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Growth Factors, Oxidative Damage and Inflammation In Exfoliation Syndrome

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Abstract

Exfoliation syndrome (XFS) produces deleterious ocular aging and has protean systemic manifestations. Local ocular production of TGF β 1 is of central importance in XFS. TGF β 1 appears to induce the expression of LOXL1 and the production of other extracellular matrix components which are known to be present in exfoliation material. Furthermore, results from several studies find that the aqueous humor of exfoliation glaucoma patients exhibits a decreased antioxidant defense and increased oxidative stress systems. Finally, studies show that the levels of interleukin IL-6 and IL-8 in the aqueous humor of XFS patients were three fold higher than in controls. Overall TGF β 1, as well as a pro-oxidative and pro-inflammatory environment seems to play an important role in XFS.

Keywords

Pseudoexfoliation glaucoma; TGF β 1; ROS; interleukin (IL)-6

Introduction

Exfoliation syndrome (XFS) is an age-related systemic disease of the elastic fibers characterized by the pathological deposition of fibrillar material in the extracellular matrix (ECM).^{1–3} In the eye, XFS is manifested by deposits of the exfoliation material (XFM) in the tissues of the anterior segment, most prominently on the lens anterior capsule at the pupillary border between the iris and the lens. The primary consequence of the presence of this material in the eye is an elevation of intraocular pressure (IOP), most likely due to the release of XFM and iris pigment particles and their deposition in the trabecular meshwork (TM), leading to physical obstruction of the aqueous humor outflow pathway in the TM. The prolonged increase in IOP leads to the development of exfoliation glaucoma (XFG), the most common identifiable cause of open-angle glaucoma worldwide, the glaucoma most associated with high IOP and the most common glaucoma in many countries. Proper characterization of the XFM components and the genetics and cellular biologic abnormalities is essential for understanding the pathophysiology of the disease.

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The composition of the insoluble material on the anterior human lens capsule has been studied using the latest technologies of mass spectrometry and atomic force microscopy.⁴⁻⁷ In addition to ECM proteins earlier identified by immunohistochemistry of the XFM deposits, such as elastin, fibronectin or laminin, a number of novel molecular components were identified by direct and imaging-coupled proteomics. Clusterin (CLU) (also known as Apolipoprotein J, ApoJ), Fibrillin-1 (FBN1), Lysyl Oxidase-Like-1 (LOXL1), Apolipoprotein E (ApoE), latent TGF β 1 binding proteins (LTBP1 and 2) and Fibulin 2 (FBLN2), were reported to be among the most abundant proteins in the deposits and were subsequently confirmed by immunohistochemistry in different laboratories. Other proteins not yet seen in XFM material would warrant consideration. Such is the case of Transglutaminase 2 (TGM2), a critical cross-linking enzyme that confers resistance to ECM breakdown. TGM2 is one of the most upregulated genes in relevant tissues (iris, lens epithelium and ciliary processes) of XFG patients.⁸ TGM2 is also upregulated by TGF β 1 in the TM⁹ and in glaucoma-derived TM cells.¹⁰ It is not well known how the XFM is formed. Based on evidence of epigenetic correlations with XFG,¹¹ one would expect that metabolic, physical, and environmental conditions would influence the biological functions of XFS-relevant proteins by changing their expression, secretion, conformation etc.

The formation of an XFM-like material has been reported in two potential animal models, porcine and mice (reviewed in¹²). The porcine model developed the material after induction of cataracts with a high sucrose diet and the material was shown to contain crystallins.¹³ The genetic mouse model, the *Lyst* mouse, exhibited in addition an iris transillumination phenotype characteristic of XFG.¹⁴ Interestingly, the material stained with anti-FBN1 antibody. However, neither of these two models showed signs of elevated IOP or degeneration of retinal ganglion cells, the hallmarks of glaucoma.

Despite the discovery of LOXL1 single nucleotide polymorphisms (SNPs) associated with an increased risk of XFGS, the high presence of these SNPs in the non-XFS population is a clear indication that a number of other factors might play a key role in the development of the disease. Very likely, not only one but a combination of hits on different XFS components will cause them to lose their equilibrium and induce the white aggregation precipitates so characteristic of the disease.

