demonstrated that GVHD patients show a white scar under their bulbar conjunctiva which could be indicative of subepithelial conjunctival fibrosis.\textsuperscript{22,23} These subepithelial fibrotic changes in conjunctiva, as a goblet cell host, most possibly can decrease goblet cell density and ocular surface mucin production, and increase tear evaporation and result in dry eye disease. Our data for the first time demonstrated in a mouse model of cGVHD that cGVHD causes conjunctival fibrosis and it is accompanied by the presence of myofibroblasts in orbital conjunctival tissue and in Tenon’s capsule. Our data support the clinical reports of white eyelid scarring in GVHD patients that are indirectly suggestive of subconjunctival fibrosis in human GVHD. Furthermore, our mouse model of GVHD recapitulates many other features of ocular GVHD manifestations, including dry eye and corneal keratopathy.
Conjunctiva has local renin angiotensin system (RAS) including renin, angiotensinogen, angiotensin-converting enzyme (ACE), and AT1 receptors\(^{24-28}\). A recent study show that components of RAS are upregulated after conjunctival injury\(^{29}\) and angiotensin treatment of conjunctival fibroblasts causes their transdifferentiation to myofibroblasts\(^{30}\). Like many other organs, we think that angiotensin II, the end effector of RAS pathway, could play a role in conjunctival fibrosis. Angiotensin II could also potentiate the profibrotic effect of cytokines released by immune cells in GVHD especially TGF-β1 to transdifferentiate the conjunctival fibroblasts to myofibroblasts. Our data in mouse model of GVHD demonstrates that conjunctival fibrosis and myofibroblast formation is accompanied an increase in local angiotensinogen and ACE levels. Our data support the previous studies that angiotensin II type 1 receptor antagonist attenuate lung, and liver fibrosis in a murine model of chronic graft-versus-host disease\(^{31}\).

It is well known that osmotic stress is an important mechanism of ocular surface damages in dry eye. Studies have demonstrated that NFAT5 is a fundamental regulator of the osmotic stress in mammalian cells activated by hyperosmolar stress and had a detrimental effect in the induction of proinflammatory cytokines and stress-induced apoptosis in human limbal epithelial cells\(^{32,33}\). GVHD-mediated damage to ocular mucins, glycocalyx and conjunctival fibrosis with a resultant increase in tear film evaporation will also potentially increase the tear osmolarity. Many clinical studies have reported an increase in tear osmolarity in GVHD patients\(^{31-35}\). Hyperosmolarity has been shown to increase the release of proinflammatory cytokines from epithelial cells\(^{36}\). Our study also demonstrated that corneal and conjunctival epithelial cells exposure to
hyperosmolar stress causes an increase in the gene expression of NFAT5 and cytokines IL-1, IL-6, TNF-α and IFN-γ. Next, we hypothesized that these proinflammatory cytokines can cause potentially cause a decrease in ocular mucins expression or can cause damage to glycocalyx as was noted in our fits study. But our data showed that these cytokines do not cause a decrease in ocular surface mucins but rather increase their levels or do not cause any damage to glycocalyx. A handful of studies have shown that proinflammatory cytokines can upregulate mucin gene expression in chronic inflammatory lung disease and intestinal cancer\textsuperscript{37-39}. Studies have also reported that MUC1 can act as a microbial scavenger to inhibit bacterial infection in the gastrointestinal tract in a mouse model and in epithelial cells\textsuperscript{40-42}. Therefore, the cytokine-mediated increase in gene expression of corneal epithelial MUC1 and MUC4 in our study could be a response to enhance the ocular surface defense against microbial stress that can increase cytokine levels. However, there has not been a study about the role of cytokine mediated changes in mucin expression in the ocular surface epithelium and our is the first study.

In summary, we investigated the effect of GVHD on tear film volume, corneal keratopathy, ocular surface glycocalyx, goblet cells, ocular surface mucins, and fibrosis of conjunctiva. We further studied whether hyperosmolar stress can modulate the release of pro-inflammatory cytokines from the ocular surface epithelial cells and if these pro-inflammatory cytokines can cause any detrimental changes to ocular surface glycocalyx and mucins. \textbf{In Chapter 2}, our data demonstrated that ocular GVHD-associated dry eye is characterized by a significant decrease in tear film volume and the onset of corneal keratopathy. Ocular GVHD caused a significant decrease in the area
and thickness of corneal glycocalyx. A significant decrease in the goblet cells, mucin 4, and mucin 5AC levels were also observed. Topical treatment with rebamipide, a mucin secretagogue, partially attenuated ocular GVHD-mediated decrease in tear film volume and significantly reduced corneal keratopathy severity. In summary, our data demonstrated that ocular GVHD has a detrimental impact on ocular surface glycocalyx, and mucins and augmentation of ocular surface mucins is a viable pharmacological approach to partially prevent ocular GVHD-associated decrease in the tear film and the severity of corneal keratopathy.

