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The Cellular and Molecular Biology of the Iris, an Overlooked Tissue

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Exfoliation syndrome and exfoliation material. What is it, where is it, where does it come from and how is it produced?

Exfoliation syndrome is a systemic disorder of elastic fibers. In the eye, it is manifested by the deposition of an abnormal fibrillar material on the extracellular matrix (ECM) of tissues of the anterior segment. Accumulation of the exfoliation material (XFM) and pigment from the iris pigment epithelium (which is disrupted by iridolenticular friction when XFM is present on the anterior lens surface) in the outflow pathway is believed to be a cause of chronic open angle glaucoma. In vivo, the pathological material appears as concentric rings of greyish fringes and flakes on the pupillary border of the iris and the peripheral lens capsule, where its deposition represents the most characteristic finding of the disorder (Figure 1A and 1B).^{1–4} In vitro, by electron microscopy, it appears as an unstructured mass, formed by aggregates of disorganized fibrils and microfibrils mixed together with electron dense material.

The composition of XFM is complex. It is comprised of a number of proteins characteristic of the elastin network and basement membrane. Immunohistochemistry has shown co-localization of XFM with elastin, tropoelastin, fibrillin1, and fibulin5, as well as with numerous glycoproteins and proteoglycans.^{5, 6} In proteomic analysis, other proteins, such as Disintegrins, Metalloproteases (ADAM) and the chaperone clusterin have also been identified.⁷ Exfoliation material has been identified in the skin and many other tissues of the body (heart, lung, liver, etc),^{1, 3} but no clear correlation of the presence of the material with a systemic disease has been established.³ In the eye, XFM is found in anterior segment tissues bathed by aqueous humor: ciliary body, iris, lens capsule, zonules, cornea and trabecular meshwork. In particular, it is very abundant and clearly seen by slit lamp examination and light microscopy on the anterior lens capsule, at the pupillary border of the iris and lens periphery.⁷ Electron microscopy of the lens capsule/lens epithelial and iris pigment epithelial cells clearly reveals that both cell types produce XFM. It is not fully clear yet how the material is produced and accumulated. It has been suggested that an excess of fibrillin in the elastic microfibrils could lead to their aggregation and formation of exfoliation material.⁸ Currently, the identification of the linkage of exfoliation glaucoma to

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the *Lysyl oxidase-like 1 (LOXLI)* gene would seem to implicate a disruption of the of the elastin network due to failure of the physiological crosslinking among its existing components.

The Iris: limited number of molecular studies

The iris is an organ of the eye located between the cornea and the lens. It is fully immersed in aqueous humor. Molecules secreted by the ciliary non-pigmented epithelium bathe the posterior face of the iris and move to the anterior chamber to cover the anterior face. Proteins secreted by the iris are carried by the aqueous humor and can affect the lens, cornea endothelium and outflow pathway. From back to front, the iris is composed of a two-cell layer, heavily pigmented epithelium (iris pigment epithelium, IPE); the dilator and sphincter muscles; a stroma of highly vascularized connective tissue containing melanocytes, melanin granules and chromatophores, and an anterior cellular border layer of irregular, individual-specific shape. The iris responds to light and controls the amount of light entering the eye. Surprisingly, the iris and the regulation of its IPE cells have been hardly studied. A review of the literature reveals an absence of reports addressing basic questions such as secretion of its cells in response to light, effect of secreted iris proteins on the lens, cornea or trabecular meshwork, effects of aqueous humor on iris gene expression, or use of the iris as a targeted reservoir to regulate production of beneficial or counteracting damaging molecules. The two layers of cells of the IPE meet apex to apex. These cells contribute to the immune privilege of the eye and suppress T-cell activation by secreting TGF β and pigment epithelium-derived factor (PEDF). IPE cells play a role in acute anterior uveitis (AAU); they express lipopolysaccharide (LPS) receptors, toll-like receptor 4 (TLR4) and secrete cytokines. The IPE shares the same embryonic origin as the retinal pigment epithelium (RPE) and conserves many functional properties of these cells, including phagocytosis, degradation of rod outer segments and synthesis of trophic factors. Much current interest on IPE cell types is centered on their use for retinal transplantation.

The iris and pseudoexfoliation glaucoma

The iris, along with the lens epithelium (LE), is an ideal tissue to study exfoliation glaucoma. Part of the XFM formed by the IPE is likely to be deposited on the lens capsule, while part would be carried by the aqueous humor to the outflow pathway. It would then be relatively easy not only to purify the material from the lens capsule location, and to examine its constituents by immunohistochemistry, but to also follow fluorescently-tagged molecules secreted by adjacent tissues. These molecules, wild-type or mutated, could be delivered by viral gene transfer to the IPE in vivo and be followed to their deposition site in the extracellular space. This approach offers a great opportunity to observe directly which IPE-secreted molecules would affect the XFM structure and/or even more relevant, how they would affect outflow facility and IOP down the road in the trabecular meshwork. The potential effect of the iris in outflow facility has been under estimated. Much as the RPE cells are essential for the maintenance of photoreceptors, the IPE cells, by regulating the secretion of numerous factors to the aqueous humor, are essential to maintain and/or to determine the health of the trabecular meshwork.