In this manuscript, we examine the relevance of growth factors, oxidative damage and inflammation as potential contributors to the formation of the XFM and/or development of the disease. In particular, we review the effects of these three conditions on the major proteins that compose the material.

Growth Factors Levels in XFG Aqueous Humor

Growth factors are proteins that regulate cellular processes involved in cell proliferation and healing. Some growth factors stimulate the development of excess fibrous connective tissue (fibrosis) by simultaneously inducing formation of ECM and inhibiting its degradation. Not surprisingly, growth factors have been extensively studied in the development of elevated IOP. An increase in the fibrotic ECM of the TM would automatically result in increased resistance to the aqueous outflow fluid and as a consequence, elevated IOP. Most likely

because of being associated with the presence and/or release of XFM in the anterior segment, XFG is always coupled with an elevated IOP. Studies on the presence of pro-fibrotic growth factors in the aqueous humor and their effects on tissues of the anterior segment are thus of maximal importance.

TGF β isoforms

In 1994, Tripathi et al. first reported increased levels of TGF β 2 in the aqueous humor of primary open-angle glaucoma (POAG) patients when compared to non-glaucomatous controls.^{15, 16} In 2001, TGF β 1 and TGF β 2 levels were measured by ELISA in the aqueous humor and serum samples from XFS patients with and without glaucoma.^{17, 18} Their results in two European populations showed that TGF β 2 levels were the same in disease and control samples. Instead, TGF β 1 isoform levels (both latent and active) were consistently higher in the aqueous humor of XFG patients. No differences were found in the serum of both groups. These findings were later confirmed in a Japanese population, where not only TGF β 1 levels were significantly elevated in XFG patients but differential levels of a third isoform, TGF β 3, were also identified.¹⁹

Microarray differential gene expression experiments of primary human TM (HTM) cells treated with TGF β 1 and TGF β 2 revealed that TGF β 1 induces more profound changes in the cells than TGF β 2.²⁰ The top category of genes induced by TGF β 1 was that of genes involved in ECM composition and regulation. For example, Chondroitin Sulfate Proteoglycan 2 (versican), a major component of the ECM and a relevant TM resistance protein²¹ was upregulated 4.8-fold by TGF β 1 and 2.7-fold by TGF β 2. ANGPL7 (CDT6), another relevant trabecular meshwork ECM protein, highly influenced by dexamethasone and elevated IOP,²² was upregulated 11.1-fold by TGF β 1 and 5.8-fold by TGF β 2. Genes involved in REDOX cycling, such as thioredoxin and several dehydrogenases were downregulated by TGF β 1 treatment, which agrees with reports of TGF β 1 induction of protein oxidative modifications in other systems.²³ Together, all these findings suggest that proteins preferably induced by TGF β 1 would be more relevant for the formation of XFM and development of XFG.

Connective Tissue Growth Factor (CTGF)

Increased levels of another growth factor, connective tissue growth factor (CTGF) were also reported in a small XFG patient population from Ireland.²⁴ This study was repeated including a larger sample with separation of the XFS group into patients with and without glaucoma. Results showed that CTGF was significantly higher in XFG patients than in XFS patients and controls, but there were no differences between the latter two groups.²⁵ In an Egyptian population, CTGF measurements in the aqueous humor of 30 XFG patients versus 30 POAG and 27 controls, showed a significant increase of the growth factor in XFG patients versus POAG or controls. In this study, the difference in CTGF between POAG and controls though was not significant.²⁶ This would indicate that overall, CTGF levels would have more of an influence in XFG than in POAG.

Basic Fibroblast Growth Factor (bFGF)

A pilot study conducted in a small Scandinavian population sample (13 XFS, 7 XFG and 17 controls)²⁷ showed that bFGF levels in the aqueous humor were higher in patients with XFG than in patients with XFS. Both types of XFS patients exhibit higher bFGF levels than non-XFS controls.

