Endoplasmic Reticulum Stress and Unfolded Protein Response in Vernal Keratoconjunctivitis

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Purpose. Vernal keratoconjunctivitis (VKC) is an ocular allergic disease characterized by a type 2 inflammation, tissue remodeling, and low quality of life for the affected patients. We investigated the involvement of endoplasmic reticulum (ER) stress and unfolded protein response in VKC.

Methods. Conjunctival imprints from VKC patients and normal subjects (CTs) were collected, and RNA was isolated, reverse transcribed, and analyzed with the Affymetrix microarray. Differentially expressed genes between VKC patients and CTs were evaluated. Genes related to ER stress, apoptosis, and autophagy were further considered. VKC and CT conjunctival biopsies were analyzed by immunohistochemistry (IHC) with specific antibodies against unfolded protein response (UPR), apoptosis, and inflammation. Conjunctival fibroblast and epithelial cell cultures were exposed to the conditioned medium of activated U937 monocytes and analyzed by quantitative PCR for the expression of UPR, apoptosis, autophagy, and inflammatory markers.

Results. ER chaperones *HSPA5* (GRP78/BiP) and *HYOU1* (GRP170) were upregulated in VKC patients compared to CTs. Genes encoding for *ER* transmembrane proteins, PKR-like ER kinase (*PERK*), activating transcription factor 6 (*ATF6*), ER-associated degradation (*ERAD*), and autophagy were upregulated, but not those related to apoptosis. Increased positive reactivity of BiP and ATF6 and unchanged expression of apoptosis markers were confirmed by IHC. Cell cultures in stress conditions showed an overexpression of UPR, proinflammatory, apoptosis, and autophagy markers.

Conclusions. A significant overexpression of genes encoding for ER stress, UPR, and proinflammatory pathway components was reported for VKC. Even though these pathways may lead to ER homeostasis, apoptosis, or inflammation, ER stress in VKC may predominantly contribute to promote inflammation.

Keywords: vernal keratoconjunctivitis, ER stress, unfolded protein response, apoptosis, autophagy

Vernal keratoconjunctivitis (VKC) is a severe and chronic ocular allergic disease characterized by an intense type 2 conjunctival inflammation and tissue remodeling.[1,2] Patients affected by all clinical phenotypes (tarsal, limbal, and mixed) complain of intense itching, photophobia, tearing, and mucous discharge for several months per year, negatively affecting their quality of life and causing psychological stress for patients and their family members.[3] Signs and symptoms are typically exacerbated by specific allergen exposure but, more frequently, by exposure to environmental non-specific conditions such as heat, pollution, sun, and hot wind, all considered conditions that can stress the ocular surface.[4] In fact, the cornea is frequently involved in the clinical aspects of epithelial punctate keratopathy, erosions, ulcers, and corneal neural inflammation.[5] We recently demonstrated in VKC significantly higher gene expression signatures related to innate and adaptive immunity, antigen presentation, T helper 2 (Th2) and Th17 priming, and inflammatory cell chemotaxis and activation compared to normal subjects.[6] In addition, we highlighted the redundant expression of pathogen recognition receptors, which, when interacting with multiple environmental, tissue, or microbial residues, might initiate or aggravate the disease.[7] Furthermore, we described in VKC an increased expression of stress-related proteins such as heat shock proteins (HSPs),[8] epithelial barrier dysfunction,[9,10] and tissue remodeling.[2,11,12] Therefore, a demand for increases in protein synthesis and folding (e.g., cytokine or mucus production) can create an imbalance in the endoplasmic reticulum (ER) and consequent ER stress, which plays a significant role in cellular survival and function.[13] ER stress and oxidative stress resulting from exposure to pollutants, irritants, and inflammatory mediators have been related in lung diseases to epithelial barrier dysfunction, development of innate

and adaptive immune responses, and airway remodeling and hyperresponsiveness,[14–16] but it may also have similar roles in ocular surface inflammatory diseases.[17] ER disfunction leads to misfolded protein, which may be harmful for cell function and the adaptive unfolded protein response (UPR).[16] To regain ER homeostasis, different compensatory responses are initiated, including increased expression of folding chaperones, inflammation, and ER-associated degradation (ERAD). ERAD is an essential process in which misfolded proteins are recognized as terminally misfolded, re-translocated into the cytoplasm, polyubiquitinated, and targeted for proteasomal degradation. The accumulation of unfolded proteins is sensed by three ER transmembrane proteins: protein kinase R (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). If these mechanisms fail, UPR results in apoptosis and cell death.[13] The objective of this study was to use clinical specimens and cell models to gain insight into upregulation of the UPR in VKC.

Methods

Subjects and Impression Cytology Samples

Fifteen VKC patients (10 males and five females) and eight healthy age-matched control (CT) subjects (five males and three females) were included. None of the control subjects used contact lenses or had inflammatory signs and symptoms or history of allergy. The study complied with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of our institution. Informed consent was obtained from the subjects (or patients), who were appropriately informed of the nature and possible consequences of the study. The diagnosis of VKC was based on the typical history, signs, and symptoms.[1] All 15 VKC patients were free of topical mast cell stabilizers and/or antihistamines for at least 3 days and free of topical corticosteroids for at least 5 days before samples were collected. Impression cytology samples were obtained for RNA isolation from all VKC and CT subjects as previously described[6] (see Supplementary Methods).

