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Involvement of ER Stress in Human Primary Pterygium

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Abstract: *Purpose:* The aim of this study was to investigate the ER stress activation in human primary pterygium. *Methods and materials:* Human primary pterygium or normal Tenon's capsule tissues were obtained from patients with primary pterygium following surgical excision or from normal human fresh cadaver eyes. The tissues were processed within 2 hours. The mRNA or protein specimens were extracted from those tissues for analysis, cryosections of those tissues were prepared for immunohistochemical staining. The mRNA levels of endoplasmic reticulum (ER) stress-related factors in those tissues were detected by qPCR analysis and the related proteins levels were detected by qPCR analysis and immunohistochemical staining or western blotting. *Results:* The ER stress-related gene transcription levels of GRP78, spliced XBP-1, ATF4 and ATF6 and the protein expression levels of GRP78, p-IRE1α, p-eIF2α and ATF6 were all increased in the human primary pterygium tissues when compared with the normal control tissues. *Conclusion:* The results in this study suggest that the three unfolded protein response pathways are all activated in the human primary pterygium tissues, which indicates that the ER stress is involved in the progression of pterygium, and also suggests a potential mechanism of ER stress-induced inflammation in the human primary pterygium tissues.

Keywords: Primary Pterygium, Endoplasmic Reticulum Stress, Unfolded Protein Response, Inflammation, UPR Pathway Activation

1. Introduction

The endoplasmic reticulum (ER) is a dynamic organelle that plays a key role in cell homeostasis [1]. One of the crucial functions of ER is protein synthesis and folding [2-4]. When the protein folding process is disturbed in the ER, which is also referred to as 'ER stress' [5], the unfolded or misfolded proteins accumulate in the ER, leading to the activation of unfolded protein response (UPR) [6]. The activation of UPR is achieved through the disassociation between the ER chaperone glucose-regulated protein 78 (GRP78) and three ER stress sensors, including inositol-requiring protein 1α (IRE1α), protein kinase RNA (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6) [1, 4].

ER stress and autophagy have been found to be involved in the induction of inflammatory response and contribute to the pathogenesis of chronic inflammatory diseases [7, 8]. In recent years, evidence has shown that inflammation is linked to ER stress and UPR in the cardiovascular system, altering gene expression and translational programs to overcome stressful conditions and to restore ER homeostasis [7-10]. ER stress is a key factor in the inflammatory response and a potential mediator of inflammation in cardiovascular diseases [11-13].

Pterygium is a chronic fibrovascular invasive centripetal overgrowth on the corneal surface that is often associated with inflammation and neovascularization. Oxidative stress is considered a major factor in the pathogenesis of pterygium, but the exact underlying mechanism remains elusive.

This study was designed to explore the involvement of ER stress in human primary pterygium tissues and, subsequently, predict the mechanism of primary pterygium targeting prolonged UPR-induced inflammation.

2. Method

2.1. Patients

This study was approved by the Human Subjects Committee of Sun Yat-sen University. We declare that all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed in this research. A total of 30 Han Chinese patients aged 30-60 years (16 men and 14 women) with intermediate-grade (Tan grade) primary pterygium participated in this study; 12 normal Tenon's capsule specimens from cadaver eyes (aged 30-60 years, 7 men and 5 women) served as controls. Each group was randomly divided into three subgroups for three repeats. An informed consent was signed by all patients prior to study initiation.

2.2. Primary Pterygium and Tenon's Capsule Tissue Collection

Human primary pterygium specimens, ~3x4mm in size, were collected following pterygium excision surgery. Human normal Tenon's capsule specimens, ~3x4mm in size, were obtained from cadaver eyes.

2.3. Immunohistochemistry on Cryosections from Pterygium and Normal Tenon's Capsule Tissues

The pterygium or normal Tenon's capsule specimens were fixed in 4% paraformaldehyde for 30 min and then cryoprotected with a series of 10-30% sucrose solutions. The tissues were sectioned vertically at 10-12 μm on a cryostat set at -20°C and immunostained using anti-GRP78 (1:600, Abcam, Cambridge, MA, USA; ab12223), anti-phospho-eIF2α (1:100, Cell Signaling Technology, Inc., Danvers, MA, USA; 119A11), anti-ATF6 (1:100, Abcam; ab11909), or anti-phospho-IRE1α (1:100, Abcam; ab48187) antibodies overnight at 4°C, followed by Cy3-conjugated secondary antibody at room temperature for 90 min. Fluorescence was visualized under an Olympus AX70 microscope (Olympus Corporation, Tokyo, Japan).

