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A thermo-responsive protein treatment for dry eyes

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A B S T R A C T

Millions of Americans suffer from dry eye disease, and there are few effective therapies capable of treating these patients. A decade ago, an abundant protein component of human tears was discovered and named lacritin (Lacrt). Lacrt has prosecretory activity in the lacrimal gland and mitogenic activity at the corneal epithelium. Similar to other proteins placed on the ocular surface, the durability of its effect is limited by rapid tear turnover. Motivated by the rationale that a thermo-responsive coacervate containing Lacrt would have better retention upon administration, we have constructed and tested the activity of a thermo-responsive Lacrt fused to an elastin-like polypeptide (ELP). Inspired from the human tropoelastin protein, ELP protein polymers reversibly phase separate into viscous coacervates above a tunable transition temperature. This fusion construct exhibited the prosecretory function of native Lacrt as illustrated by its ability to stimulate β-hexosaminidase secretion from primary rabbit lacrimal gland acinar cells. It also increased tear secretion from non-obese diabetic (NOD) mice, a model of autoimmune dacryoadenitis, when administered via intra-lacrimal injection. Lacrt ELP fusion proteins undergo temperature-mediated assembly to form a depot inside the lacrimal gland. We propose that these Lacrt ELP fusion proteins represent a potential therapy for dry eye disease and the strategy of ELP-mediated phase separation may have applicability to other diseases of the ocular surface.

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1. Introduction

The lacrimal gland–corneal axis plays a critical role in maintaining ocular surface health. While the avascular cornea serves as both a protective barrier and the main refractive element of the visual system, the lacrimal gland (LG) is the major organ secreting key proteins and electrolytes into the tear film that bathes the cornea and, through nutrient and antimicrobial proteins, sustains its function [1,2]. Dry eye disease (DED) is a multifactorial disease of the ocular surface causing visual disturbance and tear film instability [3] and can be due to either aqueous tear insufficiency originating with defects in aqueous tear production by the LG [4] or evaporative dry eye associated with meibomian gland insufficiency [5,6]. Accordingly to reports, severe DED affects approximately 5 million Americans above age 50 and its global prevalence ranges from 5% to 35% of the population [3]. Traditional approaches to treat DED include topical administration of artificial tears or the conservation of secreted tears using tear plugs [7] and eye-shields [8]. Since many cases of DED are associated with inflammation [9,10], some treatments for DED have been proposed that inhibit inflammation of the LG [11]. None of these methods are satisfactory in replacing the lost regulatory functions provided by the many components found in normal tears. To better sustain the health and homeostasis of the ocular surface there remains a need for efficient, sustained and targeted DED therapy. In humans, the inferior palpebral lobe of the LG is accessible for injection beneath the eyelid; furthermore, if coupled with a sustained release strategy this route of administration might have clinical relevance, similar to intra-vitreal injection or subconjunctival injection.

The discovery of the glycosylated human tear protein, lacritin (Lacrt), provided critical insight into the potential use of regulatory tear proteins to treat DED [12,13]. Lacrt was found in a systematic oligonucleotide screen of a human LG cDNA library and exhibited LG specific-expression [14]. Subsequent studies have proven its efficacy in stimulating peroxidase secretion in cultured rat [14], and both lactoferrin and lipocalin secretion in cultured monkey lacrimal acinar cells [15]. Lacrt also promotes constitutive tear secretion by New Zealand white rabbits and Aire KO mice via topical treatment [16,17], proliferation of transformed human corneal epithelial cells [14,18], and restored health of

Abbreviations: DED, dry eye disease; ELP, elastin-like polypeptide; Lacrt, lacritin; T_t, transition temperature; LG, lacrimal gland; LGACs, lacrimal gland acinar cells; NOD, non-obese diabetic; CCh, carbachol.