Hepatocyte Growth Factor (HGF)

In a 2001 study, HGF was measured in the aqueous humor of an American population from 72 patients (38 POAG, 17 OAG and 17 XFG) and 12 cataract controls. This factor was significantly lower in the controls versus all glaucomas. However, samples from the XFG group were significantly higher than those from POAG or OAG, which were not different between themselves. In contrast, the HGF serum levels of all groups were not significantly different.²⁸

Vascular Endothelial Growth Factor (VEGF)

An early study to determine the presence of VEGF in the aqueous humor comprised a total of 87 patients. Of them, 16 were XFG patients, 27 POAG, 8 eyes had angle closure glaucoma, and 33 patients were cataract-only controls (mixed Chinese and American populations). In this study, the VEGF levels in the aqueous humor from the control eyes were significantly lower than in all three glaucomas, but there was no significant difference among the glaucomas nor between controls of the Chinese and American populations.²⁹ A later second study from Turkey determined the levels of VEGF in 84 patients, 15 XFG, 37 XFS and 32 controls. ELISA analysis showed that VEGF levels in aqueous humor and serum from XFG and XFS were significantly higher than controls, but not different between them.³⁰

Effects of XFG-Associated Growth Factors on the Major Proteins of the XFM

Since anterior segment tissues producing XFM deposits are continuously immersed in aqueous humor, it would seem logical to presume that changes in levels of growth factors would affect the expression of the molecular components. A number of studies addressing this issue show that the growth factors found to be associated with XFG have a direct effect on the proteins forming the XFM.

Lysyl Oxidase Like 1 (LOXL1)

A report from 2011 showed that expression of LOXL1 was increased in cultured HTM cells exposed to TGF β 1, 2 & 3 isoforms.³¹ The increase occurred at the mRNA and protein levels, at 12 h and at 48 h after 5–10 ng/ml exposure to each of the isoforms. LOXL1 was induced by all three isoforms. Of all the LOX and LOXL genes assayed, LOXL1 mRNA was the highest induced by TGF β 1. When the cells were pretreated with TGF β -receptor inhibitors (LY364947, SB431542), the induction of LOXL1 was blocked.^{31, 32}

A significant intracellular LOXL1 induction was also observed in human Tenon's capsule fibroblasts cultured from XFG patients carrying high-risk haplotype G-G and treated with TGF β 1 for 48 h.³³ The microfibrillar network of the cells, stained with FBN1, appeared significantly enhanced in the treated cells. Thicker fibrils were also visible by electron microscopy. Similar TGF β 1 induction of LOXL1 occurred on cells carrying the low risk haplotype T-G. The induction was slightly higher in the lower risk haplotype, both in the untreated and treated cells, but the difference was not significant.³³ At the present time, the sample size of the study does not seem to be large enough to conclude that the presence of reduced levels of LOXL1 are influencing the high risk phenotype. Interestingly though, a recent study in a South Indian population found that LOXL1 activity in aqueous humor was significantly reduced in the XFG patients, albeit not significantly associated with the high-risk haplotype.³⁴

LOXL1 expression is also induced by exposure to the growth factor CTGF. Human transformed TM cell line NM5 exposed to a recombinant fragment of CTGF (25 ng/ml) significantly increased LOXL1 expression. Pretreatment with anti-CTGF antibody FG-3019 reverted the induction.³⁵ This finding led the authors to propose a potential FG-3019 anti-fibrotic treatment of glaucoma.

Clusterin (Apolipoprotein J) (CLU)

Clusterin is a secreted multifunctional glycoprotein that is upregulated in Alzheimer's disease and expressed ubiquitously in many tissues of the body (review in^{36, 37}). Proteomic analysis identified clusterin as one of the most abundant proteins on the exfoliation deposits.⁵ Clusterin is the most abundantly expressed gene of the human iris (<https://neibank.nei.nih.gov/index.shtml>). Interestingly, clusterin is not expressed in the iris of the monkey,³⁸ a potential reason as to why XFG has not been observed in nonhuman primates.