RNA Isolation, Integrity Measurement, and Affymetrix Assay

Total RNA was extracted from impression cytology membranes using the QIAGEN RNeasy Plus Mini Kit (QIAGEN, Hilden, Germany) and was treated with QIAGEN RNase-Free DNase, following the manufacturer's instructions. Purified RNA was stored at −80°C until testing. RNAs were quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and further processed with the Affymetrix GeneChip WT Pico Kit (Thermo Fisher Scientific). Finally, labeled cDNA was hybridized on Affymetrix Clariom S Arrays (Thermo Fisher Scientific).[18] Gene expression data for selected genes involved in ER chaperones, ERAD, ER stress, UPR, autophagy, and apoptosis were identified from a literature search and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Database (https://www.kegg.jp/pathway). The fold change for each gene was compared with the CT.

Immunohistochemistry and Western Blotting Analysis

Upper tarsal conjunctival biopsies were obtained from five active tarsal VKC patients and five healthy age-matched subjects. Tissues were snap frozen with optimal cutting temperature (OCT) compound (Histo-Line Laboratories, Pantigliate, Milano, Italy) in liquid nitrogen and maintained at −80°C until use for immunohistochemistry (IHC). Two sections from each biopsy sample were stained applying a standard IHC procedure, with antibodies specific for glucose-regulated protein 78 (GRP78)/BiP and ATF6; apoptosis markers BAX, B-cell lymphoma 2 (BCL2), and caspase-3; and inflammatory markers IL-1β and NOD-like receptor protein 3 (NLRP3), as described in the Supplementary Materials and in Supplementary Table S1. BiP tissue expression was also evaluated by western blot (WB) analysis (see Supplementary Methods). ImageJ (National Institutes of Health, Bethesda, MD, USA) was employed to determine the relative quantity of BIP in the samples, and densitometry values were normalized to those of actin.

Cells

Primary conjunctival fibroblasts were obtained after digestion of VKC tarsal biopsies with 20-U/mL type I collagenase (Worthington Biochemical, Lakewood, NJ) at 37°C for 6 hours. Immortalized Human Conjunctival Epithelial (P10780-IM; Innoprot, Derio (Bizkaia), Spain) and the human monocytes U937 (Thermo Scientific; Wilmington, DE, USA) were purchased to be used for the experiments. Conjunctival epithelial cells were cultured using the IM-Ocular Epithelial Cell Medium Kit (Innoprot), and fibroblasts were grown in complete Dulbecco's modified Eagle's medium (DMEM), both supplemented with 10% fetal bovine serum, 1% (v/v) penicillin–streptomycin, and 2-mM glutamine at 37°C, in a 5% humidified CO_2 atmosphere. The U937 cells were grown in suspension in complete Gibco RPMI 1640 Medium (Thermo Fisher Scientific) and were subcultured twice weekly by dilution using a seeding density of 10^6 cells.

Activated U937 Monocyte Conditioned Medium

U937 cells were treated with 1-µg/mL lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) for 1 hour after being differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) at a final concentration of 50 ng/mL for 48 hours. Cells were rinsed and cultured for 24 hours in complete RPMI, and the inflammatory conditioned medium (CM) was collected, centrifuged, filtered, and utilized to treat primary human cell cultures as previously reported.[19] The differentiation of monocytes to macrophages was examined under an inverted phase-contrast microscope, and the mRNA expression of the macrophage differentiation marker CD68 was analyzed by quantitative real-time PCR (qPCR; data not shown).

ER Stress, Apoptosis, and Autophagy in Conjunctival Cell Cultures and qPCR Analysis

To induce ER stress in vitro, P10780-IM epithelial cells were deprived of glucose for 24 hours or treated with U937-

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activated CM for 24 hours for further analysis. Because preliminary results showed similar effects for both stress conditions, primary fibroblasts from two different donors were grown in standard culture conditions and exposed to U937 CM for 24 hours. Each experiment was performed in triplicate. Total RNA was extracted from cell cultures, and differences in mRNA expression of *ATF6*, *IRE*, *PERK*, *IL-1β*, *BAX*, *caspase-3*, *BCL2*, light chain 3A (*LC3A*) and *LC3B*, and lysosomal-associated membrane protein 1 (*LAMP-1*) genes were analyzed by qPCR in primary conjunctival fibroblasts and epithelial cells exposed to the stress condition for 24 hours (see Supplementary Methods).

Statistical Analysis

The differential gene expression analysis was conducted using limma.[20] Genes (probesets) having adjusted $P < 0.05$ and absolute $\log_2$ fold change > 1 (ratio between the mean value of expression in the VKC group and the mean value of expression in the control group) were considered to be significantly differently expressed. In this analysis, the *P* values were corrected for multiple testing (false discovery rate criterion) using the Benjamini–Hochberg procedure. IHC scores for VKC and control biopsies were analyzed using the non-parametric Mann–Whitney *U* test and a normal approximation with a continuity correction of 0.5. PCR results in cell cultures were compared using an unpaired Student's *t*-test. The statistical significance threshold was defined as $P < 0.05$. Data are presented as means and standard deviations.