An Increase in tear osmolarity is a cardinal feature of dry eye disease. Osmotic stress activates stress-related kinases, and these kinases are responsible for increased transcription of the proinflammatory cytokines. In Chapter 3, we tested the effect of hyperosmolar stress on the changes in gene expression of NFAT5, a tonicity-regulated transcription factor and pro-inflammatory cytokines IL-1, IL-6, TNF-α, and IFN-γ using telomerase-immortalized human corneal and conjunctival epithelial cells. Our data demonstrated that hyperosmolar stress causes a remarkable increase in NFAT5 and proinflammatory cytokine gene expression in ocular surface epithelial cells. Human conjunctival and corneal epithelial cells showed a differential response to the hyperosmotic stress-mediated change in NFAT 5 and proinflammatory cytokines' gene expression, with human corneal epithelial cells being more sensitive to the hyperosmotic stress. The release of proinflammatory cytokines orchestrates the initiation and perpetuation of ocular surface inflammation. Cytokines IL-1, IL-6, TNF-α, and IFN-γ have been shown to participate in the early-stage and amplification-stage of dry eye disease. The levels of IL-6, TNF-α, and IFN-γ are known to be elevated in
patients suffering from dry eye disease. **In Chapter 4**, we tested the effect of picogram concentration of proinflammatory cytokines IL-6, TNF-α, and IFN-γ on the gene expression of membrane-tethered mucins MUC1, MUC4, MUC16, ocular surface glycocalyx, and viability of stratified cultures of telomerase-immortalized human corneal and conjunctival epithelial cells. Our data demonstrated that treatment with IL-6, TNF-α, and IFN-γ resulted in a significant increase in the gene expression of MUC1 and MUC4 in stratified human corneal epithelial cells but did not cause a significant change in the gene expression of MUC16. Unlike corneal epithelial cells, these cytokines caused no significant changes in the expression of the MUC1, MUC4, and MUC16 genes in the stratified human conjunctival epithelial cells. Further, IL-6, TNF-α, and IFN-γ exposure did not cause any notable change in the stained area of glycocalyx in the stratified human corneal and conjunctival epithelial cells. None of the tested cytokines caused any significant increase in apoptotic cell death in stratified human corneal and conjunctival epithelial cells. However, treatment with IL-6 resulted in an increase in necrotic cell death in stratified human corneal epithelial cells. In contrast, TNF-α caused a significant increase in necrotic cell death of stratified human conjunctival epithelial cells.

Finally, **In Chapter 5**, we investigated the role of myofibroblast transdifferentiation and conjunctival renin-angiotensin system (RAS) in graft versus host (GVHD) pathogenesis-associated conjunctival fibrosis. Our results demonstrated that ocular GVHD causes a significant increase in α-SMA gene expression and formation of myofibroblasts as demonstrated by the presence of α-SMA positive cells in the bulbar orbital conjunctiva of mice after allogeneic transplant. Ocular GVHD-associated fibrosis
also accompanies by a concomitant increase in the gene expression and protein expression of angiotensinogen and ACE in the subconjunctival eyelid area.

**Future Directions**

Future studies are needed to understand the mechanism of GVHD-mediated damage to ocular surface mucins and glycocalyx besides conjunctival fibrosis as shown in our study. Infiltration of ocular surface by donor T cells and T cell-mediated direct damage to corneal and conjunctival epithelial cells could be one such potential mechanism. The role of other immune cells including antigen presenting cells, and T regulatory cells and their interaction with ocular surface epithelial cells also needs to be investigated. Lastly, these immune cells especially macrophages can cause release of profibrotic cytokines such TGF-β and PDGF. The profibrotic cytokines can cause activation of other profibrotic pathways such as renin angiotensin system and transdifferentiation of fibroblasts to myofibroblasts resulting in ocular fibrosis as was observed in the present study. Polarization of macrophages from classically activated proinflammatory type to alternatively activated profibrotic healing type could be a transition in ocular GVHD from inflammatory to fibrotic phase. Future studies need to address whether GVHD causes donor macrophage infiltration into host cornea and conjunctiva and whether these macrophages undergo polarization from M1 proinflammatory type into profibrotic M2 phenotype.
References:


