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The Lacritin-Syndecan-1-Heparanase Axis in Dry Eye Disease

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31.1 Introduction

A translation of the 300 BC Salt 825 papyrus reads ‘The god Re wept and tears from his eyes fell on the ground and turned into a bee’ [1]. In another myth, Re’s tears turned into a man [1]. Today, we appreciate tears for their remarkable importance in ocular surface homeostasis. Loss of homeostasis associated with acute or chronic tear defi ciency and/or instability is known as ‘dry eye disease,’ affecting 5–7% of the world’s population including 30% of the elderly [2, 3]. Little is known of its biological basis with most attention paid to downstream inflammation.

Tears constitute a thin film of at least 1800 different extracellular proteins and numerous species of lipids [4, 5]. In this chapter, we review an effort to address the biological basis of dry eye via an approach that began as an unbiased biochemical screen and led to the discovery of homeostasis-restorative ‘lacritin‘[6], a tear protein whose active form is selectively defi cient in dry eye [5]. Lacritin targets cells via a heparanase-dependent syndecan-1- receptor complex [7]. Discovery of the lacritin-syndecan-heparanase axis brings new insight to the biology of the eye, and also potentially to the homeostasis of other organs and their diseases with lacritin peptides now detected in plasma, cerebral spinal fluid and urine [8–10].

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31.2 The Approach

31.2.1 Discovery of Lacritin

One can think of the surface of the eye as unique in multiple ways. It is arguably the most environmentally challenged wet epithelium [11], and is inclusive of the most densely innervated epithelium [12] and at the level of the cornea is the only wet epithelium that with underlying stroma is avascular [13]. Further, it is the only wet epithelium where the covering fluid is both sterile [14] and refracts most of entering light for sight [15]. As a model for epithelial and neuronal homeostasis, the surface of the eye is unmatched. Understanding precisely how homeostasis is maintained, and therefore might be restored in dry eye, is a challenge.

Our search began in 1992 using primary cultures of rat lacrimal acinar cells - taking advantage of peroxidase in secretory granules as a simple, yet sensitive enzymatic endpoint in 96 well tear secretion assays. Typically, isolated acinar cells de-differentiate and lose their capacity to respond to secretagogues, a loss we found was suppressed by plating on a 10 mM EDTA extract of Engelbreth-Holm-Swarm sarcoma matrix from mice [16]. This was indicative of a tear secretion supportive activity that appeared attributable in part to a lower molecular weight fraction against which rats were immunized for secretion blocking monoclonal antibodies. The best one was not optimal for affinity purification but gave rise to a candidate N-terminal sequence encoded by a GC-rich oligonucleotide that unfortunately failed in screens of a human lacrimal gland cDNA library. However, we were intrigued by one ‘non-specific’ but novel cDNA with very selective lacrimal and salivary gland expression that was cDNA cloned to full-length, and then manufactured as a bacterial recombinant protein taking care to ensure lack of contaminating bacterial lipopolysaccharide. Surprisingly, it enhanced acinar cell ‘constitutive’ but not ‘regulated’ tear protein secretion in a 1.4–14 nM dose-dependent manner [6]. It also triggered basal tearing without irritation on eyes of normal rabbits (80–4000 nM; [17]) and NOD.Aire$^{-/-}$ dry eye mice (4000 nM; [18]). Basal tearing is the form of tearing deficient in dry eye disease. Further, a semi-purified version from rhesus monkey tears provoked monkey acinar cell tear lipocalin and lactoferrin secretion without suppression by dry eye inflammatory cytokines tumor necrosis factor and interferon-γ[19]. When generated as an ‘elastin-like polypeptide’ fusion protein for slow release it triggered tear β-hexosaminidase secretion (10, 20 μM) by isolated rabbit lacrimal acinar cells, and tearing after injection (100 μM) into lacrimal glands of NOD dry eye mice [20]. We named this new tear protein ‘lacritin’ of the gene ‘LACRT’ on human 12q13 [6]. NCBI currently lists thirty-eight lacritin orthologs including fifteen non-primate orthologs [4] from the orders Carnivora, Chiroptera, Equidae [21], Lagomorpha, Scandentia, and Ursidae - yet none to date from Rodentia. That lacritin is effective on mice [18, 20] and rats (Hirata, Laurie, unpublished) despite lack of apparent endogenous expression implies cross-species conservation of its receptor and signaling elements, and offers potential insight into the evolution of the mammalian ocular surface. All known lacritin functions are summarized in Table 31.1.