2.4. Primary Pterygium and Tenon's Capsule Samples RNA Extraction and qPCR

Total RNA was isolated from pterygium or Tenon's capsule tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). cDNA was generated using Superscript First-strand Synthesis Kit (Invitrogen; Thermo Fisher Scientific, Inc.). cDNA samples (1.0 μl) were mixed with SYBR Green Master Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and subjected to qPCR analysis using the Bio-Rad CFX96 Real-Time system. Each reaction was set up in triplicate, and each experiment were repeated at least three times. The quantification cycle (Cq) values for each gene were normalized to the expression of 18S and analyzed using the standard curve method. The log of mean relative expression ± standard deviation is reported. The sequences of the PCR primers are listed in Table 1.

Table 1. *Gene-specific primer sequences for qPCR.*

Primer	Forward (5′-3′)	Reverse (5′-3′)
GRP78	GACGGGCAAAGATGTCAGGA	GCCCGTTTGGCCTTTTCTAC
s-XBP1	ACACGCTTGGGAATGGACAC	CCATGGGAAGATGTTCTGGG
ATF6	CTTTTAGCCCGGGACTCTTT	TCAGCAAAGAGAGCAGAATCC
ATF4	GGGACAGATTGGATGTTGGAGA	ACCCAACAGGGCATCCAAGT
18S	TCGGCTACCACATCCAAGGAAGGCAGC	TTGCTGGAATTACCGCGGCTGCTGGCA

2.5. Protein Extraction from Primary Pterygium and Tenon's Capsule Samples and Western Blot Analysis

The pterygium or normal Tenon's capsule tissues were lysed in radioimmunoprecipitation assay buffer mixed with a protease inhibitor mixture (PMSF; Thermo Fisher Scientific, Inc.) and sodium orthovanadate (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), sonicated and then centrifuged at 13,000 x g for 10 min. The supernatant was collected for determination of the protein content. The proteins were resolved on 12% sodium dodecyl sulfate polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with the appropriate antibodies, using β-actin as the internal control. The anti-GRP78 antibody was used at a 1:2000 dilution, whereas the anti-phospho-IRE1α antibody, the anti-phospho-eIF2α antibody and the anti-ATF6 antibody were all used at a 1:1000 dilution. Peroxidase-based detection was performed with Chemiluminescence Reagent (NEN Life Science, Xinhailing Company, Shenzhen, China). Each experiment was repeated three times.

3. Results

3.1. GRP78 Expression Is Increased in the Human Primary Pterygium Compared with Normal Tenon's Capsule Tissues

To investigate the GRP78 expression in the human primary pterygium, immunochemical staining was performed on cryosections obtained from pterygium and normal Tenon's capsule tissues. In this study, the expression of GRP78 in the pterygium tissues was found to be significantly increased compared with normal Tenon's capsule tissues (Figure 1A).

Based on the immunochemical staining result, GRP78 mRNA and protein expression levels were detected by qPCR and western blot analysis. Total RNA or soluble protein were extracted from pterygium tissues, with the normal Tenon's capsule tissues serving as control. We observed that the GRP78 mRNA level of the pterygium tissues was ~8.59-fold

that of the normal control (Figure 1B), and the GRP78 protein level of the pterygium tissue was ~6.85-fold that of the control (Figure 1C) These results indicate activation of GRP78 during the process of human primary pterygium formation.