Clusterin has a close and long association with TGF β 1. In cancer, clusterin interacts with the C-terminus of the TGF β receptor^{39,40} and induces expression of TGF β and Smad. In turn, prostate cancer cells treated with TGF β 1 induced clusterin expression, while treatment with a TGF β receptor inhibitor suppressed its induction. The authors also found that TGF β 1 induction of the epithelial-mesenchymal transition is mediated by secreted-clusterin.⁴⁰ Interestingly, in these studies clusterin appears to be associated with TGF β 1, not with TGF β 2.

There are no reports of CTGF or of any of the other XFG relevant growth factors effects on clusterin.

Fibrillin 1 (FBN1)

Fibrillin is a large extracellular matrix molecule which polymerizes to form microfibrils. Fibrillin controls TGF β activation. It sequesters the large latent complex (formed by latent TGF β binding protein (LTBP1), TGF β and, associated peptides), and limits the activation of TGF β . Mutations in FBN1 lead to an excess of liberated, activated TGF β in the ECM.⁴¹ Fibrillin in turn, is also induced by TGF β ,⁴² which causes fibrillogenesis in dermal fibroblasts.⁴²

In the eye, patients with XFG exhibit an enhanced staining for microfibrils, which localizes preferentially on regions where XFM deposits are detected.⁴³ TM and lamina cribrosa cells exposed to aqueous humor from XFG patients (enriched in growth factors) induced FBN1 along with other fibrotic-associated genes such as Col1A1. Pre-treatment with an anti-CTGF antibody reversed the induction.⁴⁴ Fibrillin 1 was also found to be upregulated in epithelium subtracted libraries (iris/ciliary processes/lens) of XFG versus XFS.⁸

Apolipoprotein E

The Apolipoprotein E (ApoE) gene encodes for a 299 amino acid lipid-binding protein implicates in diseases that involve pathological protein aggregation. Polymorphisms of ApoE have been associated with risk of Alzheimer disease. ApoE4 is the polymorphism with greatest risk, and ApoE2 is protective. ApoE interacts with neurofibrillary tangles in Alzheimer's plaques. It binds and retains amyloid β and thus plays a role in the accumulation of amyloid plaques. Proteomic studies of XFM showed the presence of ApoE in the XFM deposits, in particular at the pupillary area of the anterior lens capsule.⁶⁷ In the eye, in addition to being found in XFS, it is also found in drusen deposits, which increase the risk of age-related macular degeneration.

A recent study has shown that ApoE derived from astrocytes contributes to the risk of Alzheimer's disease through the TGF β /Smad2 signaling pathway.⁴⁵ In that study, delivery of TGF β to the cortex of ApoE KO mice by an adeno-associated vector markedly increased amyloid plaque formation, while delivering an inhibitor of TGF β to the WT mice, reduced it. These findings revealed that activation of the TGF β pathway is required for the effects of ApoE in the development of Alzheimer's disease. ApoE was also reported to be induced by TGF β in monocytes.⁴⁶ It is possible that a parallel mechanism could exist in the eye and that the increase of TGF β in the aqueous humor of XFG patients could contribute to a potential effect of ApoE on the accumulation and retention of the XFM deposits.

Latent TGF β 1 binding protein 1 & 2 (LTBP1 & 2)

LTBP1 is a glycoprotein that forms part of the inactive intracellular TGF β complex and helps targeting the complex to the ECM where it contributes to TGF β activation by disassociating it from the latency-associated peptides.⁴⁷ The LTBPs and fibrillins are part of a family of ECM proteins characterized for the presence of an eight cysteine repeat domain that binds to TGF β .⁴⁸ These proteins are important for both the structural integrity of the ECM and the regulation of bioavailability of TGF β . The LTBP/fibrillin family is extensively regulated at the level of transcription with tissue-specific dependent transcriptional start sites, alternative promoters and enhancers.⁴⁹ Understanding their specific regulation of expression in the cell of interest could lead to better strategies for the treatment of diseases. Recently, it has been shown that the LTBP1 protein forms filaments-like structures independent of other matrix components. The filaments bind to each other and their multimerization is stabilized by the cross-linking enzyme Transglutaminase 2.⁵⁰

An extensive early study on immunolabeling, in situ hybridization and molecular expression of LTBP1 in the eye of normal human subjects detected its mRNA and protein in practically all eye tissues.¹⁸ LTBP1 was increased in tissues from patients with XFS, with and without

glaucoma, in particular in the iris and nonpigmented epithelium of the ciliary processes. LTBP1 staining was also detected in the XFM on the iris surface.