31.2.2 Restoration of Homeostasis

Human lacritin is an N- and O-glycosylated [21] secreted protein of 119 amino acids with signal peptide excised [6]. Its 25 amino acid C-terminus is distinguished by an amphipathic α-helix that is necessary for activity [22, 23] and conserved among orthologs [4]. Truncation generates the negative control 'C-25' [22]. In addition to triggering basal tearing, we began to wonder whether lacritin can act directly on cells to promote or restore health. Exploration in the context of dry eye inflammatory cytokines interferon-γ and tumor necrosis factor and human tears, initially took advantage of the propensity of the transcription factor FOXO3 to translocate between the nucleus and cytoplasm as a simple marker of cell health. When nuclear, cells are often stressed or dying, whereas the opposite is true when cytoplasmic [24]. FOXO3 was cytoplasmic in human corneal epithelial cells treated with normal human basal tears in the presence of interferon-γ and tumor necrosis factor, reflecting the importance of basal tearing in promoting homeostasis. Surprisingly, this benefit was completely lost when basal tears were immunodepleted of lacritin [23]. One potential weakness of this experiment was that lacritin immunodepletion might have removed lacritin binding proteins responsible for the benefi t. We, therefore, tested human dry eye tears for which the active form of lacritin is selectively deficient or even absent [5]. FOXO3 was nuclear in cells treated with dry eye basal tears in the presence of interferon-γ and tumor necrosis factor. Spiking in 10 nM lacritin, but not C-25, was suffi cient for FOXO3 cytoplasmic translocation [23], suggesting restoration of health. That respective lacritin immunodepletion and add-back were sufficient to lose or regain human basal tear pro-homeostatic activity implied that no other tear protein seemed to share this property.

How does lacritin restore health? It is well known that FOXO3 as a transcription factor can induce autophagy [25], a self-catabolic process by which damaged proteins and organelles are captured in autophagosomes for lysosomal destruction to in turn restore health [26]. In 2010, Zhao et al. [27] using HCT116 colon and H1299 non-small cell lung cancer cells reported that acetylation of family member FOXO1 with stress is a prerequisite for cytoplasmic ligation of autophagy mediator ATG7 to in turn stimulate autophagy. We wondered whether autophagy might be the mechanism by which lacritin rescued stressed cells, and attempted to replicate their observation. Interferon-γ and tumor necrosis factor were suffi cient to promote stress-dependent acetylation of FOXO1, but FOXO1 surprisingly failed to bind ATG7 [23]. We then added 10 nM lacritin or C-25. Lacritin, but not C-25, promoted ligation and subsequent autophagy within minutes [23] suggesting that additional modifi cation - possibly lacritin-dependent phosphorylation of FOXO1 - was necessary. Indeed, lacritin activates the FOXO modifying kinase AKT, and no ligation was observed when lacritin was added in the presence of the AKT inhibitor 'AKTVIII' (Wang, Laurie, unpublished). Further, AKT is constitutively active in HCT116 [28] and H1299 [29] cells. With slightly faster kinetics and different mechanism, lacritin also stimulates autophagy via FOXO3. Here, lacritin- dependent acetylation of FOXO3 was necessary for ligation of upstream autophagy mediator ATG101 [23]. We followed autophagic flux by (i) monitoring conjugation of phosphatidylethanolamine to cytoplasmic LC3-I to form LC3-II in Western blots, and (ii) by loss of the EGFP signal in interferon-γ and tumor necrosis factor stressed human corneal epithelial cells expressing a mCherry/EGFP double tagged LC3 construct. Via both approaches, it was apparent that lacritin (but not C-25) transiently stimulates

autophagy within 1–10 min, and that cells return to baseline autophagy just after 24 h [23]. Further, co-expression of the mCherry/EGFP double tagged LC3 construct with toxic huntingtin mutant Htt103Q-mCFP or non-toxic Htt25QmCFP confirmed that the purpose of lacritin-stimulated autophagy is to rid cells of toxic proteins [23]. By doing so, lacritin restored oxidative phosphorylation by elevating mitochondrial 'spare respiratory capacity' through enhanced mitochondrial fusion [23]. Particularly remarkable was the relative speed by which autophagy was transiently accelerated and oxidative phosphorylation restored. It was also apparent that stress was a prerequisite of lacritin-dependent autophagy.

Validation of this activity in animal models offers promise for human dry eye. NOD.Aire$^{-/-}$ dry eye mice suffer from autoimmune disease of multiple different organs, including lacrimal and salivary glands. Like human dry eye, inflammation-associated loss of epithelial junctional complexes exposes the subepithelial stroma to topical fluorescein dye or to the food dye lissamine green as a measure of 'corneal barrier function'[30]. Epithelial crevices develop leading to an irregular corneal surface. Over three weeks of treatment, lacritin (but not C-25) restored corneal barrier function in fi ve of seven NOD.Aire$^{-/-}$ dry eye mice eyes, and diminished the number of lacrimal gland lymphocytic foci, although the total number of infiltrating CD4+ T cells did not change [18]. Similar benefi t has been observed in NOD. Aire$^{-/-}$ dry eye mice eyes treated with 19- ('Lacripep™') or 25-amino acid synthetic peptides derived from lacritin's C-terminus [Chen FYT et al., unpublished]. The severity of epithelial defects has led some to approach dry eye as a problem of corneal wound repair. In NOD dry eye mice, an algerbrush- generated 2 mm corneal defect significantly worsens over 12–24 h without treatment. Yet, eyes treated with lacritin elastin-like polypeptide repaired fully by 24 h, and at 12 h matched that of the 24 h repair by a mixture of positive control EGF and bovine pituitary extract [31]. This lacritin mitogenic activity is selective for human corneal epithelial, HEK293 and human salivary ductal (HSG)/HeLa cells. Not responsive were human epidermal (A431), pure HeLa, foreskin fibroblast (H368), fibrosarcoma (HT1080), erythroleukemia (K-562), noninvasive breast carcinoma (MCF7), melanoma (SK-MEL and WM-164), Leydig (TM3), Sertoli (TM3), mouse fibroblasts (NIH3T3) and human glioma (U-1242-MG and U-251-MG) cells [22]. Thus, the tear protein lacritin is capable of multiple functions, all of which are directed to promoting and restoring health on the surface of the eye. Reactivating basal tearing helps the eye recover, but so does transient stimulation of autophagy for restored oxidative phosphorylation, and lacritin's mitogenic activity toward epithelial repair (Table 31.1).

31.3 Cell Surface Targeting: Lacritin-Syndecan-1-Heparanase Axis

Understanding how lacritin targets cells could shed insight on disease onset. Lacritin's low nanomolar health promoting and mitogenic activities gave confidence to the attempted enrichment of biotinylated surface binding protein(s) on lacritin columns, yielding a prominent 190 kDa band identifi ed by mass spectrometry as a multimer of syndecan-1 [32]. Validating lacritin-syndecan-1 pull-downs revealed an unusual affinity for the post-heparitinase/chondroitinase ABC pellet and no affinity for syndecans-2 and −4. This differed from FGF2 in which all three syndecans pulled down equally well, and were distributed in the supernatant digest [32]. The implication, therefore, was that lacritin preferred a largely deglycanated version of syndecan-1 and that the core protein was an essential element in the

interaction. Indeed, lacritin purifi ed syndecan-1 presented as a relatively discrete band in contrast to the heterogeneous smear associated with FGF2, and distinct pools of lacritin- versus FGF2-bindable syndecan-1 could be differentiated via sequential pull-downs [32]. Heparitinase was sufficient to switch the affinity of FGF2-bound syndecan-1 to lacritin, in keeping with Sepharose CL-6B gel filtration of $Na_2{}^{35}SO_4$-labeled heparan sulfate chains from lacritin bound syndecan-1 predominantly of ~4–5 kDa versus ~40 kDa for FGF2 [32].

