Is Autophagy Dysfunction a Key to Exfoliation Glaucoma?

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Abstract: In this short report we review previous work toward the identification of the protein and cellular sources of exfoliation glaucoma and described our recent finding on dysfunction of autophagy in Tenon capsule fibroblasts obtained from exfoliation syndrome glaucoma patients at the time of surgery and discuss the potential implications of these findings for understanding the cellular sources of the disease.

Key Words: exfoliation glaucoma, Tenon fibroblasts, autophagy, microtubules, lysosomes, mitophagy

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Exfoliation syndrome (XFS) is a disorder in which protein components related to the biogenesis of elastic fibers undergo disorderly accumulation as extracellular aggregates. Elastic fiber assembly involves deamination of nascent elastin monomers (tropoelastin) by lysyl oxidase like 1 (LOXL1). The emerging aldehyde groups lead to the generation of covalent bonds between the tropoelastin monomers in a fibrous arrangement. The organization into fibers is controlled by the adapter proteins Fibulin 4 and Fibulin 5, which bind to cell surface integrins, microfibrils, and LOXL1, thereby determining the spatial positioning of tropoelastin and LOXL1 with respect to each other and to the cell surface fibrils.[1–3]

XFS is a systemic disorder and the aggregates are seen in multiple organs.[4] Yet, overt morbidity is seen primarily in the eye.[5,6] Copious deposits of flaky proteinaceous aggregates (PEX) form in the surface of all organs facing the posterior aqueous space, that is, the ciliary body, the iris, and the equatorial lens. In addition, whether independently or induced by the aggregates, the iris pigmented epithelium releases pigment granules. Although PEX itself does not appear to cause high intraocular pressure (IOP), patients presenting high IOP with PEX have a significantly higher chance of developing glaucoma (XFG) with subsequent vision loss compared with those without the presence of PEX.[7]

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Outside the eye there are multiple studies suggesting that vascular structures may also show varying degrees of deficiency in their functional parameters, yet no overt disease state can be ascribed to XFS in those organs.[4] The unique impact on eye function may reflect the fact that aggregated protein and pigment granules are incompatible with the aqueous flow function and/or that aggregates weaken the lamina cribosa independently of IOP effects.[8] The presence of such aggregates may simply have no substantial effect on organ function elsewhere. In addition, because serum albumin, a protein endowed with chaperone activity[9] is excluded from the aqueous humor, plaque growth rate may be higher in there than in other extracellular spaces where elastin is produced.

Two principal factors predispose individuals to XFS development. The first is age; XFS is rarely seen before age 55 and incidence has a steep aging curve after age 65. The second is the expression of 2 nonsynonymous single nucleotide polymorphisms coding variants of active forms of LOXL1; nearly all people diagnosed with XFS have one of these 2 variants.[10,11] However, many individuals expressing these variants do not develop XFS and the degree of association to each of the 2 variants with XFS incidence seem to be dependent on the ethnicities or geographical location of the population cohort.[12] XFS also shows susceptibility to other genetic variants located in the promoter region of LOXL1[13,14] and another in CACNA1A, a component of a calcium channels present in lysosomes.[15] The LOXL1 variant-XFS relationship suggests that individual genetic tapestry plays a major role in the initiation of the syndrome. In particular, the genetic background may influence onset via its impact on the time course of changes in age-related metabolic and cellular activity. The intersection of the individual genome with cumulative environmental insults, inflammation, UV exposure, etc., may also play a role in the pathologic outcome for LOXL1 variant carriers.[16–18] Finally, as in all age-related diseases, the effect of age suggests that the causative factors have been present from the start, but onset have been held in check by vigorous controlling mechanisms that, as many cellular functions, may weaken with age.