LTBP1 was also found to be induced by TGF β . Tenon fibroblasts from XFG patients exposed to TGF β showed that LTBP1 was the second most induced of the elastic microfibrillar components analyzed.³³ Prior studies analyzing isolated XFM deposits by proteomics and LC-MS/MS identified LTBP2, rather than LTBP1.⁷

Oxidative Damage in XFG and its influence on the major proteins of XFM

Reactive oxygen species (ROS) are accumulated inside the cells as a result of a variety of processes including byproducts of normal metabolism, environmental stresses and diseases. Oxidative stress occurs when the concentration of ROS in the cell exceeds that of antioxidant molecules such as superoxide dismutase, catalase, glutathione peroxidase and small molecules (Vitamin C, E). Oxidative stress affects a great number of diseases, including glaucoma and XFG.^{51, 52}

Koliakos et al. first found that the concentration of the antioxidant ascorbic acid was reduced in the aqueous humor of patients with XFG.⁵³ In a follow up study, the authors found that 8-isoprostaglandin F2a, a marker of oxidative stress in vivo, was elevated in the aqueous humor of XFG patients, while ascorbic acid was also reduced.⁵⁴ Oxidative stress can induce fibrogenic responses and it is involved in the pathogenesis of fibrotic disorders.⁵⁵ TGF β , a potent inducer of fibrosis, is induced by oxidative stress in Tenon fibroblasts cells from XFG patients.³³ In these experiments, oxidative stress also increased expression of XFS-associated proteins, and treatment of these cells with 50-500 μ M H₂O₂ for 48 h in serum-free conditions elevated the cDNAs of LOXL1, LTBP1, Elastin, FBN1 and FBLN2.³³

Other studies in different populations have evaluated levels of oxidants and anti-oxidants in the aqueous humor of XFG patients and controls.⁵⁶⁻⁵⁹ Results from all of them agree with the original finding that the aqueous humor of XFG patients exhibits a decreased antioxidant defense and increased oxidative stress systems. In a study that included POAG and XFG patients, Ferreira et al. showed that the total reactive antioxidant potential (TRAP) index was 64% lower in the aqueous humor of POAG patients while antioxidant enzymes, glutathione peroxidase and superoxide dismutase were significantly increased.^{56, 60} TRAP was lower in XFG than in POAG and lower in both glaucomas compared to cataracts controls. The same occurred with the reduction of ascorbic acid levels. The increase in glutathione peroxidase was higher in XFG than in POAG and both were higher than cataracts. Together these results indicate that an oxidative stress condition might play a greater role in XFG than in POAG.

The oxidative stress effects on expression of clusterin and ApoE have not been studied in XFG. However, clusterin oxidative stress related damage/protection has been studied in other diseases where oxidative injury is part of their pathology.⁶¹ For example, clusterin expression is regulated by oxidative stress in ageing and age-related diseases⁶² and it exerts a protective effect on oxidative stress-induced cell death in human corneal endothelial and neuroblastoma cells.^{63, 64} It has been suggested that the secreted clusterin may act as an extracellular chaperone scavenging misfolded proteins produced by oxidative injury.⁶²

Further, it has been proposed that clusterin has antioxidant properties by itself and can protect cells from ROS-induced cell death.⁶⁵

The influence of oxidative stress of ApoE in XFG is not known. However, the effects of stress on the regulation of ApoE have been extensively studied.⁶⁶ ApoE is involved in longevity. Because aging is characterized by a decrease in stress resistance,⁶⁷ the responses of ApoE to stresses have been investigated in vivo (KO mice) and in vitro. Results demonstrated that ApoE-KO mice show elevated markers of oxidative stress, that oxidative stress induces ApoE secretion from adipocytes, and that ApoE overexpression protects cells from hydrogen peroxide-induced damage.⁶⁶ Most interesting, the anti-oxidative properties of ApoE appeared to be isoform dependent (APOE2>APOE3>APOE4). The role of oxidative stress on the ApoE deposition on the XFM could have a parallel with that of ApoE in the amyloid plaque of Alzheimer's Disease. It is well established that oxidative stress plays a key role in the development of Alzheimer's Disease,⁶⁸ but how could oxidative stress directly influence the deposition of the XFM is not known. The rational link between the fact that ApoE is involved in antioxidants effects in the body and that it appears in the XFM deserves much investigation.