Results

ER Stress–Related Genes in Conjunctival Samples

ER chaperones were generally upregulated in VKC compared to CT samples. In general, lectin and non-classical molecular chaperones that moderate protein folding, including *HSPA5* (GRP78/BiP), hypoxia-upregulated protein 1 (*HYOU1*; *GRP170*), calreticulin (*CALR*), serpin peptidase inhibitor clade H member 1 (*SERPINH1; HSP47*), protein disulfide isomerase A4 (*PDIA4*), and *HSPB8*, were significantly upregulated in VKC samples, whereas *HSPB11* and *HSPE1* were significantly downregulated. Several other co-chaperones such as the *DnaJ* or prefoldin (*PFDN*) families were either up- or downregulated (Table 1).

Regarding the expression of genes involved in UPR signaling, eukaryotic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*) encoding for PERK, ATF4, ATF6B, and protein phosphatase 1 regulatory subunit 15A (*PPP1R15A*)/growth arrest and DNA damage-inducible protein (*GADD34*) were significantly upregulated, whereas *ERN1/IRE1*, TNF receptor-associated factor 2 (*TRAF2*), mitogen-activated protein kinase kinase kinase 5 (*MAP3K5/ASK*), *MAP2K7*/mitogen-activated protein kinase kinase 7 (*MKK7*), and X-box binding protein 1 (*XBP1*) were unchanged and *MAPK6/JNK* was downregulated (Table 1).

The expression of genes involved in ERAD was also altered. Even though ER degradation-enhancing alpha-mannosidase-like (*EDEM*) proteins 1, 2, and 3 and derlin-1 (*DERL1*), as well as translocation factor SEC genes, were normally expressed, genes associated with misfolded protein degradation (*PDIA4*, *OS9*, *ERMan1/MAN1A1*, *HSPA1*, *DNAJA1*, *HSPH1*) and proteasomal degradation-related genes (*UBQLN2*, *USP3*, *USP32P2*, *USP18*, *USP7*, *USP9Y*) were upregulated (Table 1).

Apoptosis, Autophagy, and NF-κB–Related Genes in Conjunctival Samples

The genes related to ER stress–induced apoptosis (*BAX, BCL2, CASP3, CASP7, CASP12*, and *DDIT3/CHOP*) and cell death–related genes (*TRIB3* and *CEBPB*) were not upregulated, but synovial apoptosis inhibitor 1 (*SYVN1*) was upregulated, suggesting that apoptosis and cell death are inhibited in VKC. In contrast, several genes related to autophagy (*ATG*, *MAP1LC3A*, *MAP1LC3B*, and *LAMP3*), members of the nuclear factor-κB (*NF-κB*) gene family, and several genes encoding for proinflammatory cytokines, such as *IL-1*, *IL-6*, *IL-32*, *CXCL8*, *NLRP3*, and *NLRP6*, were significantly upregulated (Table 2).

IHC and WB Analysis

IHC analysis of the selected proteins in conjunctival biopsies showed significantly increased immunostaining for ATF6 and BiP in VKC conjunctival tissues compared to the healthy controls (Figs. 1A–1C). BiP protein in VKC was confirmed by western blotting (Fig. 1D). The levels of proteins involved in apoptosis, BAX, BCL2, and cleaved caspase-3 were similar for both the VKC group and the healthy control group (Figs. 1E, 1F). IHC expression of IL-1β and NLRP3 were also significantly higher in VKC patients compared to the healthy controls (Figs. 1G, 1H).

Cell Cultures and qPCR Analysis

Conjunctival epithelial cell cultures deprived of glucose for 24 hours (Fig. 2A) or treated with U937-activated CM (Fig. 2B) showed a significantly increased expression of *ATF6*, *IRE1*, and *PERK* by qPCR compared with untreated control cells. The same cells treated with U937 CM showed significantly increased expression of *IL-1β* and apoptosis markers *BAX* and caspase-3 but not *BCL2* (Fig. 2C) or the autophagy marker *LAMP-1* (Supplementary Fig. S1A) compared with the controls. Similarly, in the same experimental conditions, conjunctival fibroblasts treated with U937-activated CM showed significantly increased expression of *ATF6*, *IRE1*, *PERK* (Fig. 3A), *IL-1β, BAX*, and *caspase-3* but not *BCL2* (Fig. 3B) or autophagy markers *LC3A* and *LC3B* (Supplementary Fig. S1B).

Discussion

ER stress and UPR have been described to play a pathogenetic role in several inflammatory disorders,[13] including allergic asthma, for which it is related to clinical severity and inflammatory phenotypes.[15,21,22] Respiratory airways and ocular surfaces exposed to pro-inflammatory stimuli may experience recurring cycles of damage and repair; therefore, they may express high levels of ER and UPR. In fact, multiple non-specific environmental factors such as sunlight, heat, pollutants, and ultraviolet exposure can trigger inflammation in VKC due to poorly characterized mechanisms.[4] We previously reported a possible role of autophagy in

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Table 1. Expression of Genes Encoding for ER Chaperones and Genes Involved in UPR Signaling and ERAD in VKC Conjunctival Samples Compared to Control Samples

Gene Symbol	Gene Name	Fold Change	*P*	Adjusted *P*
ER chaperones and co-chaperones				
HSPA5	Heat shock 70-kDa protein 5 (glucose-regulated protein, 78-kDa) (GRP78/BiP)	1.98	0.011*	0.107
HSP90B1	Heat shock protein 90-kDa beta (GRP94), member 1	1.54	0.241	0.563
HYOU1	Hypoxia upregulated 1 (GRP170)	1.96	0.000*	0.023*
CANX	Calnexin	1.27	0.108	0.378
CALR	Calreticulin	1.91	0.022*	0.158
SERPINH1	Serpin peptidase inhibitor clade H (HSP47), member 1	1.85	0.042*	0.227
ERP29	Endoplasmic reticulum protein 29	1.29	0.196	0.513
PDIA2	Protein disulfide isomerase family A, member 2	1.01	0.965	0.989
PDIA3	Protein disulfide isomerase family A, member 3	1.45	0.189	0.504
PDIA4	Protein disulfide isomerase family A, member 4	2.14	0.001*	0.037*
PDIA5	Protein disulfide isomerase family A, member 5	1.05	0.794	0.930
PDIA6	Protein disulfide isomerase family A, member 6	−1.05	0.749	0.911
HSPA1A	Heat shock 70-kDa protein 1A	1.81	0.015*	0.127
HSPA1B	Heat shock 70-kDa protein 1B	1.83	0.023*	0.160
HSPB8	Heat shock 22-kDa protein 8	21.12	0.001*	0.036*
HSPB11	Heat shock protein family B (small), member 11	−2.53	0.001*	0.038*
HSPE1	Heat shock 10-kDa protein 1	−2.27	0.002*	0.048*
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	1.56	0.026*	0.173
DNAJC11	DnaJ (HSP40) homolog, subfamily C, member 11	1.59	0.001*	0.032*
DNAJC19	DnaJ (Hsp40) homolog, subfamily C, member 19	−2.66	0.000*	0.012*
PFDN4	Prefoldin subunit 4	−3.03	0.000*	0.009*
PFDN5	Prefoldin subunit 5	−2.48	0.000*	0.004*
PFDN6	Prefoldin subunit 6	−1.49	0.010*	0.100
UPR signaling				
EIF2AK2	Eukaryotic translation initiation factor 2-alpha kinase 2 (PERK)	2.74	0.002*	0.047*
ATF4	Activating transcription factor 4	1.89	0.007*	0.083
ATF5	Activating transcription factor 5	1.27	0.333	0.656
ATF6	Activating transcription factor 6	1.23	0.251	0.574
ATF6B	Activating transcription factor 6 beta	1.82	0.016*	0.133
PPP1R15A	Protein phosphatase 1, regulatory subunit 15A (GADD34)	3.67	0.037*	0.214
NFE2L2	Nuclear factor erythroid-derived 2-like 2 (NRF2)	1.37	0.089	0.343
ERN1	Endoplasmic reticulum to nucleus signaling 1 (IRE1)	1.38	0.123	0.404
TRAF2	Tumor necrosis factor (TNF) receptor-associated factor 2	−1.17	0.322	0.646
MAP3K5	Mitogen-activated protein kinase kinase kinase 5 (ASK1)	1.63	0.128	0.414
MAP2K7	Mitogen-activated protein kinase kinase 7 (MKK7)	1.59	0.053	0.260
MAPK8	Mitogen-activated protein kinase 8 (JNK)	−1.88	0.012*	0.110
ORMDL3	ORMDL sphingolipid biosynthesis regulator 3	1.13	0.626	0.853
XBP1	X-box binding protein 1	1.25	0.440	0.741
ERAD				
EDEM1	ER degradation enhancer, mannosidase alpha-like 1	−1.03	0.886	0.963
EDEM2	ER degradation enhancer, mannosidase alpha-like 2	1.17	0.258	0.581
EDEM3	ER degradation enhancer, mannosidase alpha-like 3	1.35	0.156	0.458
MAN1A1	Mannosidase, alpha, class 1A, member 1	1.93	0.001*	0.036*
MAN1A2	Mannosidase, alpha, class 1A, member 2	1.08	0.656	0.869
MAN1B1	Mannosidase, alpha, class 1B, member 1	1.51	0.125	0.408
DERL1	Derlin 1	−1.11	0.631	0.856
OS9	Osteosarcoma amplified 9, endoplasmic reticulum lectin	2.09	0.024*	0.165
HSPA1A	Heat shock 70-kDa protein 1A	1.81	0.015*	0.127
HSP90AA1	Heat shock protein 90-kDa alpha family (cytosolic), class A member 1	1.26	0.104	0.370
DNAJA1	DnaJ (HSP40) homolog, subfamily A, member 1	1.56	0.026*	0.173
HSPH1	Heat shock protein 105-kDa	1.87	0.005*	0.069
UBQLN2	Ubiquilin 2	1.82	0.016*	0.132
UBE2G2	Ubiquitin conjugating enzyme E2 G2	1.58	0.013*	0.120
SEC61A1	SEC61 translocon alpha 1 subunit	1.45	0.224	0.543
SEC62	SEC62 homolog, preprotein translocation factor	−1.18	0.372	0.688
SEC63	SEC63 homolog, protein translocation regulator	−1.03	0.916	0.973

* Statistically significant.

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Table 2. Expression of Genes Involved in Apoptosis, Autophagy, and Inflammation in VKC Conjunctival Samples Compared to Control Samples

Gene Symbol	Gene Name	Fold Change	*P*	Adjusted *P*
Apoptosis				
BAX	BCL2-associated X protein	1.13	0.721	0.898
BCL2	B-cell chronic lymphocytic leukemia (CLL)	1.28	0.373	0.689
BCL2L1	BCL2-like 1	1.15	0.698	0.887
BCL2L11	BCL2-like 11 (apoptosis facilitator)	3.44	0.001*	0.026*
CASP3	Caspase-3	1.31	0.366	0.683
FAS	Fas cell surface death receptor	−1.03	0.927	0.977
FASLG	Fas ligand (TNF superfamily, member 6)	−1.09	0.620	0.851
PIDD1	p53-induced death domain protein 1	1.18	0.294	0.621
ENDOG	Endonuclease G	−1.01	0.968	0.990
TRIB3	Tribbles pseudokinase 3	1.25	0.095	0.353
CEBPB	CCAAT/enhancer binding protein beta	−1.13	0.440	0.741
HRC	Histidine-rich calcium binding protein	1.19	0.210	0.528
DDIT3	DNA damage-inducible transcript 3 (C/EBP homologous protein [CHOP])	1.11	0.669	0.875
TP53	Tumor protein p53	1.06	0.775	0.923
SYVN1	Synovial apoptosis inhibitor 1, synoviolin	2.51	0.003*	0.050*
Autophagy				
LAMP1	Lysosomal-associated membrane protein 1	1.75	0.011*	0.109
LAMP2	Lysosomal-associated membrane protein 2	1.18	0.427	0.731
LAMP3	Lysosomal-associated membrane protein 3	4.52	0.005*	0.067
BECN1	Beclin 1, autophagy related	1.10	0.532	0.801
SQSTM1	Sequestosome 1 (p62)	1.42	0.087	0.339
MAP1LC3A	Microtubule-associated protein 1 light chain 3 alpha	1.86	0.050*	0.265
MAP1LC3B	Microtubule-associated protein 1 light chain 3 beta	2.75	0.003*	0.057
ATG16L1	Autophagy related 16-like 1	1.42	0.004*	0.058
ATG2A	Autophagy related 2A	1.62	0.082	0.327
ATG4A	Autophagy related 4A, cysteine peptidase	2.30	0.000*	0.014*
ATG5	Autophagy related 5	−1.37	0.080	0.324
ATG7	Autophagy related 7	1.57	0.043*	0.231
ATG10	Autophagy related 10	−1.90	0.043*	0.232
ATG12	Autophagy related 12	−1.06	0.747	0.911
NRBF2	Nuclear receptor binding factor 2	1.15	0.364	0.681
CTSA	Cathepsin A	3.45	0.000*	0.007*
CTSB	Cathepsin B	1.81	0.065	0.291
CTSC	Cathepsin C	4.13	0.000*	0.025*
CTSD	Cathepsin D	2.12	0.085	0.334
CTSL	Cathepsin L	1.12	0.489	0.772
NF-κB				
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	1.29	0.209	0.527
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49)	2.41	0.000*	0.008*
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3.15	0.003*	0.054
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	1.59	0.059	0.276
NFKBID	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, delta	−1.08	0.675	0.877
NFKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	1.55	0.046*	0.240
NFKBIL1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	1.17	0.265	0.589
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	3.39	0.001*	0.033*
Proinflammatory cytokines				
IL1β	Interleukin-1 beta	2.32	0.182	0.494
IL1R1	Interleukin-1 receptor, type I	3.08	0.003*	0.050*
IL6	Interleukin-6	4.02	0.012*	0.111
CXCL8	Chemokine (C-X-C motif) ligand 8/IL-8	6.00	0.025*	0.170
IL18BP	Interleukin-18 binding protein	2.10	0.015*	0.129
IL32	Interleukin-32	2.55	0.002*	0.040*
NLRP3	NLR family, pyrin domain containing 3	1.57	0.001*	0.034*
TNFAIP1	Tumor necrosis factor, alpha-induced protein 1 (endothelial)	1.79	0.047*	0.245
TNFAIP2	Tumor necrosis factor, alpha-induced protein 2	7.63	0.000*	0.021*
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	9.75	0.001*	0.033*

* Statistically significant.