Beyond genetics there have been substantial efforts to characterize the composition of the aggregates by proteomic approaches[19,20] and by real time polymerase chain reaction and immune-histochemical staining of the anterior eye segment using age-matched control eyes without glaucoma as the contrasting specimen.[3] Completion of these pioneered studies required considerable efforts to identify cadaver specimens displaying XFS particulates. Differences in gene and protein expression were identified, in particular deficits in antioxidant defense, endoplasmic reticulum–related stress response, and DNA repair suggested that induction of a persistent proinflammatory state and activation of the profibrotic growth factor transforming growth

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factor (TGF)β1 may be precipitating factors in the onset of XSF.[21]

Further progress in unraveling the etiology of XFS is likely to require experimentally pliable cellular or animal models, such as transgenic mice and/or cell cultures. Unfortunately, at this point a proven XFS animal model is not available. However, small amounts of XFS tissue can be derived from Tenon capsule and from the iris during trabeculectomy surgery. Correspondingly, Ritch et al[22] and Schlötzer-Schrehardt[3,18,23] pioneered a cellular approach. They established fibroblasts cultures from the Tenon capsule biopsies and characterized the production and distribution of components related to exfoliation syndrome in XFS and primary open angle glaucoma (POAG)-derived cells. Although it holds that these cells are not located in the intraocular space, the systemic nature of the XFS aggregates makes them a valid model for investigation of XFS etiology. These earlier studies indicated a role for a stress-induced, spatially, and temporally restricted subclinical inflammation in ciliary body epithelial cells with involvement of TGFβ. Treatment of Tenon capsule fibroblasts (TFs) with TGFβ, oxidative stress, UV light, and hypoxia induced a significant increase in expression levels of LOXL1 and other elastic proteins in both control and XFS populations.[18] But cellular content and secretion of LOXL1 from cells expressing the non-XFS and XFS-associated LOXL1 variants showed only small differences. Regarding potential activity differences, in vitro assay of deaminase activity showed no difference between the high-risk sequence and a no/low risk LOXL1 variant.[24]

Recently, following Schlötzer-Schrehardt work we endeavored a comprehensive comparative study of TFs derived from XFS and POAG patients.[25] Soon after initiation of the study we were intrigued by the fact that under our particular culturing conditions of XFS and POAG, TFs displayed differences in gross morphologies. POAG TFs resembled the fibroblasts derived from other organs in their spindle shape and their self-aligning behavior, XFS TFs were less organized, displayed a wider body spread in multiple directions, features that were preserved even as cells approached confluence. In addition, flow cytometry analysis of relative cell size by the forward scatter parameter demonstrated that the XFS were not only more spread on a culture substratum but were larger than the POAG congeners. Furthermore, differential interference contrast (Nomarski) illumination and electron microscopy suggested that the XFS cells were replete with large vacuolar structures not visible in the POAG cells. XFS-derived TFs also proliferated at a slower rate than the POAG-derived cells. Hence, we undertook a comparative analysis of the mechanisms involved in macromolecular and vesicular cellular clearance, focusing primarily on macroautophagy, the principal cellular clearance mechanism for macroscopic detritus.

Figure 1 is a schematic description of known cellular pathways designed to process misfolded proteins and metabolic detritus. When proteins start misfolding during translation, refolding is aided by specialized chaperones. Misfolded proteins that prove resilient to this initial attempt at structural correction are ubiquitinated. This modification targets them to degradation in the proteasome complex. Polypeptides that escape the 2 early quality control mechanisms are disposed of by autophagy. This process consists of a continuous de novo formation of autophagasomes, double membrane vesicular structures that engulf large particulate material. Autophagosomes undergo centripetal transit along microtubules and normally concentrate at a dense juxta nuclear microtubule organizing center (MTOC). The adapter protein LC3 is central to these processes. A cytosolic form, LC3-I, undergoes lipidation to become LC3-II. The latter becomes immediately incorporated into the early autophagosomal membrane and controls the late autophagosome transit and interactions.[26] Mature, cargo-carrying autophagosomes fuse with lysosomes to form the autolysosome, an autolytic structure where the autophagosome and its contents, including LC3, are fully degraded to their amino acid components. Numerous studies have shown that another cargo organelle, the endosome, can either deliver its cargo for degradation directly to lysosomes or, in a more circuitous manner, fuse with the mature autophagosome before its fusion with the lysosome.[27] Autophagy has been implicated in the function of the outflow pathway at the trabecular meshwork and the survival of retinal ganglion cells in hypertensive glaucoma.[28–30]

