Glutathione and Its Transporters in Ocular Surface Defense

HOVHANNES J. GUKASYAN, PHD,[1*] KWANG-JIN KIM, PHD,[2-5,8] VINCENT H.L. LEE, PHD, DSC,[1,6‡] AND RAM KANNAN, PHD[2,7]

ABSTRACT **Glutathione (GSH) is an abundant antioxidant ubiquitous in nearly all cell types. Deficiency of GSH has been linked to ocular disease and viral infection. Other established vital roles of GSH include detoxification and immunoprotection. Endogenous GSH plays a protagonist's role in safeguarding active transport processes compartmentalized at the interface between conjunctival mucosa and the tear film. Optimal electrokinetic transport across the conjunctival epithelium requires the mucosal presence of GSH. Glutathione is the most abundant known endogenous antioxidant molecule in tear fluid, mainly derived from conjunctival secretion. Conjunctival GSH transport, a major kinetic component of GSH turnover, occurs through multiple functionally distinct mechanisms. Cell membrane potential regulates conjunctival GSH efflux, while conjunctival GSH uptake requires extracellular Na^+. Significant modulation of GSH, its constituent amino acids, and functions of associated transporters occurs in the conjunctival epithelium with viral inflammatory disease. Topical conjunctival delivery of GSH, its metabolic precursors, or pharmacologic stimulation of endogenous conjunctival GSH secretion carry potential in alleviating viral-inflammatory conjunctivitis.**

KEY WORDS **cysteine/cystine, cystic fibrosis transmembrane conductance regulator protein (CFTR), epithelial transport mechanisms, glutathione (GSH), ocular surface defense, oxidative stress**

I. INTRODUCTION

Age-related ocular diseases characteristically exhibit an inability of afflicted tissues to offset oxidative insult, a feature that may play a chronic role in injury of epithelia found at the corneo-conjunctival tear fluid boundary. This epithelial barrier provides local defensive organization as a last obstacle against penetration into deeper tissues by toxic, microbial, and mechanical insults from the outside environment. Tears are the first line of defense for the ocular surface, concentrating locally released biochemical mediators, and they play a major role in the immunology of the pre-ocular milieu. Glutathione (**GSH**) is the most abundant biological antioxidant found in mammalian tear fluid. Vital roles of GSH include detoxification of electrophiles, maintenance of protein thiol status, free radical scavenging, and participation in immunoprotection. Conjunctival mucin and fluid secretion, driven by intricate subcellular transport machinery, plays a supplementary defensive role by partially contributing to the tear film structure and function. In this setting, conjunctival GSH metabolism and transport are essential in the maintenance of homeostasis, both in the tissue epithelium and stroma, with an emerging immunopharmacological importance for the ocular surface. Recent studies on conjunctival GSH turnover have generated great interest in ocular surface protection and maintenance during inflammatory disease and oxidant stress.

II. A COMPARTMENTALIZED PERSPECTIVE OF CONJUNCTIVAL EPITHELIAL TRANSPORT MECHANISMS

A. Solute Transport

The conjunctiva is a thin, mucus-secreting, vascularized tissue that covers the inner surface of the eyelids and the anterior sclera where the cornea begins.[1] In terms of surface

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From the departments of [1]Pharmaceutical Sciences, [2]Medicine, [3]Physiology and Biophysics, [4]Biomedical Engineering, [5]Molecular Pharmacology and Toxicology, and [6]Ophthalmology; [7]Doheny Vision Research Center; and [8]Will Rogers Institute Pulmonary Research Center, Schools of Medicine, Engineering, and Pharmacy, University of Southern California, Los Angeles, CA, USA. Current affiliation: *Pfizer Global R&D, La Jolla Laboratories, San Diego, CA, USA. ‡The Chinese University of Hong Kong, School of Pharmacy, Faculty of Medicine, Hong Kong.

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Single copy reprint requests to: Ram Kannan, PhD (address below).

Corresponding author: Ram Kannan, PhD, University of Southern California, Keck School of Medicine, Department of Ophthalmology, 1355 San Pablo Street, DVRC 308, Los Angeles, CA 90089-9224, USA. Tel: (323) 442-6715. Fax: (323) 442-6688. Email: RKannan@doheny.org

OUTLINE

area, the conjunctival epithelium occupies 80% of the ocular surface, functioning as a protective barrier and participating in the maintenance of tear film stability by means of the mucus secreted from resident goblet cells.[1-3] The dynamic physiological nature of the conjunctiva is demonstrated through the identification of several active transport mechanisms for Na^+/substrate- and H^+/peptide-absorption, as well as Cl^-/anion-secretion.[4] Electrophysiological measurements and isotopic flux studies indicate that a majority of this active ion transport by the epithelial cells is composed of net serosal to mucosal Cl^- secretion, while the remainder is attributed to a net mucosal to serosal Na^+ absorption.[5] Notably, the existence and apical localization of cystic fibrosis transmembrane conductance regulator protein (**CFTR**) has been confirmed and validated in the pigmented and albino rabbit and rat, as well as in porcine conjunctival epithelia.[6,7] It has been suggested that the mucosal exit of Cl^- from conjunctival epithelial cells (**CEC**) may be coupled to the parallel secretion of fluid and GSH.[8,9]

A quantitative assessment of the relative and individual contributions of various active sodium and chloride transport processes in ocular surface ion and fluid secretion for tear film homeostasis was recently proposed as an iterative mathematical model.[10] Functioning in concert, molecular transport mechanisms of ions and substrates partly sustain the tear film, coating the immediate microenvironment that lines the mammalian ocular surface (Figure 1).

B. Ion Transport

An automatic voltage clamp apparatus has been used to collectively measure all active and electrogenic biophysical processes that occur in the conjunctiva.[11] Conjunctival short circuit current, I_{sc}, represents the cumulative sum of all active (and electrogenic) current conveyed by various ions transported across the conjunctiva. Surgically excised pigmented rabbit conjunctival tissues exhibit a spontaneous potential difference (**PD**) of 15.5 ± 1.5 mV (mean ± SEM) across (tear side negative) when mounted in a modified Ussing chamber.[5,11-13]

Strong temperature dependency and the inhibitory effect of serosal ouabain on the rabbit conjunctival I_{sc} are characteristic of active Na^+,K^+-ATPase driven ion transport in this tissue.[11] While oxidation may have a direct, yet nonspecific, effect on basolateral Na^+,K^+-ATPase pumps in the conjunctiva, elevated endogenous H_2O_2 levels can mediate the reduction in tissue GSH level and trigger secondary mechanisms that are responsible for processing of oxidized, damaged, and nonfunctional membrane proteins.

Age-related ocular diseases usually manifest an inability to sufficiently cope with oxidation, a feature that may contribute to the accumulation of damaged protein(s) in ocular tissues like the conjunctiva. A decline in the ability to respond toward oxidative stress with time of cellular self-repair and protein turnover mechanisms, such as ubiquitination and proteosomal activity, is associated with tissue injury.[14,15] Whether or not similar pathways play a role in oxidant and GSH-mediated modulation of ion transporters in the pigmented rabbit conjunctiva is a crucial topic for further investigation.

Measurements of tissue I_{sc} (or comparable whole cell ion currents and conductances) have been validated as suitable endpoints to characterize the effect of oxidative stress on ion transport across a number of epithelia. For example, H_2O_2 and GSH modulation of specific Cl^- channels in the human retinal pigment epithelium (**RPE**)[16] and t-butylhydroperoxide-induced changes in permeability and ion permeation-selectivity in rat tracheal epithelia[17] were investigated to correlate the role of redox balance as it relates to fundamental epithelial transport processes participating in regulation of cellular pH, volume, or levels of extracellular fluid.[18,19] Hydrogen peroxide elicited asymmetric effects on active ion transport rates and transepithelial resistance to passive solute flow in rat alveolar epithelial cell monolayers on permeable supports.[20]

In alveolar epithelium, although the addition of H_2O_2 to either apical or basolateral fluid decreased I_{sc} gradually in a dose-dependent manner, the basolateral treatment was ~100-fold more potent in terms of the effective concentration of H_2O_2 at which I_{sc} was decreased by 50% (IC_{50}). Furthermore, the sensitivity of I_{sc} to apical H_2O_2 was inversely dependent on catalase (a ubiquitous heme protein that catalyzes the dismutation of H_2O_2 into water and molecular oxygen) activity, whereas that of the basolateral treatment was not.[20]

Recent results show that mucosal GSH is crucial for the maintenance of proper ion transport activity in isolated pigmented rabbit conjunctival tissues. Conjunctival net GSH secretion may be the primary mechanism of counter-

acting mucosally generated peroxides present within the immediate microenvironment of the tear film in contact with this tissue. Mucosally applied GSH (or analogs) provide functional protection of Na^+,K^+-ATPases in the serosal membranes, maintaining the physiological activity of conjunctiva under oxidative stress. Glutathione may be important in the modulation of cellular responses that lead to the processing of oxidized, damaged, and nonfunctional membrane proteins found in age-related ocular diseases under oxidant stress.[21]

C. Critical Role of Antioxidants in Tear Film

Its unique location and immediate proximity to the surrounding environment expose the conjunctiva to oxidative insult emanating from light, heat, chemicals, atmospheric (noxious) gases, or physical abrasion. Although several antioxidant molecules are known to be present within tear fluid secretions, their exact source is unclear.[22] For example, ascorbate and riboflavin are present at substantial levels in various tissues at ocular surfaces and tear fluid, while their interaction in the presence of light (or trace amounts of unbound metals in the absence of light) spontaneously generates H_2O_2 as ascorbate is consumed.[23] The dual nature of ascorbate as a pro- and anti-oxidant may partly contribute to the nonspecific defense mechanisms present in the mucin network of the tear film, utilizing the natural bactericidal properties of "autologous" H_2O_2.

Within the tear film, serving as a front line of chemical defense for the conjunctiva and cornea, endogenous antioxidants and enzymes with antioxidative activity are continuously regenerated. Conjunctival mucosal GSH secretion is critical to the maintenance of ocular surface redox homeostasis.[24] As stated earlier, GSH not only serves multiple, vital, detoxifying functions, but also participates in the maintenance of essential thiol status of proteins, providing an intracellular reservoir for amino acid cysteine and modulating critical cellular processes ranging from DNA synthesis to immune function in the conjunctiva.

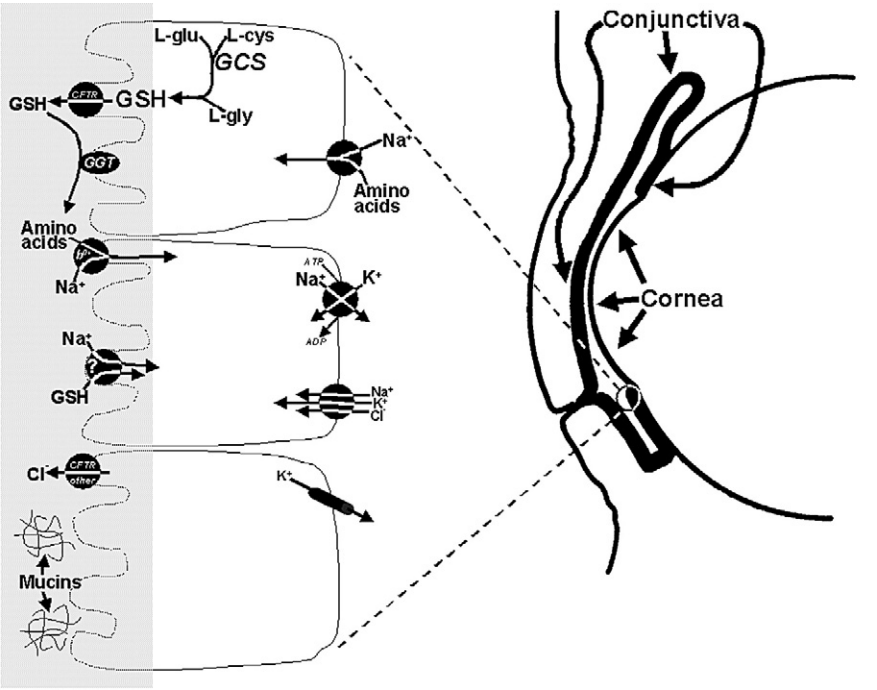

Figure 1. Conjunctival GSH turnover: biosynthesis and transport. Bulbar, fornicial, and tarsal regions of conjunctiva are indicated by arrows, and the cornea is highlighted for contrast. The enlarged section shows conjunctival epithelial and tear fluid microenvironment. Key active transporters are shown, ie, serosal Na^+,K^+-ATPase pump and K^+-channel, Na^+:K^+:$2Cl^-$-cotransporter, as well as mucosal CFTR, working in concert with GSH and its constituent amino acid transport and metabolic processes.

III. CONJUNCTIVAL GSH TURNOVER

A. Biosynthesis

Glutathione (*γ-L-glutamyl-L-cysteinyl-glycine*) is synthesized in virtually all known cell types, including conjunctival epithelial cells, from the precursor amino acids L-cysteine, glycine, and L-glutamic acid through a two-step pathway involving the rate-limiting enzyme γ-glutamylcysteine synthetase (**GCS**) and glutathione synthetase.[25,26] The availability of sulfur amino acid precursor L-cysteine is critical for maintenance of intracellular GSH. An amino acid transport system highly specific for L-cystine (the oxidized, -S–S, form of L-cysteine) and L-glutamate, operating in a Na^+-independent manner, has been described in many cell types including human RPE.[27]

In addition to the widely distributed X_c^- system, other known transporters for L-cystine like $b^{0,+}$ and X_{AG} exist.[28] Transcriptional modulation of these transporters from oxidative insults can result in reduced levels of cytoplasmic GSH. In immortalized human RPE cells, nitric oxide (**NO**) causes adaptive induction of the X_c^- amino acid transport system and increases L-cystine uptake, elevating intracellular GSH levels.[27] Nitrosative stress also causes a parallel induction of L-cystine uptake and the key enzyme GCS in the conjunctiva. However, this response may be heterogeneous, involving more than one specific amino acid transporter system.[29]

Functional transport mechanisms for L-cyst(e)ine in the conjunctival epithelium are multifactorial. While conjunctival tissue L-cyst(e)ine may be of systemic or intraocular origin, it may also arise from the catabolism/metabolism of GSH itself. After GSH is released into extracellular space, it is subsequently degraded via a two-step enzymatic process[30] that releases one equivalent of L-cysteine, which is then salvaged by active reuptake into the conjunctiva (Figure 1). Expression of γ-glutamyl transpeptidase (**GGT**), an enzyme that cleaves the γ-glutamyl bond of GSH, has been shown in excised conjunctival tissue and primary CEC utilizing

freshly isolated conjunctival epithelial cells, suggesting that the breakdown and re-synthesis of GSH within the tear film may be an important component of overall GSH metabolism in this tissue.[21,24,29]

B. Transport

The superficial cell in multicell-layered conjunctiva largely dictates the electrochemical transport properties of the conjunctiva, due to its moderate-to-low tight junctional conductance. Exact enumeration of tight junctional conductance relative to overall tissue conductance of conjunctiva has not been reported to date. It is necessary to model the conjunctiva as a simplified single-cell-layered membrane with a parallel shunt in order to illustrate directionally asymmetrical fluxes of GSH with all other operating, electrochemically connected transport processes. However, since the conjunctiva is a complex multi-layered tissue, such a simplistic consideration might disregard possible bilaminar unstirred layers. Outer (overlying cells) and inner (in between cells) tear fluid lamina of the superficial conjunctival cell layer are stagnant due to their proximity to the stationary cells, and could act as diffusion barriers that influence paracellular movements among other transport phenomena (Figure 1). However, the estimates of paracellular GSH flux would only involve basal rates, but would not be predictive of modulation in transcellular transport.

GSH export into the extracellular space contributes predominantly to its turnover. A facilitative, Na^+-independent GSH efflux system that mediates GSH transport from high-millimolar tissue concentrations to micromolar levels in vascular fluid has been characterized in several cell types.[30,31] Efflux of cytoplasmic GSH occurs in its reduced and oxidized form, or as a chemical conjugate with toxins. Export of reduced GSH into the luminal space lined by polarized epithelia of intestine, kidney, upper airway, and conjunctiva has been shown.[8,24,32-34] Evidence has been presented for a role of the ATP-binding cassette (**ABC**) transporter family as GSH carriers. Organic anion transporter protein (**oatp1**), hepatic basolateral organic solute transporter, and Mrp(1 or 2), ATP-dependent multidrug resistance-associated plasma membrane transport proteins (basolateral or canalicular) were all shown to mediate GSH efflux.[31,35,36] Evidence for the presence of Na^+-dependent GSH uptake transporters in tissues such as the conjunctiva, lens, and liver have also been reported in recent studies,[37,38] although their identity and regulation at the molecular level is not fully understood.

IV. THE CONJUNCTIVA SECRETES GSH INTO TEAR FILM: POSSIBLE INVOLVEMENT OF CFTR

A. Mechanistic Links Between Cl^- and GSH Efflux

It is believed that the efflux of GSH may be dependent on the efflux of chloride. For example, abnormal transport of GSH may be caused by CFTR mutation in cells or tissues obtained or originated from cystic fibrosis (**CF**) patients.[8,9,39] Pathology in CF disease is linked to a mutation in the CFTR Cl^- channel leading to diminished efflux of cytoplasmic GSH into the extracellular space from certain cells that do not express other Cl^- channels. In this abnormal transport-related illness, total GSH in epithelial cells is at normal levels, but the cells lack proper anion secretory capability, creating a chronic and progressive extracellular deficit of GSH.[8]

The secretion modality of GSH into the tear fluid by conjunctiva may be subject to similar pathophysiological regulatory mechanisms. Tear fluid concentrations of Na^+, K^+, Cl^-, and Ca^{2+} were estimated at various flow rates for normal and CF subjects. Steady-state levels of the ions in tear fluid are abnormal in patients with CF, and high tear Ca^{2+} and low tear Na^+ are prevalent characteristics.[40-43] Although tear film GSH content has not been evaluated systematically in CF to date, other factors such as age, gender, and the environment did not seem to account for the corneal pathology of CF patients.[40,43]

Studies suggest that CFTR may have an additional function (albeit it is not clear if CFTR per se conducts GSH), namely, to facilitate GSH export from cells.[9,44] For example, cells expressing mutant CFTR (ΔF508) showed decreased GSH efflux compared to cells expressing wild type CFTR.[45]

Gao et al hypothesized that some of the lung pathology associated with CF might be due to inadequate GSH transport into the airway epithelial lining fluid.[8] This lining fluid normally contains high levels of GSH, approximately 400 µM, compared with blood plasma levels of only 5-20 µM.[39,46,47] The GSH content of airway epithelial lining fluid from CF patients is approximately one third of that in normal humans.[48] Similarly, CFTR knockout mice have lower levels of GSH in their airway epithelial lining fluid,[49,50] and this deficiency may render the lung epithelia more susceptible to oxidative damage from chronic infection and inflammation.[8]

Interestingly, Cantin et al, using a model cell line, recently demonstrated that the expression of CFTR protein is tightly downregulated by oxidant stress. Sublethal doses of oxy-radicals markedly decreased CFTR in a time-dependent manner with a parallel reduction in γ-GCS catalytic subunit level. In this regard, it may be surmised that suppression of CFTR expression represents an adaptive response of mucosal epithelium to an exogenous oxidant stress.[46]

With use of biological and electrophysiological techniques, CFTR has been identified in conjunctival tissues of multiple species, including the pigmented rabbit (Figure 1).[6,7] Findings of decreased secretion of chloride, fluid, and GSH, along with oxidant stress and Ad5-infection of the conjunctiva, may suggest a close interaction between the GSH and chloride transporters, including CFTR.[21,51] Biochemical or molecular-biological information on the presence of other organic anion transporters in the conjunctiva, or the effect of viral infection on their expression and function, is not available at the present time. The activity (and perhaps abundance) of CFTR and the level or rate of GSH secretion may be coupled together via yet unknown mechanisms. There is also the additional possibility, as shown by recent studies,[9] that CFTR itself directly mediates the efflux of GSH.

B. The Role of CFTR in GSH Efflux into Tear Fluid

Two theories regarding the influence of CFTR on the transport of GSH can be postulated.

1. Theory 1: Direct Mediation of GSH Transport by CFTR

According to the first theory, CFTR can directly mediate GSH transport (Figure 1). Structurally analogous multidrug resistance protein 1 (**MRP1**) substrates were shown to block the CFTR channel, suggesting that MRP1 and CFTR may share GSH-like substrates.[9,46] CFTR was originally shown to allow passage of GSH using the patch clamp technique, but whether the imposed experimental conditions were physiologically relevant is debatable.[9] Seemingly direct transport of GSH by CFTR was observed in membrane vesicle and proteoliposome experiments.[44] ATP binding and hydrolysis in CFTR regulates channel gating, suggesting that ATP may alter substrate affinity of CFTR.[52,53] The presence of GSH alters ATPase activity of CFTR, similar to what is known for MRP1, suggesting that GSH inhibits CFTR ATPase activity[54] and that this inhibition can change the properties of CFTR, favoring GSH over chloride ions.[44] Because CFTR is the only MRP family member that is functionally polarized to the apical membrane of epithelial cells, there is a high probability that it may in fact function as a GSH transporter or channel. Assuming that CFTR is a major route of GSH efflux from epithelial cells, oxidant-mediated increases in cellular (but not in extracellular) GSH levels[21] suggest that suppression of CFTR function represents an adaptive mechanism contributing to the preservation of intracellular GSH during such stress conditions.[46]

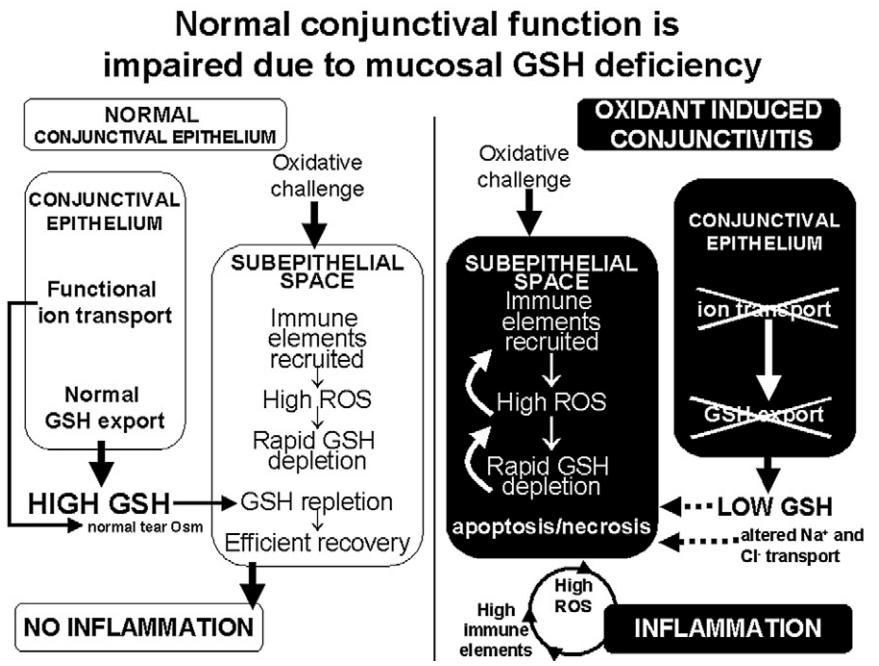

Figure 2. Impairment of conjunctival function due to GSH deficiency. Delineating cascades of tissue and cellular response following exposure to oxidative challenge: normal (left panel) and inflamed (right panel) conjunctivas. Healthy (left panel), actively ion transporting epithelial cells maintain a high GSH secretion rate, while presence of basal levels of mucosal GSH allows for efficient electrophysiological activity of the conjunctiva and continuous repletion of intracellular GSH. Inflamed and oxidant-challenged conjunctivas (right panel) are not able to maintain functional ion transport to support GSH secretion, leading to inefficient recovery and cellular damage.

2. Theory 2: Modulation of GSH Transport Via Alteration of Cl^- Gradients

The second theory proposes that CFTR does not mediate direct transport of GSH, and instead modulates GSH transport (occuring via other transporter proteins) through its ability to alter Cl^- gradients. Epithelial cells expressing mutated CFTR regain GSH secretion when treated with Cl^- ionophores,[39] suggesting that the restoration of chloride flux is sufficient to stimulate GSH transport. Additionally, a decrease in mRNA level for MRP1 has been linked to cells with mutated CFTR and shown to be even lower when basal Cl^- conductance is severely compromised.[55] These reports suggest that expression of MRP1 may be dependent on Cl^- gradients, and that mutations in CFTR can inhibit GSH transport via MRP1. CFTR influences the function of outwardly rectifying Cl^- channels, potassium channels in renal outer medullary ducts, and amiloride-sensitive epithelial sodium channels,[56] and, therefore, they may also participate in modulation of MRP1 or perhaps other GSH transporter activity.

V. CONJUNCTIVAL GSH AND ION TRANSPORT IN AN ADENOVIRUS TYPE 5 (AD5) RABBIT OCULAR INFECTION MODEL

Studies on GSH metabolism have become more important with the emerging immuno-pharmacologic potential of GSH.[57] Thus, the role of metabolism/transport of GSH in protecting the conjunctival tissue and in maintaining the redox state of the conjunctival cells during inflammatory disease and oxidant stress is a topic of great interest.

GSH was found to have antiviral properties and attenuated viral replication in keratitis and conjunctivitis.[58] The mechanisms of reduction of virally-induced infection and inflammation are complex and have not been fully elucidated. It was suggested that the formation of sub-epithelial infiltrates in the Ad5-infected ocular model is likely to be immune-based,[59] and antiviral drugs may block the formation of these infiltrates (Figure 2).

Clinical and in vivo studies suggested involvement of proinflammatory cytokines in allergic conjunctivitis. Models of allergic conjunctivitis have shown a late-phase allergic response,[60] indicating the possible involvement of inflammatory mediators. In fact, it was shown recently that hu-

man conjunctival epithelial cells, when stimulated by lipopolysaccharide, secreted several cytokines, such as TNF-α, interleukin (**IL**)-6, IL-8, and granulocyte-macrophage colony stimulating factor (**GM-CSF**), which were undetectable under normal conditions.[61] Thus, conjunctival epithelial cells may contribute to the pathogenesis of human ocular diseases by producing pro-inflammatory cytokines, and this finding may be helpful in designing targets of therapy.

Although pharmacological manipulation of GSH homeostasis in ocular tissues and its effect on cytokine-mediated responses are essentially unknown, unraveling the biochemistry of redox-linked pathways could be a reasonable clinical approach. This concept is supported by various studies of cytokine-GSH interaction in other tissues.[62,63]

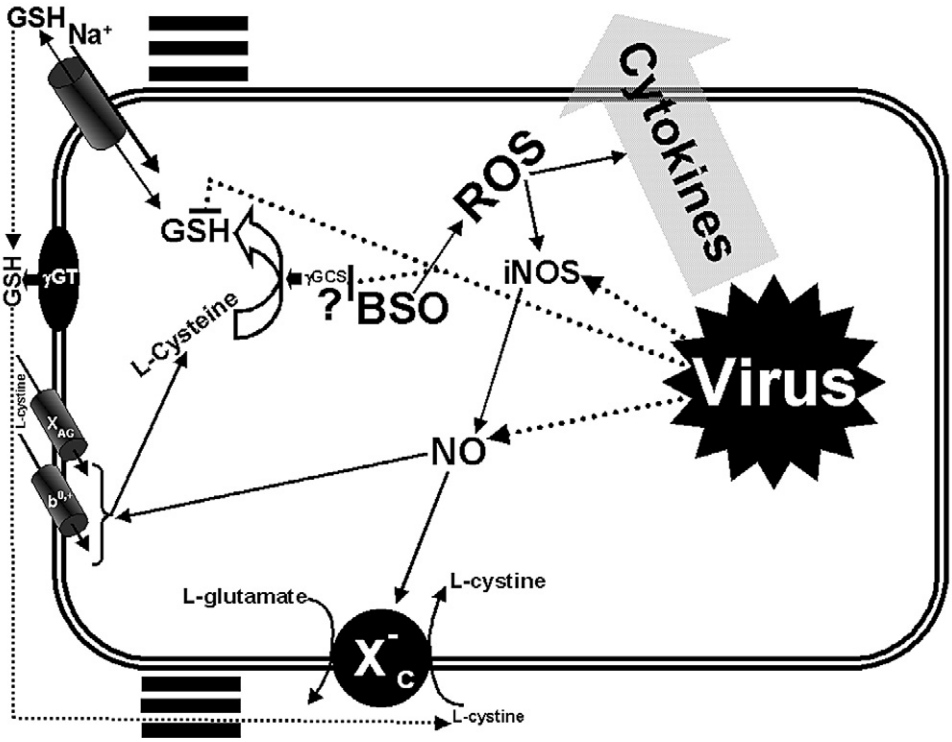

Figure 3. Proposed cellular mechanisms of virally induced perturbations in GSH homeostasis and potential inflammatory response triggers. ⊢---- indicate a biochemical inhibition mechanism, while ◄—/◄---- indicate a stimulation or downstream response effect. Cytokine secretion from epithelial cells triggers elevation of free radicals and antioxidant depletion. Antioxidants and GSH precursors (ie, L-cystine) have been shown to downregulate cytokine activation of downstream inflammatory processes, while buthionine sulfoximine (BSO, an agent that depletes GSH by inhibiting the rate-limiting synthetic enzyme γGCS, marked with a "?"), has the potential to enhance cytokine secretion by upregulating ROS pathways.

Interleukin-1 induced responses occur through modulating redox-equilibrium.[64] In addition, reactive oxygen species (**ROS**) signaling that regulates the transcription of IL-4, IL-6, IL-8, and TNF-α occurs through a thiol-dependent mechanism.[64,65] Antioxidants and GSH precursors have been shown to downregulate cytokine synthesis, activation, and downstream processes. On the other hand, buthionine sulfoximine (**BSO**, an agent that depletes GSH by inhibiting the rate-limiting synthetic enzyme (γGCS), has the potential to enhance cytokine secretion by upregulating ROS pathways (Figure 3).[65]

Many studies stress the importance of GSH in the conjunctiva, demonstrating that GSH supplementation leads to attenuation of conjunctivitis and keratitis caused by viral infection.[58] For example, we reported that adenovirus type 5 infection of rabbit eyes decreases conjunctival GSH levels.[51] Moreover, fluid and Cl^- secretion across Ad5-infected conjunctival epithelium are also severely impaired in this model.[66] Since inflammatory events, such as cytokine and ROS production, are recognized as major participants in ocular pathophysiology, they may define the protective role of GSH under these conditions. Elevated levels of conjunctival lipid peroxidation, up-regulation of transporters that supply the conjunctiva with L-cyst(*e*)ine (a rate-limiting process in cellular GSH homeostasis), and diminished net mucosal secretion of reduced GSH are processes closely regulated either directly by viral infection (ie, expression of viral proteins) or through other pathologic consequences of viral infection (ie, induction of NOS2). (See Figure 3.)

Active ion transport in the pigmented rabbit conjunctiva is accomplished by Cl^- secretory component(s) driven by the activity of the basolateral Na^+,K^+-ATPase. A family of mucosal Cl^- channels in the conjunctiva mediate the secretion of this anion, in parallel with that of GSH, from blood to tear side.

The complex Cl^- secretory mechanism(s) and activity of basolaterally-localized pumps have their own level of susceptibility to oxidative damage or inflammation. It is reasonable to postulate that the degree of vulnerability to oxidative damage may be related to abundance and oxidizable features of given transporters or other cellular components; the adverse effect of oxidative agents on conjunctival physiological function is likely to be decreased when the epithelial cells are treated mucosally with pharmacological levels of GSH. Specifically, we hypothesize that physiological levels of GSH present in mucosal surfaces are enough to maintain a proper redox balance at the healthy conjunctival epithelium, and pharmacological GSH levels can restore this balance in oxidation-induced conjunctival disease(s). Suppression of CFTR function due to genetic defects or via natural design for defense can represent mechanisms contributing to the preservation of intracellular GSH during

stress conditions. Although CFTR-mediated Cl^- and GSH secretion across conjunctival epithelia is defective due to mutation(s) in CFTR, the epithelium retains capacity to biosynthesize GSH and secrete (in parallel with Cl^-) via alternative pathways. Modalities via these pathways to restore the secretion of conserved stores and/or direct topical delivery GSH to the ocular mucosa may offer methods to restore redox balance and healthy conjunctival function.

The proposal that conjunctival function will be impaired due to GSH deficiency is depicted schematically in Figure 2. When both normal (left panel) and inflamed (right panel) conjunctivas are exposed to oxidative challenge, recruitment of inflammatory elements and oxidative damage are the known outcome. Rapid depletion of intracellular GSH can put the conjunctival epithelium at risk for further damage. Healthy (left panel), actively ion-transporting epithelial cells are able to maintain a high GSH secretion. The presence of an adequate level of mucosal GSH allows for efficient electrophysiological activity of the conjunctiva and subsequent (and perhaps continuous) repletion of intracellular GSH. By contrast, in inflamed and oxidant-challenged conjunctivas (right panel), epithelial cells are not able to maintain proper functional ion transport and cannot continue to provide GSH secretion, leading to inefficient recovery and cellular damage. ROS and stress signals are released in the milieu, leading to exacerbated inflammation and damage to tissue structure and (sub)cellular assembly, favoring infection and obstruction (Figure 2).

