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Potential role of stromal collagen in cystine crystallization in cystinosis patients

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ABSTRACT

Cystinosis is a genetic disease that leads to the accumulation of intracellular cystine crystals in all organs including cornea due to the loss of cystine efflux transporters in the lysosome of the cells. While the mechanism for formation of intracellular cystine is well understood for most organs, it does not explain many observations for crystal accumulation in the cornea of cystinosis patients. First, the crystals in cornea are extracellular and needle-like with several hundred microns length which is in sharp contrast with the rectangular or hexagonal crystals found throughout other organs. Second, these crystals are arranged parallel to the stromal collagen, which is a unique to the cornea. Third, crystal growth in the cornea reaches a saturation point after where no further crystallization occurs. We propose a hypothesis supported by in vitro and ex vivo data to explain these observations. We hypothesize that the stroma crystals form extracellularly due to the ionic interactions between the cystine diffusing into the eye and collagen fibrils present in the stroma. We examine cystine crystal growth both with in vitro polymer solutions and ex vivo in rabbit cadaver eyes to show that negatively charged polymers lead to the formation of more cystine precipitation in aqueous solution and that needle-like cystine crystal formation can be observed only in presence of certain polyelectrolytes including collagen. This proposed mechanism explains many of the yet unanswered questions but it needs further support from in vivo studies. The improved understanding could lead to improved treatment of corneal cystinosis.

1. Introduction

Cystinosis is a metabolic disease characterized by intracellular accumulation of crystals of cystine, the disulfide of the amino acid cysteine (Gahl et al., 2002). The disease is caused by genetic mutations that result in loss of the cystine efflux and accumulation above the solubility limit of 0.1 mg/mL leading to crystal formation in lysosome and eventual cell death (Gahl et al., 1982). Cystinosis patients appear normal at birth, but exhibit growth retardation and renal complications and hypothyroidism (Gahl et al., 1982, 2000, 2002; Nesterova and Gahl, 2008; Tsilou et al., 2007). Cystine crystals accumulate in various tissues including cornea and other ocular tissues such as iris, conjunctiva, retinal pigment epithelium (Bishop, 2017; Gahl et al., 2002; Labbe et al., 2009; Liang et al., 2015; Tsilou et al., 2007). Cystinosis patients begin showing ocular symptoms at the age of 16 months and without appropriate treatment, the entire peripheral stroma and epithelium can be packed with crystals eventually resulting in serious complications (Gahl and Kaiser-Kupfer, 1987; Nesterova and Gahl, 2008; Schneider et al., 1990).

The cornea is the part of the eye most affected by cystinosis crystal formation. The cornea comprises of five layers— epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium. Recent work has also suggested a sixth layer, called Dua's layer, which occurs between the stroma and Descemet's membrane (Dua et al., 2013; Dua and Said, 2016). A majority of the crystals form in stroma, which is comprised mostly of collagen and keratocytes at an approximate density of 20,500 cells/mm^3 (Patel et al., 2001). These keratocytes comprise 96% of all cells in the cornea (Hippert et al., 2012). The keratocytes play a crucial role in general repair and maintenance. Upon injury to the cornea, cells in the epithelium release IL-1α and TGF-β2, which enters through the wound into the stroma (Ljubimov and Saghizadeh, 2015). IL-1α induces keratocytes to either undergo apoptosis or become active and begin synthesizing matrix metalloproteinases that assists in tissue remodeling, and TGF-β2 causes keratocytes to transform into myofibroblasts that secrete extracellular matrix material to repair the stroma. Once the epithelium has healed, the flow of signaling molecules stops, resulting in the deactivation of stromal keratocytes. Keratocytes play a role in cystinosis as, similar to leukocytes and fibroblasts, they uptake proteins, leading to elevated levels of cysteine and cystine (de Graaf-Hess et al., 1999). Deficiencies in cystine transport in keratocytes

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can then lead to intracellular cystine crystal formation (Brigham et al., 1960; Crawhall et al., 1968).

Cystinosis is commonly treated with cysteamine (β-mercaptoethylamine) (Cantani et al., 1983; Gahl et al., 1985; Iwata et al., 1998; Jones et al., 1991; Kimonis et al., 1995; Simpson et al., 2011; Tavares et al., 2009; Thoene et al., 1976; Tsilou et al., 2003) which reacts with intralysosomal cystine to produce mixed disulfide cysteine-cysteamine dimers and the amino acid cysteine (Bradbury et al., 1991; Kaiser-Kupfer et al., 1987, 1990; MacDonald et al., 1990). These are transported out of the lysosome via the lysine transport system, bypassing the damaged cystinosis transporter (Labbe et al., 2009). In the eyes though, a majority of the cystine is extra-cellular, i.e., it is dispersed in the water like stoma layer of the cornea. The products from the cysteamine-cystine reaction can diffuse from the stroma both into the tears and the aqueous humor, where these are cleared through tear and aqueous humor drainage, respectively.