To obtain statistical confirmation of any differences or similarities between POAG and XFS-derived TFs, we based our study on the comparison of the first 4 TF cultures successfully derived from XFS-related trabeculectomies against the first 4 age-matched POAG cultures. Immunofluorescence was used to localize lysosomes, endosomes in their different functional/maturation stages, autophagosomes and microtubules, the cellular filaments used by these membrane-bound organelles to transit throughout the cell. Proteins related to elastin fiber assembly, including Fibulin 5 and LOXL1, were examined both by immunocytochemistry and immunoblotting. Flow cytometry methods were used to compare (a) cell size; (b) cytophagic flux with Cyto-ID; and (c) mitochondrial membrane potential (MMPT) with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, respectively. Comparisons were made under basal (serum-rich) cell culture conditions, after the acceleration of autophagic flux induced by fetal bovine serum (FBS) removal (aka, starvation) or after long-term culture under conditions that favor collagen fiber production (3D culture).

Overall the studies demonstrated visible and statistically significant differences in structure and function. In POAG cell microtubules converged into a dense juxta nuclear MTOC. The MTOC showed a marked condensation upon induction of autophagy by starvation. These are structural-features and functional responses common to most healthy cells. In XFS, in contrast, an MTOC was barely distinguishable whether under basic or starvation-induced conditions. Consistent with the role of microtubules in organelle traffic, in the POAG TFs induction of autophagy led to the relocalization to the MTOC-perinuclear area of the great majority of all the organelles studied. In XFS, in contrast, a major fraction of the organelles remained dispersed throughout the peripheral cytosol. Given the intrinsic dependence of fusion events on direct apposition, it is easy to see how the inability of endosomes, autophagosomes and lysosomes in XFS cells to tightly coalesce in and around a well-developed MTOC may hinder the ability of these cells to accomplish efficient autophagy. An independent measurement of autophagic flux with the proprietary tracer Cyto-ID yielded results consistent with this latter premise; upon withdrawal of serum, a statistically significant increase in autophagic flux took place in the POAG cells compared with the XFS cells.

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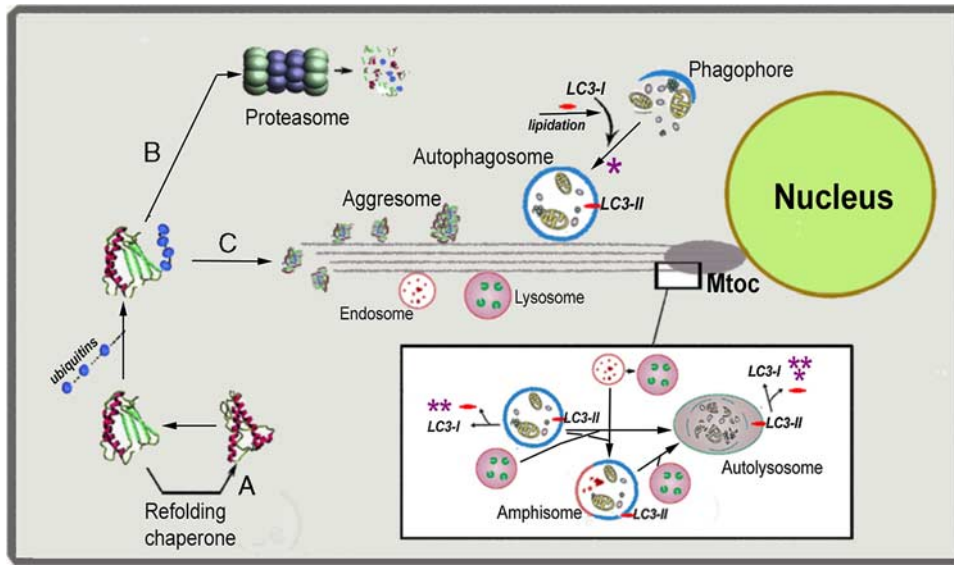