Lacritin triggers calcium signaling for mitogenesis within 20 seconds in a pertussis toxin inhibitable manner [22], and FOXO3 acetylation within 1 min [23]. Appreciating that lacritin signaling was much more rapid than, and unlike, that usually associated with syndecan-1, we wondered whether syndecan-1 was capable of mediating lacritin function. To examine this, competition and siRNA knockdown studies were coupled with cell proliferation assays. The bacterial recombinant syndecan-1 ectodomain construct HS1ED (as soluble competitive inhibitor), and siRNA knockdown of syndecan-1 (but not syndecan-2) each abrogated lacritin-dependent ‘human salivary gland’/HeLa cell proliferation in a dose-dependent manner [32]. Thus, syndecan-1 is essential and likely can pair with a $G\alpha_i$ or $G\alpha_o$ coupled receptor(s), per the inhibitory capacity of pertussis toxin.

Deglycanated syndecan-1 is unstable [33] and not detectable as an immature, intracellular form [32]. Since heparanase is expressed by corneal epithelial cells [34] and detectable in tears (Romano, Laurie, unpublished), the involvement of heparanase was explored by siRNA in ‘human salivary gland’/HeLa cells. siRNA knockdown of heparanase, but not heparanase 2 mRNA erased lacritin-dependent cell proliferation in a dose-dependent manner that was rescued by addition of exogenous heparitinase or heparin-purifi ed heparanase [32]. Thus, heparanase serves as an ‘on-switch’ for lacritin-syndecan-1 ligation (Fig. 31.1). Although secretion of active heparanase is ATP-dependent [35] in a 25 mM glucose-dependent manner [36, 37], regulation of heparanase activity in the context of the lacritin-syndecan-1-heparanase axis has not yet been explored.

With the affinity of lacritin for syndecan-1 heparanase-regulatable, further attention was paid to the mutual ligation site. Truncation analysis narrowed lacritin binding to syndecan-1’s fifty N-terminal amino acids [32], with further focus on N-terminal amino acids 20–30 inclusive of two heparan sulfate substitution sites and the hydrophobic sequence ‘GAGAL’ [7]. The corresponding sequences in syndecans-2 and −4 are respectively GADED and GDLDD which are less hydrophobic by the Kyte & Doolittle scale. Swapping GAGAL out for ‘GADED’ or ‘GDLDD’ in syndecan-1 largely abrogated lacritin binding, indicating that GAGAL is the core protein specifier, as per its conservation among orthologs [7]. We wondered whether α-helicity of lacritin’s C-terminal amphipathic α-helix might be influenced by GAGAL. As monitored by circular dichroism, this was indeed the case with interaction involving lacritin hydrophobic face residues leucines-108, −109 and phenylalanine- 112 whose joint affi nity for syndecan-1 was absent after each had been mutated to serine [7]. Other interactions were suggested by loss of affinity of lacritin E103S/K107S and K111S for syndecan-1 [7]. These might interact with a 3-O-sulfation group on the heparanase generated heparan sulfate stub and with a short chondroitin sulfate chain substituted in place of heparan sulfate at syndecan-1’s N-terminus, as per the blocking capacity of single chain anti-heparan HS4C3 and chondroitin sulfate IO3H10 antibodies [38]

and point mutation of heparan and chondroitin sulfate substitution sites [7]. Thus, heparanase is the ‘on-switch’ for lacritin targeting of syndecan-1. It exposes two of three elements for ligation: (i) the syndecan-1 specifi c sequence GAGAL that interacts with the hydrophobic face of lacritin’s amphipathic α-helix, and (ii) likely 3-O-sulfation of the heparanase-generated heparan sulfate stub. A third binding element is an N-terminal chondroitin sulfate that is uncommonly substituted in place of heparan sulfate [39]. This requirement would be expected to diminish the availability of syndecan-1’s for ligation.