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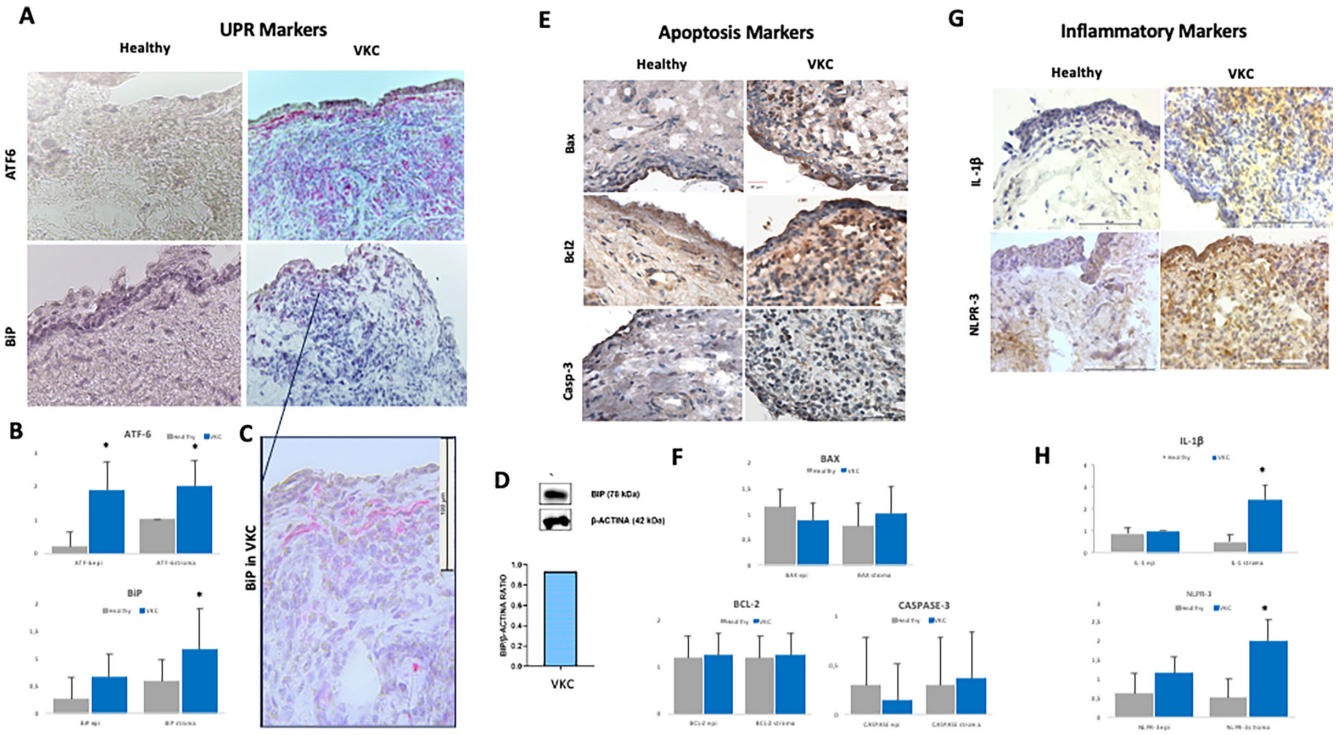

Figure 1. (**A**) Immunohistochemistry for UPR markers BiP and ATF6 in healthy normal subjects as controls (healthy) and in VKC patients. (**B**) Immunostaining for ATF6 was significantly higher (*) in VKC epithelium ($P = 0.006$) and stroma ($P = 0.01$) compared to the healthy controls, but BiP was significantly higher in VKC stroma ($P = 0.04$). (**C**, **D**) Higher magnification (100×) of BiP positive reactivity (**C**) and western blotting expression (**D**) in tissues from one representative VKC patient. (**E**) Immunostaining for the apoptosis markers BAX, BCL2, and caspase-3 in VKC patients and healthy controls showed no significant differences in staining scores. (**F–H**) Significant increase of the immunostaining for the inflammatory marker IL-1β and NLRP3 in VKC stroma compared with healthy controls.

VKC pathogenesis with increased expression of autophagy markers[23] and an overexpression of several HSPs in VKC conjunctival tissues,[8] possibly as a non-specific response to a wide variety of unfavorable physiological and environmental conditions. In the present study, we suggest that initial pro-inflammatory stimuli in VKC may induce ER stress, misfolded protein formation, and a UPR,[24] leading to inflammation rather than apoptosis. In fact, we found that genes related to protein folding were normally expressed or overexpressed and that several chaperones and genes related to the UPR were overexpressed in VKC. Because genes related to ERAD were generally upregulated, we believe that recognized misfolded proteins may be re-translocated into the cytoplasm to regain ER homeostasis or targeted for proteasomal degradation. In addition, several genes related to autophagy were upregulated but not the one related to apoptosis. These data were validated by the IHC results in VKC tissues, in which the UPR markers BiP and ATF6 were overexpressed compared to normal tissues, and the apoptosis markers were similarly expressed. Moreover, pro-inflammatory and *NF-κB* genes were upregulated, suggesting that ER stress and UPR in VKC are not promoting cell death but rather induce degradation of misfolded proteins and inflammation. Similarly, in a recent paper, the expression of apoptosis markers BAX and BCL2 were reduced in VKC patients together with increased expression of the inflammatory marker IL-6, suggesting that the absence of apoptosis contributes to the inflammation and severity of VKC.[25]

In a previous study, we highlighted downregulation of the ribosomal proteins (RPs) in VKC and suggested that several pathways involving mRNA translation, initiation, elongation, and silencing and rRNA processing in the nucleus and cytosol were significantly downregulated. It is possible that RPs, functioning as RNA chaperones, stabilize rRNAs and promote their correct folding for the assembly of ribosomal subunits.[26] Downregulation of the RPs may be responsible for the abnormal response to cellular stress.