Because of the strong association of certain geographic locations with a high incidence of XFS, the response to light of the iris cells at the molecular level (gene expression, protein release etc) would be an important area of study. A number of questions such as the identification of IPE genes that could regulate the production/ induction of XFM, or which IPE genes would be induced under exfoliation conditions need to be addressed. A first important study searched for genes that are differentially expressed in exfoliation clinical samples from different anterior segment tissues compared to those of matched controls.⁹ Results from this study have provided the first tools to be able to follow identified genes functionally, and to determine their relevance to XFS both in IPE cells in vitro and in living rodents. Among the upregulated mRNAs in the iris of exfoliation patients the authors found *Latent TGF- β binding protein1 and 2 (LTBP1 and LTBP2)*, MMP1 inhibitor *TIMP2* and *Transglutaminase 2*. Among the down regulated genes they found MMP1 inhibitor *TIMP1* and *Clusterin*. Finally, it would also be interesting to study the iris, in particular IPE cells, to address the disturbance of iris pigmentation reported to occur in patients with exfoliation glaucoma.^{2, 3}

Genes expressed in the iris: Clusterin

The sequence of an iris cDNA library was conducted at NEI, NIH, published in 2002 and reanalyzed in 2011¹⁰ (<http://neibank.nei.nih.gov/index.shtml>). The library was constructed from iris tissue pooled from 10 individuals (4 to 80 years old). It was unamplified and unnormalized, thus reflecting the true abundance of each gene. From the 2,000 clones sequenced, 1,263 non-redundant genes were identified. A table of the 25 top genes present in the iris showed that *Clusterin* was the most abundant gene. Other potentially relevant genes include *Glutathione peroxidase* (3th), *Opticin/oculoglycan* (6th), *Prostaglandin D2 synthase* (7th) and *Tissue inhibitor of metalloproteinase 3 (TIMP3)* (20th).¹⁰

Clusterin is a ubiquitous, multifunctional protein. It plays a role in many cellular processes ranging from lipid transport, to chaperone, to cellular proliferation and death. It is secreted as a complex glycoprotein form of 70 to 80 kDa.¹¹ The gene encoding this protein is also known as *ApoJ* and it is induced by heat, oxidative and mechanical stress.¹¹ *Clusterin* appears to be subjected to post-transcriptional regulation.¹² An analysis (at writing) of the NEI bank libraries of eye tissues (<http://neibank.nei.nih.gov/index.shtml>) showed that the iris was the tissue where clusterin was the most abundantly expressed gene. Clusterin ranked first in the iris, 15th on the lens, 27th in cornea, and 29th in the retina. It was not listed as present in the trabecular meshwork. Curiously, clusterin was not detected in the iris of monkeys.¹³

Clusterin has been associated with exfoliation glaucoma. It was found to be present in exfoliation deposits on anterior lens capsules.⁷ As mentioned above, *Clusterin* was reported to be consistently downregulated in the iris of 3 patients with exfoliation glaucoma.^{3, 9, 14} Despite downregulation of its mRNA, *Clusterin* protein seemed to be more prominent in the extracellular space of the exfoliation patients, where it co-localized with the XFM.¹⁴ This finding would be consistent with the presence of a post-transcriptional regulation for this gene, which has been described in the endometria.¹²

Lens capsule and lens epithelium. Their role in exfoliation glaucoma

The lens capsule is a modified basement membrane of the LE cells.¹⁵ Even if the posterior lens does not contain a lens epithelium layer, the capsule surrounds and completely encloses the lens. This basement membrane has a structural, protective and signaling role for the lens cells. It contains growth factors which are involved in the differentiation of LE to fiber cells, selectively filters metabolites and intermediate size molecules, and it is not permeable to viruses.^{15, 16} It has been observed by electron microscopy that cells from both LE and IPE cells are involved in the production of XFM.³ Whether the lens capsule material contains only its secreted XFM or, whether it contains exfoliation molecules originating in the IPE cells is not known. It would seem logical to think that at locations such the pupillary border, the material seen is a combination of both. Nor is it known whether a combination of secretions from IPE and LE would contribute to augment the deposits observed on the anterior surface of the capsule. Experiments aimed to study the formation and composition of XFM would greatly benefit from set ups that include having LE and IPE cells physically close together, whether in vitro or in vivo situation.