Inflammatory responses in XFG

The presence of pro-inflammatory cytokines in the aqueous humor plays a key role in the physiology and pathophysiology of the eye. These cytokines regulate a number of relevant parameters such as ECM remodeling, vascular permeability and IOP. They are upregulated under inflammatory conditions such as uveitis and the resulting imbalance can affect tissues of the anterior segment. Profiles of cytokines in the aqueous humor are currently being used as biomarkers of several diseases such as glaucoma, macular edema and choroidal neovascularization.^{69–72} The recently available multiplex bead immunoassay has opened the door to the analysis of cytokines in very small samples, thus allowing to assay and quantify multiple ones in each aqueous humor sample.^{73, 74}

XFS and XFG involve low grade inflammation and several authors have looked at the differential levels of inflammatory cytokines in the aqueous humor of XFG patients compared to controls. It is becoming more and more clear that in some diseases like cancer, a cytokine-enriched environment leads to the remodeling of the tumor's ECM.⁷⁵ A chronic inflammation state in XFG could then contribute to an increased ECM deposition and further hamper aqueous humor flow. Studies on patients with XFS showed that the levels of interleukin (IL)-6 and IL-8 in the aqueous humor were three fold higher than in controls in the early stage of the disease but not in the late stage or in XFG.⁷³ In addition, the treatment of transformed non-pigmented ciliary epithelial cells with IL-6 but not with IL-8, induced the expression of FBN1 and LTBP1, essential components of the XFM.⁷³ Primary Tenon fibroblasts cells derived from XFS patients treated with IL-6 significantly induced the expression of elastin, FBN1, LTBP1 and TGFβ1, but not FBN2 nor LOXL1.³³

Although there are no studies to evaluate the effect of the aqueous humor cytokines on the expression or secretion of ApoE and Clusterin, there are numerous systemic studies which provide a window into what such effect could be on the development and progression of

XFG. Clusterin has been associated with cytokines. While studies on clusterin have focused primarily on its chaperone function of protecting against stresses,^{36, 76} Clusterin is able to induce secretion of TNF α in macrophages and to up- and downregulate the expression of chemotactic cytokines.^{77, 78} We do not know which of the clusterin functions would be more relevant for its contribution to the formation of the XFM, but it is likely that such function could be affected by the elevation of pro-inflammatory cytokines in the aqueous humor of XFG patients.

Regarding ApoE, it is believed that there is a cross-talk between ApoE and cytokines.⁷⁹ That, in addition of its well-established role as a lipid transport, ApoE is able to modulate inflammatory and immune responses and in turn, pro-inflammatory cytokines can either up or down-regulate ApoE isoforms in a tissue specific manner. It would be logical to think then that cytokines could also have an effect on the potential ApoE function on XFG.

Concluding thoughts

After reviewing the XFS/XFG literature for this mini-review, one cannot but think that the relevance of this disease and its impact on the general aging population are just too great to be ignored. Enormous advances have been made during the last ten years on all the fields of clinical research, genetics and molecular biology. The evidence at every turn seems to point out towards a central master control in the hands of TGF β 1. Every pathway associated with XFG, from the components of the XFM deposits to their expression and physiology affected by the environment, seem to involve TGF β 1. All the basic knowledge accumulated during this period should be able to carry us to the next step of finding a cure for the disease. The vision and investment of The Glaucoma Foundation towards this goal is invaluable. Their strategy of continuously bringing outside experts on every new discovered biological and clinical aspect of the disease, will undoubtedly bring us closer to the understanding, management and cure of XFS/XFG in a not so distant future.

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