The cystine crystals in most organs including kidneys are intracellular and polygonal in shape, while those in the stroma are predominantly needle-like, and likely extracellular. The most compelling evidence for the extracellular cystine in the stroma is based on a randomized clinical trial comparing the free thiol cysteamine with its disulfide cystamine (Iwata et al., 1998). A randomly chosen eye of each of the 14 patients was treated with 0.5% cysteamine while the other eye of the same patient was treated with cystamine. The mean reduction in crystal density for cysteamine treated eyes was 1.02 units compared with 0.07 units for cystamine-treated eyes. This was a very interesting and surprising result because previous studies had demonstrated that cystamine depletes cystine in fibroblast although not as effectively as cysteamine itself (Iwata et al., 1998). Also mechanistically the low pH in the lysozyme is expected to reduce cystamine to cysteamine and make it an effective cystine-depleting agent. The authors concluded that the most likely reason for the low efficacy of cystamine is that the crystals are outside rather than inside the cells. There is also direct evidence for extracellular crystals based on multiple in vivo confocal microscopy (IVCM) studies (Shams et al., 2014; Simpson et al., 2011). In vivo confocal microscopy of a nine year old boy with diagnosis of nephropathic cystinosis showed needle shaped crystals in stroma that were highly variable in length with some as long as 100 μm (Alsuhaibani et al., 2005). Since keratocytes in stroma are only a few microns in size (West-Mays and Dwivedi, 2006) and lysosomes are only about a few hundred nm in size, the long needle shaped crystals must be extracellular.

To our knowledge the reasons for the formation of extracellular, needle-like cystine crystals in the cornea are not understood. The presence of extracellular crystals is counter-intuitive as cystinosis patients are noted for not having elevated levels of cystine in plasma—matching the roughly 1 mg/100 mL found in healthy patients (Brigham et al., 1960; Crawhall et al., 1968). This suggests that corneal levels of dissolved cystine should be near that of normal patients. This means that keratocytes should still play a role in the formation of extracellular crystals, but that the continued existence of those crystals must require additional mechanisms; otherwise, the extracellular crystals should dissolve with normal drainage into the aqueous humor. An additional question on corneal cystine crystals arises from their unique needle shape. Most cystine crystals formed in the body are of hexagonal or rectangular shape, including even the conjunctiva, which has both intracellular and extracellular crystals (Tsilou et al., 2007). The needle-like stromal crystals are oriented parallel to the corneal stromal lamellae (Alsuhaibani et al., 2005), suggesting that the structure of the collagen in the stroma plays a role. It is also interesting that the crystal formation in the cornea reaches a saturation point at around 12 years of age, after which crystal growth in the cornea stops (Labbe et al., 2009) but if the cornea is treated followed by a period of no treatment, crystal formation resumes (MacDonald et al., 1990).

All these observations suggest that a different mechanism may be responsible for the formation of cystine crystals in the cornea. It is known that presence of polymers in solution can impact the shape of crystals of many different molecules (Klapwijk et al., 2016; Shao-Feng et al., 2010; Tian et al., 2009). These papers point that any kind of molecular interaction can lead to an effect on crystal formation, with the suggested mechanism that when the adsorption of an additives occurs more on one crystal face, that face will have slower growth, altering the normal aspect ratio of the crystal (Jones and Ogden, 2010). Backed by this knowledge on crystallization, we hypothesize that the presence of highly packed collagen lamellae in the stroma play an important role in the formation and shape of external crystals in the cornea. To explore this hypothesis, we first conduct in vitro experiments to observe the effect of various polymers including collagen on the shape of cystine crystals and the solubility limit. Next, we incorporate cystine crystals into cadaver rabbit eyes and compare the morphology of crystals with those in vivo. Finally, we propose a mechanism that we believe could play at least some role in the crystal formation in the stroma in tandem with the normal lysosomal precipitation route.

The overall goal of this work is to try to determine why the cystine crystals in eyes are needle-shaped while those in other organs are polygonal. By combining in vitro and ex vivo experiments, we examine the possibility that collagen fibers in the stroma play a key role in the formation of the needle shaped crystals. Additionally, we try to understand the mechanisms through which the collagen and potentially other polyelectrolytes can impact the shapes of the cystine crystals.

2. Materials and methods

2.1. Materials

L-Cysteine (> 97%), polyethylene glycol (PEG 2 kDa), polyvinylpyrrolidone (PVP, 360 kDa), and sodium hydroxide (NaOH, 1 M), were purchased from Sigma-Aldrich, L-Cystine, Cysteamine (98%), and hydrochloric acid (HCl 1 M) were purchased from Fischer Scientific, Phosphate buffered saline (PBS), 1x, without calcium and magnesium, was purchased from Mediatech, Inc., Darocur TPO was purchased from Ciba, frozen rabbit eyes were purchased from Pelfreeze Biologicals, carboxymethylcellulose sodium salt (CMC, 90 kDa, DS = 0.7) was purchased from Acros Organics, Collagen Type I was purchased from MP Biomedicals, LLC.

2.1.1. Equipment

20 mL vial and 50 mL glass beakers and 120 mL polypropylene sealable containers were purchased from Fisher Scientific. Thermospectronic Genesys 10S UV–Vis was used for all UV–vis spectrophotometry. Ultraviolet transilluminator UVB-10 (Ultra·Lum, Inc.) was used for UV curing of hydrogels. Amscope T490 was used for microscope imaging. MP Select Mini 3D Pinter, V2 (IIIP) was used for 3D printing (poly-lactic acid).

2.2. Methods

2.2.1. In vitro crystal formation in presence of polymer

60 mg of polymer was dissolved in 6 mL HCl (1 M) followed by dissolution of 300 mg of cystine. For Type I collagen, only 6 mg was added to the 6 mL HCl (1 M) due to its lower solubility, followed by dissolution of 300 mg of cystine. Control cystine solutions were also prepared without any polymer. Each solution was stable due to the high solubility of cystine in 1 M HCl. 1 M NaOH was then added to the solution in 50 μL drops every minute until the solution reached a neutral pH. The change in pH reduced the cystine solubility causing crystals to form rapidly. After settling, crystals were withdrawn using a transfer pipette, dried on a microscope slide and imaged at 10× magnification.

2.2.2. Measuring precipitate mass of cystine solution in presence of polymer

Solutions of polymers in DI water were prepared at concentrations