Figure 1

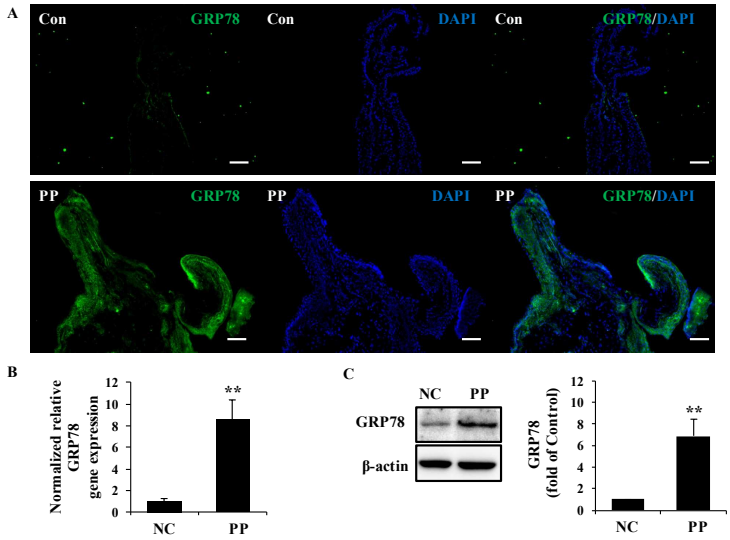

Figure 1. *Increase of GRP78 mRNA and protein levels in primary pterygium tissues. (A) Human primary pterygium (PP) and normal Tenon's capsule (Con) cryosections were immunostained with anti-GRP78 antibody (green). DAPI was used for staining of the cell nuclei (blue). RNA and protein were extracted from primary pterygium (PP) and human normal Tenon's capsule (Con) tissues. (B) qPCR was performed to detect the GRP78 gene expression level, using 18S as the internal control gene (mean ± SD, n=3); (C) the protein level of GRP78 was determined by western blotting, using β-actin as the internal control.* $^{*}P<0.05$; $^{**}P<0.001$.

3.2. Activation of the Unfolded Protein Response IRE1/XBP-1 Pathway in Human Primary Pterygium

To investigate the activation of the IRE1/XBP1 pathway in human primary pterygium, pterygium and normal Tenon's capsule tissue cryosections were analyzed by immunohistochemical staining. We observed that the expression of the phosphorylated IRE1α (p-IRE1α) was significantly increased in the pterygium compared with that in the normal Tenon's capsule (Figure 2A). To confirm this result, we then determined the transcription level of the spliced X-box binding protein 1 (XBP1s) by qPCR assay and the protein level of p-IRE1α by western blot analysis. The protein and RNA samples were extracted from human primary pterygium and normal Tenon's capsule tissues. In the primary pterygium group, the mRNA level of XBP1s was significantly upregulated (4.65-fold) compared with the normal control (Figure 2B); the p-IRE1α protein level was also upregulated (~12.34-fold) compared with the normal control (Figure 2C).

Figure 2

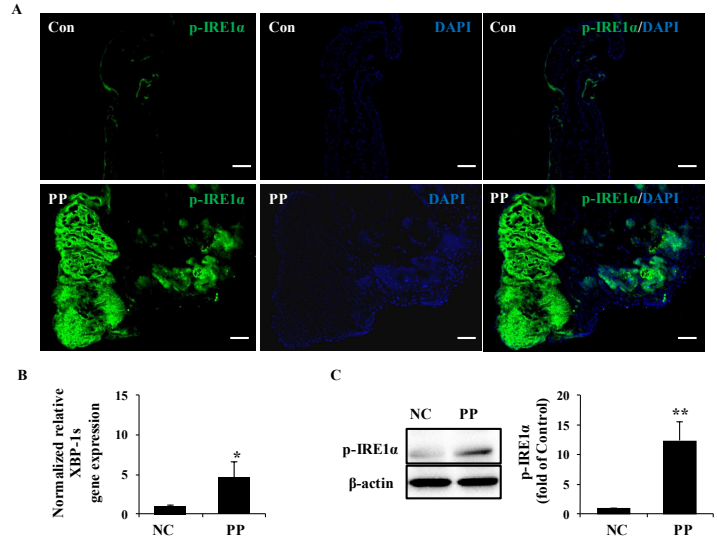

Figure 2. *P-IRE1α protein and spliced XBP1 gene expression levels were increased in human primary pterygium tissues. (A) Cryosections of human primary pterygium specimens (PP) and human normal Tenon's capsule specimens (Con) were immunostained with anti-p-IRE1α antibody (green). (B) qPCR was performed to detect the relative gene expression levels of spliced XBP1 in each group (mean ± SD, n=3); (C) The protein level of p-IRE1 was detected by western blot analysis, using β-actin as the internal control.* $^{*}P<0.05$; $^{**}P<0.001$.

3.3. Activation of the PERK/eIF2α/ATF4 Pathway in Human Primary Pterygium

Immunohistochemical staining for phosphorylated eIF2α (p-eIF2α) was performed on primary pterygium and normal Tenon's capsule tissue cryosections, and the results revealed a markedly increased p-eIF2α level in the primary pterygium group compared with that in the normal control (Figure 3A). qPCR and western blot analyses were then applied to confirm the PERK/eIF2α/ATF4 pathway-related gene or protein expression level. The results revealed the same trend between ATF4 gene expression and p-eIF2α protein level. The ATF4 mRNA level of the primary pterygium group was ~15.11-fold higher compared with that of the control (Figure 3B), and the p-eIF2α protein level of the primary pterygium group was ~8.06-fold higher compared with that of the control (Figure 3C).