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transformed human corneal epithelial cells, primary human corneal epithelial cells [19] and primary monkey lacrimal acinar cells [15] that had been stressed with the inflammatory cytokines interferon-γ and tumor necrosis factor. Interestingly, Lacrt displays growth factor-like behavior; however, its specificity for target cells of the ocular surface system results from a unique 'off–on' switch controlled by heparanase deglycanation of the cell surface protein, syndecan-1 [20], which both exposes and generates a Lacrt binding site [21] as a prerequisite for mitogenic signaling. Confirmed by 2-D electrophoresis, mass spectrometry and surface-enhanced laser desorption/ionization studies, Lacrt [22] is down regulated in blepharitis (chronic inflammation of the eyelid) vs. normal tears [23], and most aqueous deficient dry eye [24]. Whether down regulation of Lacrt provokes disease is a key unresolved question, but its prosecretory and corneal mitogenic activity suggest that it might have activity as a protein therapeutic for ocular surface diseases.

Great strides have been made to improve the bioavailability and simplify the administration of existing drugs, which include depot formulations that deliver short peptides such as leuprolide and bioadhesive polymers used in buccal drug-delivery systems [25]. Recently, stimuli-responsive polypeptides have emerged as an attractive controlled release strategy. One such type of biomaterial is the elastin-like-polypeptides (ELPs) [26]. Biologically inspired from human tropoelastin, ELPs are composed of a pentapeptide repeat $(VPGXG)_n$, where the 'guest residue' X can be any amino acid and n determines molecular weight. One unique property of ELPs is their inverse temperature phase transition behavior. ELPs are soluble in aqueous solutions below their transition temperature (T_t) and self-assemble into various-sized particles above T_t [27]. T_t can be precisely modulated by adjusting the number of pentapeptide repeats, n, and the hydrophobicity of the guest residue, X, which can determine whether the ELP remains a soluble macromolecular drug carrier [28], assembles a nanoparticle [29], or phase separates into micron-sized coacervates [30] at physiological temperature. With their distinctive thermo-responsive, elastic, and biocompatible properties, ELPs have impacted fields such as protein purification [31], stimuli responsive hydrogels [32], tissue engineering [33,34], and targeted cancer treatment [35,36]. Yet, the application of ELPs in ophthalmology has just started [37,38].

To explore the concept of a thermo-responsive reservoir drug as a potential novel treatment for DED [7], we generated a novel Lacrt–ELP fusion with T_t below physiological temperature. The construct exhibits thermo-responsiveness of the parent ELPs while retaining prosecretory efficacy of native Lacrt, as demonstrated by its ability to stimulate dose-dependent β-hexosaminidase secretion from primary rabbit lacrimal gland acinar cells (LGACs). Moreover, the Lacrt–ELP fusion enhanced tear secretion from the non-obese diabetic (NOD) mouse model of autoimmune dacryoadenitis when given via intra-lacrimal injection. This treatment formed a depot that lasted over 24 h inside the LG, which was confirmed by confocal laser scanning microscopy. Finally, we captured the intracellular trafficking and transcytosis of exogenous Lacrt in LGACs using time-lapse confocal fluorescence microscopy, which was prolonged by fusion to the ELP. These findings support the potential enhancement of Lacrt therapeutics via the linkage to a thermo-responsive ELP, which may have broader implications in the treatment of DED.

2. Material and methods

2.1. Animals

In vitro studies were conducted using LG from Female New Zealand White rabbits (2.2–2.5 kg) obtained from Irish Farms (Norco, CA). In vivo studies were conducted using LG isolated from 12-week old male/female C57BL/6 (Jackson Labs, Bar Harbor/ME, USA) or in house bred non-obese diabetic (NOD) (Taconic Farms, Germantown/NY, USA) mice. All procedures performed were in accordance to the university approved IACUC protocol.