VI. POTENTIAL PROSPECTS FOR GSH-MEDIATED PHARMACOTHERAPY IN OPHTHALMIC DISEASE(S)

Animal models based on viral infection intended for studying pathogenesis of conjunctival diseases have been developed and validated.[59] The importance of GSH in cell protection in ocular tissues is also established.[58] Our laboratory has presented evidence for the importance of transport of GSH and its sulfur amino acid precursor L-cystine in CEC. We also demonstrated that in conjunctiva under physiological conditions, apical GSH secretion is the primary pathway of GSH turnover.[24] Since the tear fluid contains >100 μM GSH (about an order of magnitude higher than that in blood plasma), it is likely that an impairment of GSH secretion can cause a decrease in tear GSH and, thus, may compromise tear film function and protective properties.

The antiviral activity of GSH in some epithelial cell models has been reported. For example, administration of GSH inhibited viral replication of Sendai Virus, HSV-1, and human immunodeficiency virus (**HIV**) infections.[67-69] GSH in corneal tissue of rabbits decreased in HSV-1-induced keratitis, and topical administration of GSH reduced the severity and progression of keratitis and conjunctivitis.[58] The mechanism(s) for the decrease in GSH in viral infection or for its elevation during topical GSH treatment are not known precisely to date. The metabolism of GSH in pathological conjunctival tissue is also not understood.

Ocular inflammatory diseases due to viral infection are common. Among these, the Ad5 replication model of ocular disease has received a great deal of attention, particularly with reference to studies showing suppression of viral replication and inflammation with steroidal and non-steroidal antinflammatory drugs.[70] Increases in production and release of cytokines in adenovirus infection have also been described.[61] Our previous reports also show increased synthesis and secretion of the key proinflammatory cytokines with Ad5 infection in CEC.[51,71-73] Since cytokine activation would lead to increased ROS and oxidant stress, it is expected that cellular antioxidants would play a favorable role in attenuating conjunctival inflammation (Figure 3). Indeed, a beneficial effect of GSH and GSH prodrugs in several viral infections have been recently reported,[58,68,74] although GSH effect on Ad5-induced viral inflammation has not been studied systematically to date.

Because our previous studies[51] showed that conjunctival GSH decreases significantly even before maximal viral replication (day 3) occurs in the Ad5 infection model of the pigmented rabbit, and that GSH depletion in conjunctival epithelium leads to increased production of ROS, we recently investigated the putative protective role of GSH and the mechanisms involved in suppression of conjunctival inflammation, utilizing Ad5-infected rabbits. The endpoints measured represented two common clinical parameters—Schirmer's I test for measurement of tear production rates and tear break-up time test with fluorescent dye for estimation of tear film stability. Initial scores suggested that supplementation with GSH led to maintaining both of these parameters at levels close to those scored in paired uninfected control eyes.[71]

Based on literature reports on antiviral properties of GSH and its analogs in other tissues and cell types, it is reasonable to predict that treatments of infected conjunctival tissue with GSH, GSH ester, and precursor prodrugs are likely to suppress viral replication and reduce conjunctivitis. The potency and mechanism may, however, be different among the various GSH derivatives and may also depend on the efficiency of virus infection (Ad5 vs other viruses). Glutathione ester is highly permeable and provides an efficient way to build up intracellular levels of GSH due to endogenous esterase activity, and, thus, may prove to be more effective in this regard. How the efficacy of GSH-caused attenuation of conjunctival inflammation will compare with the known modalities of antiviral drugs in current use is not clear at this time. However, GSH (being an endogenous antioxidant) may offer some advantages with respect to cytotoxicity and other side effects associated with exogenous drug treatments. On the other hand, with respect to the function of other antiviral or anti-inflammatory drugs in the suppression of virally-induced conjunctivitis, it is not known whether up-regulation of cellular GSH accompanies protection offered by these drugs. The mechanism of action of these drugs may involve blocking of viral DNA synthesis and may occur via improvement of the cellular thiol status. Finally, therapeutic approaches

described herein may find application in other ocular diseases (eg, corneal disease) associated with Ad5 or other viruses such as HSV-1.

A decrease in GSH (possibly due to rapid efflux) and a decrease in intracellular pH were suggested as important consequences of virus infection in many different cell types, including the rabbit cornea.[61,67,75] Virus infection, as well as chemical insults, lead to production of lipid peroxides and oxygen free radicals, necessitating the studies of oxidant stress on the regulatory mechanisms of GSH metabolism.[76] During an acute stage of inflammation, an intense local oxidation process takes place at the inflammatory center through the production of free radicals. The extent of tissue damage in acute conjunctival inflammation would be the result of balance between the free radicals generated and the cellular defense system. Thus, antioxidant therapy can alleviate the change in GSH levels of cells subjected to inflammatory disease and could have a prominent role in the therapy of acute conjunctival inflammation.

The role of epithelial cells is less well defined with respect to their participation in relieving conjunctival inflammation, but they may very well respond to local inflammatory stimuli by synthesis and secretion of cytokines. It is likely that the chemokine and cytokine secretion from epithelial cells could be the trigger for the elevation of oxygen free radicals and the resultant antioxidant depletion and tissue damage (Figure 3). Proinflammatory cytokines TNF-α, IL-6, IL-8, and granulocyte-macrophage colony stimulating factor (**GM-CSF**) are secreted by human CEC upon stimulation by endotoxins.[61] Induction of oxidant stress and secretion of pro-inflammatory cytokines with Ad5 infection in freshly excised conjunctiva were reported from our laboratories, in part by showing the synthesis and release of TNF-α and IL-6 in Ad5 infected rabbit CEC.[51,71-73,77] However, more confirmatory evidence using a tissue culture model is needed to establish a contributory role of epithelial cells per se, and applicability of other models of oxidant stress need to be investigated.