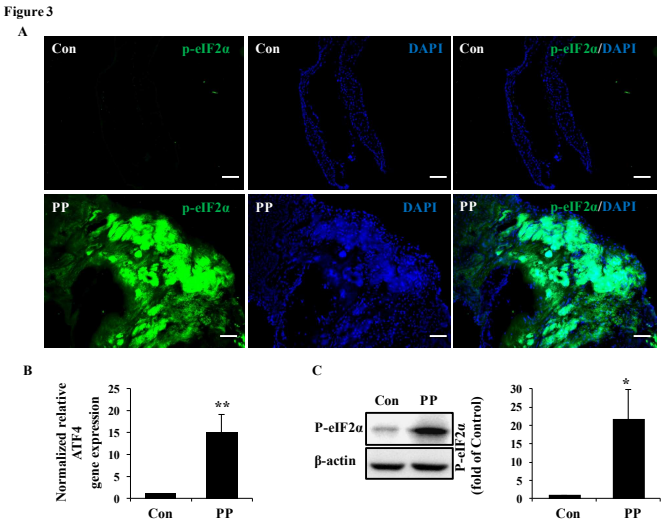

Figure 3. *P-eIF2α protein and ATF4 gene expression were found to be upregulated in human primary pterygium. (A) Cryosections of primary pterygium tissues were immunostained with anti-p-eIF2α antibody (green). (B) qPCR was performed to detect the relative gene expression levels of ATF4 (mean ± SD, n=3); (C) the protein level of p-eIF2α was determined by western blot analysis. *P<0.05; **P<0.001.*

3.4. Activation of the ATF6 Pathway in the Human Primary Pterygium Group

Based on the finding that two pathways of UPR were activated in human primary pterygium tissues, we aimed to determine whether the ATF6 pathway was activated as well. Immunohistochemical staining on primary pterygium tissue sections revealed notable induction of ATF6 (Figure 4A). The qPCR assay revealed an ATF6 mRNA level ~2.97-fold higher compared with that of normal control (Figure 4B). Western blot analysis revealed a similar trend of the ATF6 protein (~2.4-fold) compared with that of normal control (Figure 4C).

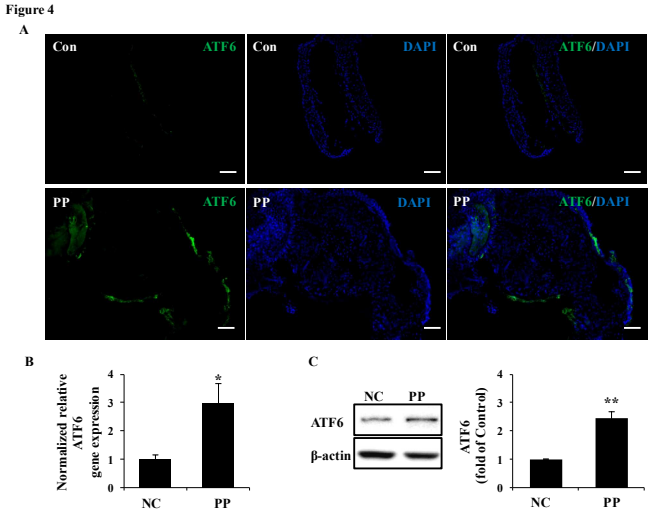

Figure 4. *ATF6 expression was activated in human primary pterygium. (A) Cryosections of primary pterygium (PP) tissues were immunostained with anti-ATF6 antibody (green). RNA and protein were extracted from the human PP specimens and human normal Tenon's capsule specimens (Con). (B) qPCR was performed to detect the relative gene expression levels of ATF6 in each group (mean ± SD, n=3); (C) Western blot analysis was performed to detect the ATF6 protein level. *P<0.05; **P<0.001.*

4. Discussion

We herein present the activation of UPR in human primary pterygium. The UPR primarily aims to restore the ER homeostasis [14]; however, prolonged ER stress may cause excessive UPR activation, thereby initiating inflammatory responses [15, 16]. Accumulating evidence indicates that UPR activation is directly linked to inflammation [17]. Inflammation induced by desiccation [18], antigens by solar radiation-denatured proteins [19] or microtrauma [20], were hypothesized to be the factors underlying the pathogenesis of pterygium. Clinical evidence also suggests that persistent inflammation is an important risk factor of postoperative recurrence [21]. This suggests that UPR activation may contribute to the pathogenesis of pterygium.