FIGURE 1. Primer on different mechanisms involved in the disposition of nascent misfolded protein. Refolding by specialized chaperones (A); full degradation of ubiquitinated misfolded proteins by processing by the proteasome complex (B); disposal via macroautophagy (C). This later complex system involves (i) packing of misfolded protein into aggresomes; (ii) biogenesis of autophagosomes, from precursor aggregophores, a process fully dependent on attachment of the lipidated form of LC3 (LC3-II) to nascent autophagosome; (iii) aggresome and decaying mitochondria engulfment by autophagosomes. These processcess occur simultaneously with centripetal dyenin-dependent traffic along microtubules toward the juxta nuclear microtubule organizing center (MTOC). Normally, it is there, as described in the insert representing the augmented MTOC zone, that late endosomes either transfer their cargo to lysosomes for full degradation or fuse with mature autophagosomes to establish an amphisome containing cargo of both vesicular structures. Both autophagosomes and amphisomes then, in turn, fuse with lysosomes to form transient autolysosomes, where both the vesicular cargo and the intrinsic vesicular components is lytically degraded into basic biochemical building blocks by the lysosome's acidic hydrolases. The tight accumulation of these organelles in a single spot is likely to greatly enhance both aggresome uptake and intervesicular fusion. Defects of autophagosome maturation, lysosome acidity of the activity of its enzymes or of microtubule kinetic function of spatial organization, will result in reduction of autophagic flux. Purple astericks indicate autophagy steps which may relate to the increased LC3-I levels observed upon autophagy induction by starvation as discussed in Figure 2 and its related narrative.

The differences in autophagy between POAG and XFS cells are also highlighted by the differential effects of starvation on the amount of total and free and bound LC3 protein and LC3 cellular staining patterns (Fig. 2). As all or part of the LC3 undergoes degradation in the autolysosome, induction of autophagy involves an essential increase in the rate of LC3 synthesis to sustain LC3 levels. Thus, whether total cellular LC3 increases, decreases, or remain constant upon autophagy induction is primarily a function of the ratio between enhanced synthesis and enhanced degradation. Figure 2A shows, respectively, LC3 immunoblots for 3 POAG and 3 XFS cell lines before and 48 hours after induction of autophagy by starvation. Figure 2B collects the quantification (mean ± SD) for LC3-I, LC3-II, and total LC3 (LC3-T = LC3-I + LC3-II) for each triplicate result, with *P* values for paired comparison of means between the intensities for all 3 parameters in complete medium (FBS^+) and after transference to the starvation medium (FBS-). A text bar in italics above the graphs provides the FBS^-/FBS^+ intensity ratios (R).

Acceleration of autophagy by starvation caused changes in LC3 protein in opposite directions between the 2 cell types. In the POAG cells the main effect of starvation was a >60% statistically significant decrease in LC3-II (Fig. 2B, middle). LC3-I underwent only a minor increase (Fig. 2B, left). The result was a small decrease in total LC3 (LC3-T) in the cells (Fig. 2B, right). These changes are compatible with an increase in autophagic flux large enough to process and digest nascent autophagosomes at a rate exceeding their formation as limited by upstream factors. In contrast to the results in the POAG, in the XFS cells, starvation induced a statistically significant, >3-fold increase in LC3-I and a moderate increase in LC3-II as well. Together, these changes yielded a statistically significant 1.8-fold increase in total LC3 cell content (Fig. 2B, right), that is, a change in the opposite direction to the change observed in the POAG cells. We also noted that, while in serum the protein expression of both LC3-I and LC3-II were not statistically different, between XFS and POAG, whereas after starvation XFS contained between 2 to 4 times more LC3 [indicated by the 3 inter-type (higher) statistical bars in Fig. 2B]. These data may reflect a number of possible autophagy disturbances. First, a reduced clearance of autophagosomes (ie, LC3-II) in XFS cells may not be able to match the increased synthesis (see *, Fig. 1). Second, a slower autophagosome/amphisome fusion with lysosomes (**, Fig. 1) may increase the chances of recovery of LC3-I by delipidation (***, Fig. 1). The fact the LC3-positive (LC3-II) vesicles appear much larger in the XFS cells than in the POAG cells (Fig. 2C) would seem to be consistent with this mechanism, as a slower fusion event with lysosomes may allow autophagosomes to continue growing. Finally, a reduced rate of protein degradation in autolysosomes (eg, due to less acidity) could also be a source of LC3-I accumulation because a diminished rate of