Conjunctival mucosal secretion of GSH carries great physiological and pharmacological significance. The baseline secretion of GSH into the tear film covering the conjunctiva can play an important protective (serving as a biochemical defense against oxidative insult) as well as regulatory role (where it may modulate the function of various enzymes that are either bound to conjunctival surface or residing in tear film). Furthermore, the evidence showing negligible GSH secretion from CEC during injury or disease suggests that supplementation with exogenous GSH may prove beneficial. Additionally, there are a number of pharmacological ways to achieve higher GSH in tears or ocular epithelium during disease (metabolic feeding, prodrugs, molecular GSH, to name a few). The presence of GSH transport mechanisms, the immediate local availability of the target in ocular surface disease by topical administration, and the ease of preparation of pharmacological concentrations of active GSH are all in favor of GSH-based therapeutics.

Glutathione offers unique advantages as a pharmacological agent for treatment of a number of epithelial cell disorders. It is relatively safe, as high doses of GSH to cells or tissues by extracellular administration have not been found to be harmful.[21,51,58,68,74] This may raise questions about the potency of GSH as a therapeutic entity. The wide range of pharmaceutical and therapeutic indications for mucosal administration of reduced GSH compensate required supraphysiological concentrations at the site of action. While pharmacodynamics of GSH in epithelial cells are not entirely unequivocal, as a natural antioxidant GSH may have inherent exemptions from common toxicological considerations associated with synthetic xenobiotics. Various physical-chemical factors relevant for formulation development, including pH and osmotic challenge, do not present a difficulty in developing GSH as a pharmaceutical product. Furthermore, most epithelial tissues susceptible to pathological disorders are endowed with relatively high tolerance to pharmacological doses of GSH.[8,58,67,74,78]

For example, the ocular epithelium is unique in its ability to handle high, pharmacological doses of topical ophthalmic GSH.[21] Anatomy of the mammalian eye plays an important role in the maintenance of tear film homeostasis,[1] while utilization of topically applied GSH within the tear film milieu is largely non-problematic, in part due to anatomical characteristics of ocular tissues. Basal tear production and drainage, as well as tear film buffering capacity, play a regulatory role in disposition of exogenous doses of topical GSH.[72] All these factors act in a coordinated fashion to sustain steady-state conditions of ocular surface fluids.

Volume of the tear film covering the eye is about 7.5-10 µL under nonstimulated conditions, and a GSH concentration range of 70-110 µM is found within this compartment at steady state, sustaining a total amount of 160-340 ng of this antioxidant.[24] Based on principles of ocular pharmacokinetics and conventional topical liquid dosage forms intended for ophthalmic use, the administration of a 5 times daily dose of 1550 µg GSH, formulated as eyedrops of 50 µL volume containing 100 mM GSH in phosphate buffered saline at pH 7.4, was found to be viable in an Ad5-infection model of pigmented rabbits.[71] Furthermore, a prodrug of GSH, GSH-monoethyl ester, was also tolerated at 50 mM when administered in a regimen identical to that of molecular GSH. Beneficial endpoints were determined by estimates of tear break-up time (slit lamp exams with topical fluorescein) and tear production (Schirmer's I test). Moreover, the animals tolerated these high, pharmacological doses of reduced GSH without showing any stress identifiable by immediate reflexes to the administered eyedrops or subsequent visual examination methods evaluating conjunctival irritation.[71]

The advantage of using GSH prodrugs is threefold and warrants further discussion. Prodrugs of GSH may be designed to have improved physicochemical properties (ie, stability and solubility) and additional passive diffusive penetration efficiency across lipid bilayers of epithelial cells. In fact, mono- or di-alkyl esters of GSH have been synthesized utilizing the two free carboxylic acid end groups on

the molecule, and these successfully elevated intracellular GSH levels in artificially GSH-depleted cells.[21,79] Possibilities exist for making novel prodrugs of GSH that can offer proprietary opportunities. Based on current understanding, one advantage of using cell membrane-permeable analogs of GSH as prodrugs is that this could bypass feedback inhibition in *de novo* GSH biosynthesis (a well-known phenomenon that occurs when the cytoplasmic milieu is overloaded with exogenous GSH), since the analogs are not recognized as substrates for GSH synthetase nor for γ-glutamyl cysteine synthetase. Novel prodrugs of GSH may be chemically engineered to possess intrinsic properties that allow their metabolic longevity in GSH-secreting mammalian epithelia (ie, conjunctiva).

Reduced GSH appears to be the active ingredient that produces therapeutic improvement in ocular disease as a topically administered agent. Because GSH is constitutively secreted from epithelial cells of various mammalian tissues, including the conjunctiva, and it is present in all cells, it will be challenging to identify specific molecular targets that GSH protects. Through the studies presented in this review, the protective role of mucosally secreted or administered GSH in maintaining proper functioning of active ion transport that occurs across the conjunctival epithelium is linked to specific molecular targets of GSH protection (which may be numerous in nature, although the Na^+,K^+-ATPase appears to be a likely prospect).

The formulation of topical ophthalmic GSH may be challenging from a pharmaceutical point of view. Stability by formulation design needs to achieve resistance to auto-oxidation, reduce odor (a common characteristic to all thiol-containing small molecules), lengthen residence time in the conjunctival sac after application, and control for pH and osmotic modalities. The topical ophthalmic delivery of GSH can also be improved by using prodrugs (ie, the carboxy-esters of GSH are available with better cell-permeating properties). Finally, biosynthesis of GSH from metabolic precursors may offer a third method of endogenously elevating ocular epithelial GSH content.[79]

In summary, conjunctival GSH metabolism and transport play a key role in the maintenance of homeostasis, protecting the intricate innate subcellular transport machinery of this tissue. With an emerging immunopharmacological importance, ocular surface GSH contributes to the complexity of tear film makeup and function, where potential therapeutic benefits necessitate further research to gain greater understanding.

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