31.4 Clinical: Deficiency or Absence of Active Lacritin Monomer in Dry Eye

When tears are blotted for lacritin, several bands are noted: (i) ~9, 10 and 12 kDa C-terminal fragments, (ii) ~25 kDa monomer and (iii) dimer and trimer of 50 and 75 kDa, respectively or even larger multimers [40]. Dimer, trimer, and multimers develop as a consequence of constitutive tissue transglutaminase cross-linking, largely involving lysines 82 and 85 as donors and glutamine 106 as acceptor [41]. Since glutamine 106 resides in the syndecan-1 binding domain, dimer, trimers, and multimers are incapable of binding syndecan-1 or do so with low efficiency [41], and are inactive (Romano, Laurie, unpublished). Tissue transglutaminase expression is elevated in human dry eye [42], as is transglutaminase 1 in a mouse desiccating stress model of dry eye [43], and is the most likely reason for lacritin monomer deficiency or absence in dry eye. Proteomic analyses have documented selective lacritin deficiency in tears of individuals suffering from aqueous defi cient dry eye, aqueous deficient dry eye with meibomian gland disease, blepharitis, climatic droplet keratopathy, contact lens-related dry eye, Fusarium keratitis and primary Sjögren’s syndrome dry eye [4]. Its deficiency in primary Sjögren’s syndrome tears can be particularly striking [44] McKown, Romano, unpublished]. Thirty-nine other tear proteins (of ~1800) are deficient in dry eye diseases, but none are known to share lacritin’s properties. Since lacritin is a basal tearing secretagogue, the absence or defi ciency of some tear proteins may be a consequence of the unavailability of lacritin monomer. For example, secretion of lipocalin-1 and lactoferrin is in part lacritin-dependent [19], and defi ciency of both has been reported in aqueous deficient and Sjögren’s Syndrome dry eye, as well as in meibomian gland disease [5]. To test the hypothesis that dry eye might be in part a lacritin deficiency disease, ‘A Double-Masked, Randomized, Multi-Center Phase 2 Study to Evaluate the Effi cacy and Safety of Lacripep™ in Subjects with Dry Eye Associated with Primary Sjögren’s Syndrome’ (NCT03226444) was initiated in the summer of 2017 with full enrollment now complete (results not available at time of writing). ‘Lacripep™‘, a 19-amino acid synthetic peptide representing lacritin’s C-terminal amphipathic α-helix appears to be equally active as lacritin, and was tested at two concentrations versus vehicle.

31.5 Concluding Remarks

In an effort to address the biological basis of dry eye, an unbiased biochemical secretion screen was initiated in 1992 that, with considerable serendipity, made possible the discovery of lacritin. As a tear protein that contributes to basal tearing and ocular surface health and yet is selectively deficient in dry eye, lacritin offers a paradigm shift in our appreciation of

how homeostasis of the eye surface may be regulated, and disease initiated. Exploration of its cell surface interactions uncovered a previously unknown heparanase ‘on-switch’ mechanism by which lacritin targeting of syndecan-1 is dependent, and about which there is much to learn. Although our focus is on the eye where lacritin expression predominates, expression in invasive breast cancer has been suggested [45], and mass spectrometry has detected lacritin C-terminal fragments in plasma, cerebral spinal fluid and urine [8–10]. Thus, the lacritin-heparanase-syndecan-1 axes may have wide relevance.