2.2. Instruments and reagents

Terrific broth dry powder growth medium was purchased from MO BIO Laboratories, Inc. (Carlsbad, CA). Isopropyl β-D-1-thiogalactopyranoside, OmniPur*. 99.0% min. was purchased from VWR (Visalia, CA). Amicon Ultra concentrators were purchased from Millipore (Billerica, MA). Thrombin CleanCleave™ Kit, carbachol (CCh) and insulin–transferrin–sodium selenite media supplement were purchased from Sigma-Aldrich (St. Louis, MO). 4–20% Tris-Glycine PAGEr gels were purchased from LONZA (Allendale, NJ). Cell culture reagents were from Life-Technologies (Carlsbad, CA). Peter's Complete Medium (PCM) consisted of 50% Ham's F-12 plus 50% DME (low glucose) supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), glutamine (4 mM), hydrocortisone (5 nM), transferring (5 μg/ml), insulin (5 μg/ml), butyrate (2 mM), linoleic acid (0.084 mg/l), carbachol (1 μM), laminin (5 mg/l) and insulin–transferrin–sodium selenite (ITS) media supplement (5 μg/ml).

2.3. Biosynthesis of Lacrt–ELP fusions

A sequence encoding human Lacrt without a secretion signal peptide was designed using the best *Escherichia coli* codons in EditSeq (DNAStar Lasergene, WI) [39]. A thrombin cleavage site was encoded between the Lacrt sequence and ELP tag via insertion at the *BseRI* site. A custom gene flanked by *NdeI* and *BamHI* restriction digestions sites at the 5′ and 3′ ends was purchased in the pIDTSmart-KAN vector from Integrated DNA Technologies (IDT) as follows: Q5

5′-**CATATG**GAAGACGCTTCTTCTGACTCTACCGGTGCTGACCCGGCTC
AGGAAGCTGGTACCTCTAAACCGAACGAAGAAATCTCTGGTCCGGCTG
AACCGGCTTCTCCGCCGGAAACCACCACCACCGCTCAGGAAACCTCTG
CTGCTGCTGTTCAGGGTACCGCTAAAGTTACCTCTTCTCGTCAGGAAC
TGAACCCGCTGAAATCTATCGTTGAAAAATCTATCCTGCTGACCGAAC
AGGCTCTGGCTAAAGCTGGTAAAGGTATGCACGGTGGTGTTCCGGGTG
GTAAACAGTTCATCGAAAACGGTTCTGAATTCGCTCAGAAACTGCTGA
AAAAATTCTCTCTGCTGAAACCGTGGGCTGGTCTGGTTCCGCGTGGTT
CTGGTTACTGATCTCCTC**GGATCC**-3′.

The gene encoding for V96 was synthesized by recursive directional ligation in a modified pET25b(+) vector as previously reported [40,41]. The Lacrt-thrombin gene was subcloned into the pET25b(+) vector between the *NdeI* and *BamHI* sites. LV96 gene fusions were synthesized by ligation of a gene encoding for the ELP V96 via the *BseRI* restriction site, resulting in placement of the thrombin cleavage site between Lacrt and ELP. Correct cloning of the fusion protein gene was confirmed by DNA sequencing. The amino acid sequences of ELPs used in this study are described in Table 1.

2.4. Expression and purification of Lacrt ELP fusion protein

Plain ELP V96 and the Lacrt fusion LV96 were expressed in BLR (DE3) *E. coli* (Novagen Inc., Milwaukee, WI). Briefly, V96 was expressed for 24 h in an orbital shaker at 37 °C at 250 rpm. For LV96, 500 μM IPTG was added to the culture when the OD 600 nm reached 0.5, at which point the temperature was decreased to 25 °C for protein expression for 3 h. Cell cultures were harvested and re-suspended in phosphate buffer saline (PBS). Proteins were purified from clarified cell supernatant by inverse transition cycling [39] until ELP purity was determined to be approximately 99% by SDS-PAGE stained with $CuCl_2$. Due to partial proteolysis of LV96 during biosynthesis, fusion proteins were further purified to homogeneity using a Superose 6 (GE Healthcare Bio-Sciences, Piscataway, NJ) size exclusion column at 4 °C. After equilibration with PBS (pH 7.4), 10 mg LV96 was loaded onto the column and washed out by isocratic flow of PBS at 0.5 ml/min. P1, representing LV96 (Supplementary Fig. S1), was collected and concentrated using an Amicon Ultra concentrator (10 kD). When desired, free Lacrt was

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