To confirm that these mechanisms can be induced in the conjunctiva, we exposed conjunctival epithelial cells and conjunctival fibroblasts to an inflammatory stress condition, which showed that three ER stress sensors (*ATF6, IRE*, and *PERK*) were upregulated in both cell type cultures. In addition, the inflammatory marker IL-1β, the apoptosis markers *BAX* and *caspase-3*, and the autophagy markers *LC3A* and *LC3B* (in epithelial cells) and *LAMP-1* (in conjunctival fibroblasts) were upregulated, confirming that the stress can lead to both inflammation and cell death differently from the in vivo VKC environment, where inflammation and protein degradation pathways (but not apoptosis) were activated. We previously demonstrated in VKC the activation of multiple inflammatory processes, remodeling mechanisms,[27] and proteases, including matrix metallopeptidase 9 (MMP-9),[28] and the activation of autophagy

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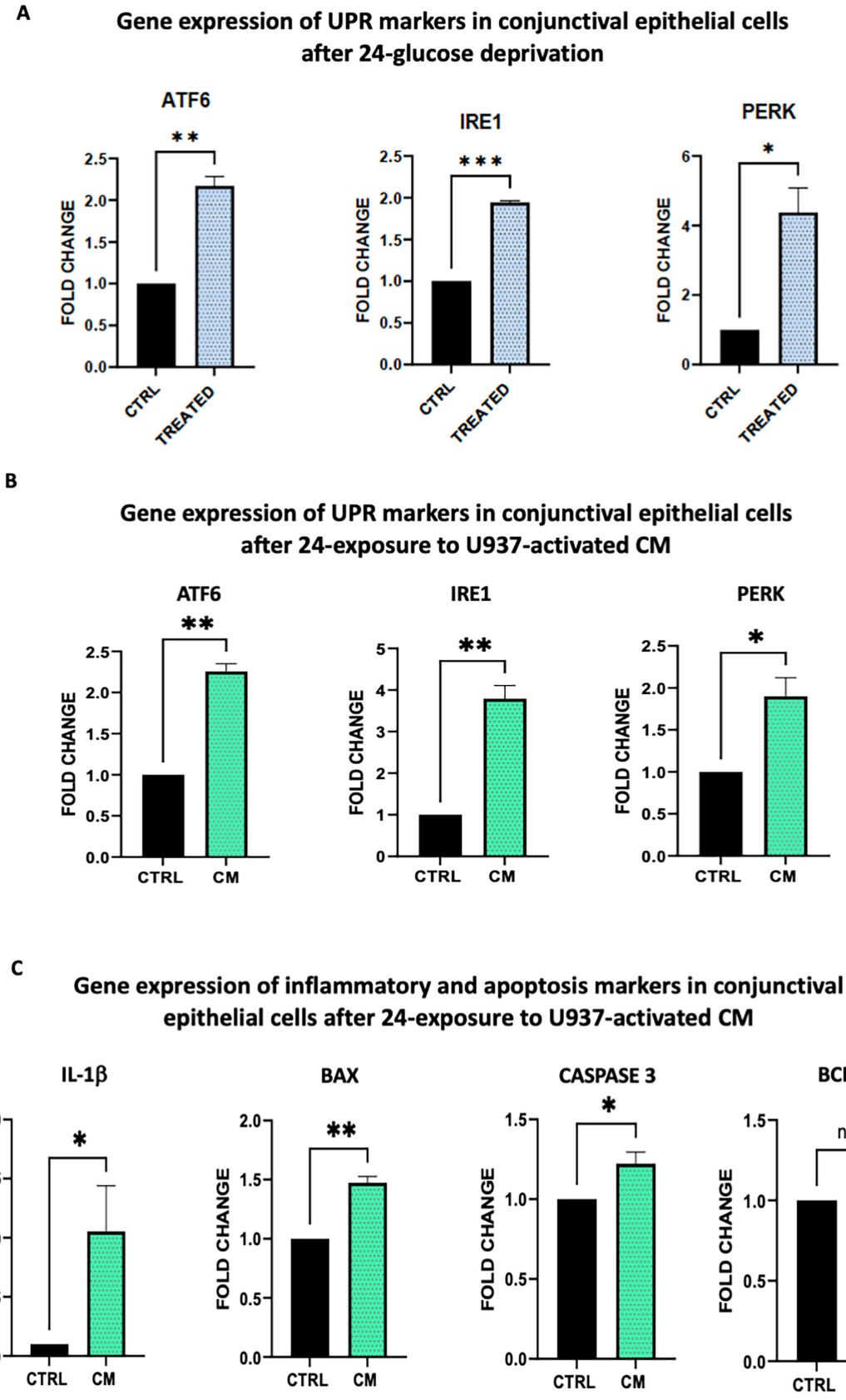

Figure 2. (**A**, **B**) Increased expression of *ATF6*, *IRE1*, and *PERK* by qPCR in conjunctival epithelial cells deprived of glucose for 24 hours (treated) (**A**) or after 24-hour exposure to U937-activated conditioned medium (CM) (**B**). Conjunctival epithelial cells showed a significant increase of *ATF6*, *IRE1*, and *PERK* in both stressed conditions compared with untreated cells (CTRL). (**C**) Increased expression of the proinflammatory factor *IL-1β* and the apoptosis markers *BAX* and *caspase-3* after 24-hour exposure to U937-activated CM. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$.