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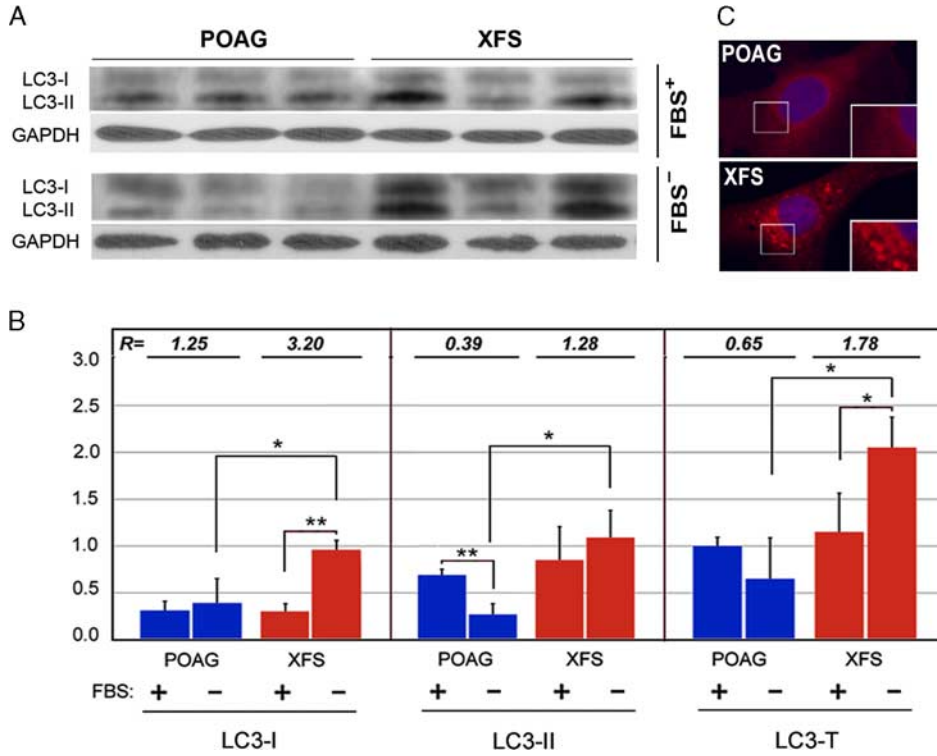

FIGURE 2. LC3 expression and distribution in POAG and XFS Tenon capsule fibroblasts in FBS-rich medium and 48 hours after FBS withdrawal to induce the starvation response. A, Results for 3 POAG (I to III) and 3 XFS (I to III) cell lines. All conditions are indicated in the graphs. LC3-I is the cytosolic form of the protein, LC3-II is the lipidated, membrane-bound forms (Fig. 1). Staining for glyceraldehyde 3-phosphate dehydrogenase confirmed that protein loading was similar for all bands. B, Mean and SD values for POAG (blue bars) and XFS (red bars) cells in FBS$^+$ and FBS$^-$ media, respectively, and the Student *t* test *P* limits for interband relationships, $*P<0.05$; $**P<0.01$. LC3-T = LC3-I + LC3-II. All band intensities have been normalized to LC3-T for POAG, which equals 1. Note that the main effects of starvation are (A) a >3-fold increase in LC3-I increase in XFS; B, a decrease of >60% in LC3-II in the POAG cells and, as a result of the opposite direction of these and other changes, statistically significant higher amounts of LC3-I, LC3-II, and LC3-T in XFS respective to POAG. C, Representative LC3 immunostaining of POAG and XFS cells 2 days after introducing starvation conditions. Note the large size of LC3-stained vesicles in the XFS cells relative to the POAG controls. FBS indicates fetal bovine serum; POAG, primary open angle glaucoma; XFS, exfoliation syndrome.