Lysyl oxidase-like 1 (LOXL1) and the elastin network

LOXL1, an extracellular protein involved in the crosslinking of elastin during the formation of the elastin network, has been found to be associated with higher risk of exfoliation glaucoma.^{17–19} LOXL1 belongs to the family of Lysyl Oxidases, extracellular copper-requiring enzymes that catalyze the oxidative deamination of lysine residues to aldehydes in collagen and elastin. The LOXL1 enzyme is required for elastin fiber homeostasis.²⁰ Of the two domains of the protein, the C-terminal region contains the elements required for catalytic activity and is very highly conserved among all members of the protein family. The N-terminal is more variable and it is released from the entire protein after secretion and processing by a procollagen C-proteinase (PCP). As a consequence, the processed C-terminal is enzymatically active (Figure 2).

Formation of the elastin polymer requires fibrillin-containing microfibrils, fibulin 5, LOXL1 and tropoelastin. Fibulin 5 interacts with microfibrils and binds tropoelastin at its N-domain and LOXL1 at its C-domain. This binding brings LOXL1 enzyme and its substrate tropoelastin close together for activation of the tropoelastin by LOXL1. Activated tropoelastin units associate with one another to form a polymer (Figure 2).²⁰ Thus, LOXL1 serves both as a cross-linking enzyme and an element of the scaffold during mature elastin formation. Experiments delivering tagged LOXL1 full length protein or each of the domains to rat lung fibroblasts showed that the C domain alone is unable to associate with the elastin at the ECM, while both the full length form and the N domain of the protein target easily to the elastin network.²¹

Interestingly, all exfoliation-linked single nucleotide polymorphisms (SNPs) described until now map to exon 1, which comprises the N-terminal domain of the protein. It would be very interesting to determine whether full length LOXL1 proteins containing the SNP changes in the N-domain, are able to direct the altered LOXL1 protein to the elastin network in the same way as the wild type protein does.

Targeting the iris by gene transfer

Gene transfer to the different tissues of the eye is accomplished by the use of replication-deficient viral vectors. Of all the viral vectors available, adeno-associated viruses (AAV) are the ones of choice for gene therapy of the eye. These vectors, either in their single-stranded DNA form, or in the second generation double-stranded (scAAV) transduce very efficiently all cell types of the eye. They have early onset, long-term expression and low immunogenicity. A single intraocular injection can confer transgene delivery for at least 2 years.²² Targeting the IPE by these vectors is very efficient and it has already been shown to occur in rats and monkeys (Figure 1C).²² The availability of this technology would allow the transfer of any of the candidate genes or their domains to the iris in vivo, and consequently observe their effect on the elastin network of the iris/lens region and on outflow facility. Overexpressing or silencing these candidates could add a new insight into the development of exfoliation glaucoma.

Concluding insights: the iris, from an overlooked to a preferred tissue

The time has come to look at the iris/lens capsule/lens epithelium as a preferred site to understand and potentially treat exfoliation glaucoma. While the trabecular meshwork is the ultimate target, it is quite clear that the formation of a considerable amount of material occurs upstream of the outflow pathway. An important key study has identified candidate proteins by proteomics analysis of lens capsule material.⁷ This finding was followed by the landmark discovery that an elastin crosslinking gene, LOXL1 was associated with the disease.¹⁷ With these discoveries, we are beginning to understand that the generation of the XFM might not be only due to the presence of new proteins, or to the relative abundance of existing ones, but perhaps to the inability to form a correct conformation of elastin due to mutated cross-linker proteins.^{8, 21} We now need to go further. Most technologies to address the questions raised here are available. Among them, rational gene transfer to the IPE cells could be the beginning. Finally, part of these ideas started to consolidate upon observing laboratory data of the high tropism of our viral vectors for transgene delivery to the IPE of the rat, and upon reexamining our own published data in the monkey.²² The excellent studies of the last few years on the cellular, molecular and genetics of XFS have paved the way to begin a new approach, which would consider the iris as a very important element in the search for a treatment of exfoliation glaucoma.

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Figure 1. The iris and exfoliation material

Composite of images bringing the relevance of the iris in the formation of XFM, and of its potential as a target tissue for exfoliation treatment. 1A: Magnification of original hand drawings by John Lindberg, who in 1917 first observed greyish fringes at the pupillary border of the iris in older patients, especially in those with glaucoma.^{2, 23} 1B: Images from exfoliation patients obtained by direct in vivo observation and ultrasound microscopy.⁴ 1C: Targeting a reporter transgene to the Iris Pigment Epithelium after an intracameral injection.²²

Figure 2. Diagrams of the structure and function of the LOXL1 protein

Top: LOXL1 gene exons and their correspondence to translated domains of the protein. PCP site for cleavage of the protein into the N and C-terminal (active) domains. *Bottom:* Functional model of the LOXL1 role in the development of elastogenesis (adapted from²⁰).