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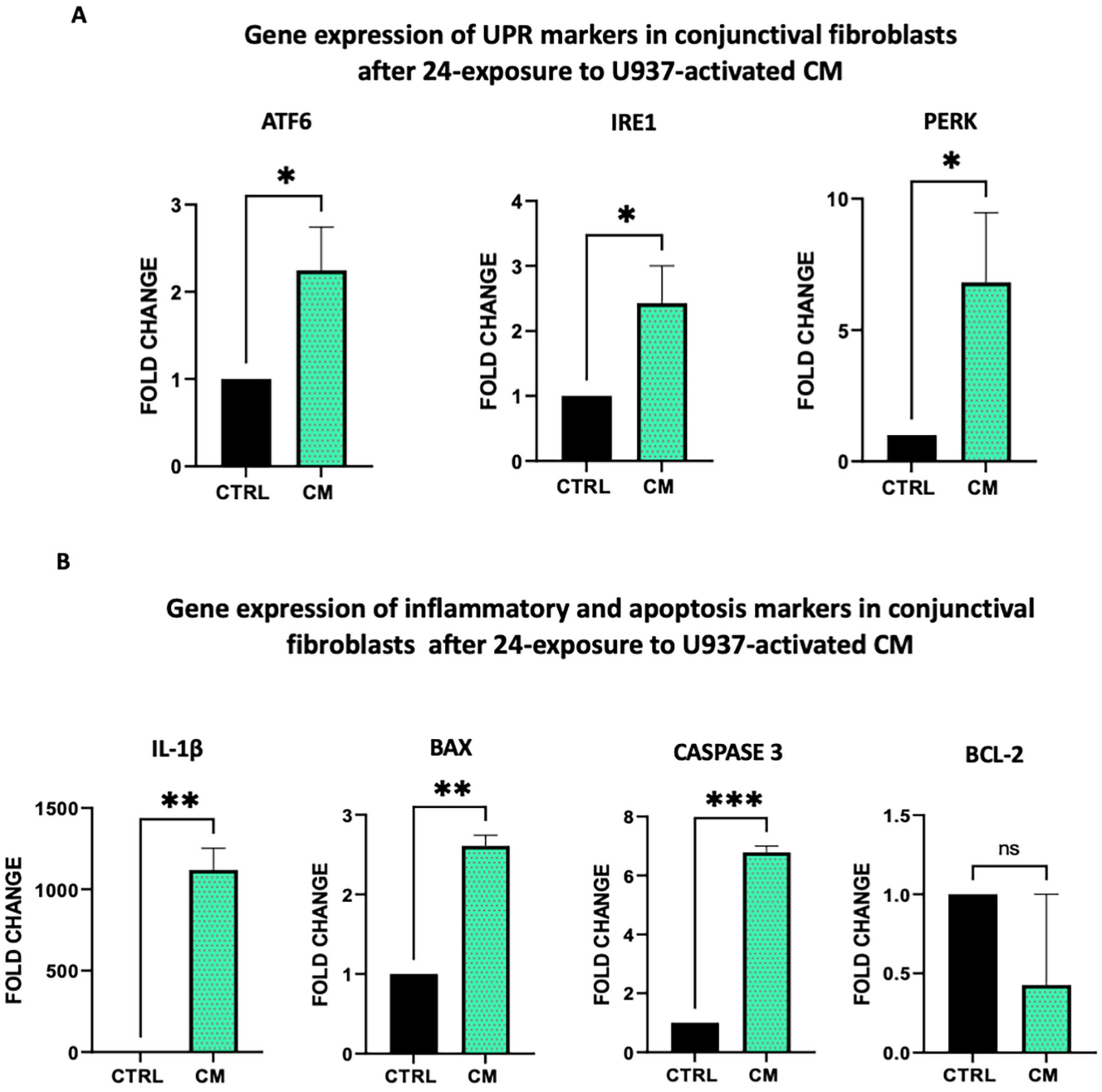

Figure 3. (**A**) Significantly increased expression of *ATF6*, *IRE1*, and *PERK* by qPCR in conjunctival fibroblasts after 24-hour exposure to U937-activated CM. (**B**) Conjunctival fibroblasts showed a significant increased expression of *IL-1β*, *BAX*, and *caspase-3* in the presence of U937-activated CM compared with untreated cells (CRTL). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

as a potential protective mechanism.[23] Similarly, a potential association between ER stress and regulation of the proteolytic microenvironment in ocular surface diseases has been suggested based on increased expression of the molecular chaperone GRP78/BiP and MMP-9 in pathological specimens.[17]

In conclusion, we demonstrated that conjunctival tissues from VKC patients exhibit high levels of ER stress. Activation of ER stress, promoting misfolded or unfolded proteins, engages the UPR machinery, which, in VKC, instead of leading to cell death, may lead to further inflammatory processes.

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