GRP78 is a major ER chaperone and usually binds to the three UPR sensors, namely IRE1, PERK and ATF6, when the ER is under homeostasis conditions. Once the unfolded or misfolded proteins overaccumulate in the ER, GRP78 dissociates from the three UPR sensors and binds to the improperly folded proteins, helping to eliminate them from the ER through triggering the three UPR pathways [22]. Thus, GRP78 induction is also known as a crucial marker of ER stress or UPR activation [23, 24]. According to our findings, the transcription and protein levels of GRP78 were increased in human primary pterygium tissues, which indicates UPR activation.

The direct evidence of UPR induction is the activation of three UPR pathways [22]. These three pathways are cross-linked as a network or may be induced separately. The IRE1α/XBP1 branch is considered as the conserved core of UPR [14]. When the IRE1α/XBP1 pathway is activated, IRE1α is autophosphorylated to its p-IRE1α form and then splices a 26-nucleotide intron from the XBP1 mRNA, which results in a frameshift of the XBP1 gene and produces a more stable form, referred to as spliced XBP1 (XBP1s) [25]. XBP1s is also a potent activator of UPR [26, 27]. In our research, the results revealed significant upregulation of the p-IRE1α protein level and the XBP1s gene expression level in primary pterygium tissues, indicating activation of the IRE1/XBP1 branch. In addition, the upregulated XBP1s can induce downstream genes, including genes encoding ER chaperones, such as GRP78 [28], which accelerates the induction of GRP78 and UPR.

The activation of the PERK/eIF2α/ATF4 branch results in PERK autophosphorylation to its p-PERK form. Subsequently, p-PERK phosphorylates the α-subunit of eIF2α to its p-eIF2α form. The p-eIF2α then induces another transcription activator, ATF4. In this study, we observed increased expression of ATF4 and p-eIF2α, indicating activation of the PERK/eIF2α/ATF4 pathway. Furthermore, ATF4 then induces a subset of UPR genes, such as XBP1, and subsequently accelerates the activation of UPR.

Release from BiP enables ATF6 to be transported to the Golgi apparatus from ER, where it is cleaved to an activated form (p50ATF6) [29]. The cleaved ATF6 then moves to the nucleus to induce the expression of a subset of UPR target genes [30]. In this study, we detected increased protein levels of cleaved ATF6 and ATF6 gene expression levels in the pterygium group, suggesting ATF6 branch activation.

It has been established that the three branches of the UPR also crosstalk with the inflammation networks [10]. Under conditions of sustained ER stress, three UPR branches exert strong effects on the NFκB pathways of systemic chronic inflammation [10, 31]. IRE1 activates the NFκB-IKK pathway by binding to the IKK complex promoting the degradation of inhibitor IκB; PERK activates the NFκB pathway by reducing its inhibitor IκB, and induces the pro-inflammatory gene expression of TNF and interleukin-1 (IL-1) by translocation of the dimer to the nucleus; and ATF6 plays role on the activation of the NFκB-IKK pathway through its involvement in the transient phosphorylation of protein kinase AKT [31-33]. Some stressors, such as oxidative insults causing ER stress-induced inflammation, may be an important mechanism underlying primary pterygium formation. Furthermore, anti-inflammatory measures are crucial following pterygium excision to prevent recurrence.

5. Conclusion

In summary, these results demonstrated that the three pathways activate UPR in human primary pterygium tissue. The findings of the present study indicate that the anti-inflammatory effect may be improved through treatments targeting ER stress; therefore, anti-ER stress measures may be a novel approach to preventing the progression of pterygium at its early stages, or to preventing recurrence following pterygium excision surgery. Further research focused on the association between the two is recommended in the future.

Declarations

Ethics Approval and Consent to Participate

This study was approved by the Human Subjects Committee of Sun Yat-sen University. Informed consent was obtained from the patients before the study was initiated.

Availability of Data and Materials

Data can be shared upon request.

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Declaration of Interest

The authors report no conflicts of interest. The authors

alone are responsible for the content and writing of the paper.

Authors' Contributions

SZ contributed to immunohistochemistry and real-time PCR experiments, participated in the writing of the manuscript. JY participated in the design of the study, data analysis and drafting the manuscript.

The first two authors (Sheng Zhou and Jing Yang) contributed equally to the work and therefore should be considered equivalent authors.

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List of Abbreviations

ER stress: endoplasmic reticulum stress
UPR: unfolded protein response
ER: endoplasmic reticulum
GRP78: Glucose-regulated protein 78
IRE1α: inositol-requiring protein 1α
PERK: protein kinase RNA (PKR)-like ER kinase
ATF6: activating transcription factor 6
XBP1: X-box binding protein 1
XBP1s: spliced X-box binding protein 1

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