LC3-II degradation will increase the chance for LC3 release as its delipidated form (Fig. 1).

The fundamental function of autophagosomes is to clear the cells from aging or damaged organelles. Conceivably a cell subjected to cytotoxic effects may display an elevated rate of organelle decay, for example, due to an increase in oxidative stress. But even if a negative influence is not present, a slowing of autophagy rate resulting from elevated protein particulate load could affect the status of the organelle population due to delayed clearance; that is, a bystander effect. The most critical organelle cleared by autophagy is the mitochondria. Mitochondria are numerous in cells, have intrinsic high turnover rates, their physiological activity generates potentially damaging reactive oxygen species (ROS) species and autophagy is the only route of clearance for its aging units. When we performed a comparative XFS–POAG TFs analysis it was found that XFS cells had an abnormally high percent of low MMPT cells. Degraded mitochondrial function implicit in the low MMPT introduces a double risk to cells. First, decaying mitochondria may not be able to properly contain their intrinsic ROS production thereby increasing ROS release to the cytosol. Second, the loss of energy production may reduce the rate of housekeeping functions that depends on ATP. Thus, once a deficit in mitochondrial function develops, it may become a major contributor to a chronic disease state.

In summary, our recent results strongly point to a relationship between age-related XFS pathology and autophagy dysfunction and provide intriguing mechanistic clues that could serve as a basis to further investigation of autophagic dysfunction in XFS cells. Understanding the sources of this autophagic dysfunction or finding ways to reduce the magnitude of functional consequences may prove to be instrumental for developing treatment for XFS glaucoma. In this respect, one significant observation made in our studies is the permanence on the dysfunctional state in the XFS cells through many generations of growth in culture, where any external driver of the disease, for example, a sustained in vivo proinflammatory state, could be expected to wane away. The permanence suggests that the causative agent for deficient autophagy is intrinsic to the cells. Neurodegenerative diseases are believed to result from intracellular protein aggregates (ie, "aggregopathies") deriving from an aging cell's inability to continue handling its intrinsic load of nascent misfolded polypeptide.[31] The possibility that certain misfolded proteins, propagate misfolding to neighbor polypeptides (the 'bystander' effect) is

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being considered as well. In this context, the possibility that certain LOXL1 variants associated with XFS, even though enzymatically active when normally developed, increase the misfolding load in the XFS cells is tantalizing.

In addition, it is of note that while the primary clinical aim in most age-related neurological diseases is the prevention of cell death, in XFS glaucoma the aim is to prevent extracellular aggregate development. Our findings in XFS fibroblasts resemble in many aspects features that are observed in the major age-related neurological diseases, such as Alzheimer, Huntington, and Parkinson which include deficits in autophagic flux, mitochondrial health, and accumulation of denatured proteins. Indeed, a new recently discovered pathway involving the expulsion of undigested misfolded proteins from cells may also be important to understand the mechanisms that produce XFS pathology.[32] Thus, the differences in clinical focus notwithstanding, the resemblance in cellular phenotypes provide for unique opportunities for progress in XFS research and treatment by piggy backing on the present and future discoveries in the large field of research and treatment of these major age-related aggregopathies. Conversely, since in XFS cell death is not apparent neither in vivo or in cell culture, the study of these versatile proliferating fibroblasts provides unique opportunities for the investigation of abnormalities in autophagy and the development of age-related aggregopathies at large.

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