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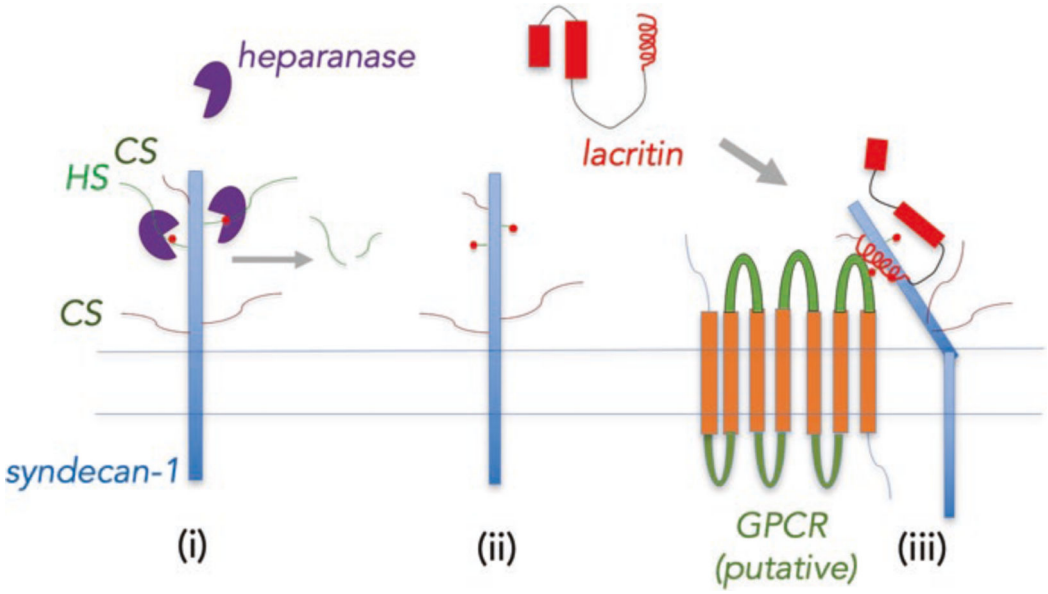

Fig. 31.1.

Lacritin-syndecan-1-heparanase axis. (**i**) Heparanase deglycanation of syndecan-1 gives rise to (**ii**) syndecan-1 with heparan sulfate proteoglycan stubs of ~4–5 kDa with 3-O sulfation groups (red circle). A short chondroitin sulfate chain substitutes in place of heparan sulfate on syndecan-1's N-terminus. (**iii**) Lacritin's C-terminal amphipathic α-helix targets the exposed core protein sequence GAGAL, 3-O-sulfation group(s), and the N-terminal short chondroitin sulfate chain. Pertussis toxin inhibits lacritin dependent calcium signaling, suggesting the involvement of a G-protein coupled receptor (GPCR)

Table 31.1

Application site

Lacritin function	Lacrimal gland	Eye	Refs
Tear secretion	Peroxidase secretion by rat lacrimal acinar cells. Lipocalin-1 and lactoferrin secretion by monkey lacrimal acinar cells; no interference by interferon-γ and tumor necrosis factor. β-Hexosaminidase secretion by isolated rabbit lacrimal acinar cells. Tearing by NOD mice after injection into lacrimal glands.	Basal tearing by normal rabbits. Tearing by NOD.Aire$^{-/-}$ dry eye mice (combined basal and reflex tearing since controls and lacritin treated also receive IP pilocarpine).	[6, 17, 18, 20]
Restoration of homeostasis	Diminished number of lacrimal gland lymphocytic foci in NOD.Aire$^{-/-}$ dry eye mice.	Restored corneal barrier function on NOD.Aire$^{-/-}$ dry eye mice eyes. On interferon-γ and tumor necrosis factor stressed human corneal epithelial cells, transiently stimulated autophagy to restore oxidative phosphorylation by mitochondrial fusion. This benefit was lost when C-terminal 25 amino acids were removed (‘C-25’) from lacritin, or reduced following I98S, F104S, L108S/L109S/F112S or F112S point mutation, or following preincubation in xyloside. Using the same interferon-γ and tumor necrosis factor stressed human corneal epithelial cells, normal human tears rescue, but not normal human tears depleted of lacritin. Dry eye tears do not rescue, in contrast to dry eye tears spiked with lacritin but not lacritin C-25.	[18, 23]
Cell proliferation	Acinar cells from embryonic day 14.5–17.5 mouse lacrimal gland explants (Makarenkova and Laurie, unpublished).	Corneal wound healing of NOD mice. Proliferation of subconfluent human corneal epithelial cells.	[22, 31]