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Chaperone patterns in vernal keratoconjunctivitis are distinctive of cell and Hsp type and are modified by inflammatory stimuli

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Abstract

Background: Vernal keratoconjunctivitis (VKC) is a severe ocular allergy with pathogenic mechanism poorly understood and no efficacious treatment. The aims of the study were to determine quantities and distribution of Hsp chaperones in the conjunctiva of VKC patients and assess their levels in conjunctival epithelial and fibroblast cultures exposed to inflammatory stimuli.

Methods: Hsp10, Hsp27, Hsp40, Hsp60, Hsp70, Hsp90, Hsp105, and Hsp110 were determined in conjunctiva biopsies from nine patients and nine healthy age-matched normal subjects, using immunomorphology and qPCR. Conjunctival epithelial cells and fibroblasts were cultured and stimulated with IL-1β, histamine, IL-4, TNF-α, or UV-B irradiation, and changes in Hsp levels were determined by Western blotting.

Results: Hsp27, Hsp40, Hsp70, and Hsp90 levels increased in the patients' conjunctiva, whereas Hsp10, Hsp60, Hsp100, and Hsp105 did not. Double immunofluorescence demonstrated colocalization of Hsp27, Hsp40, Hsp70, and Hsp90 with CD68 and tryptase. Testing of cultured conjunctival cells revealed an increase in the levels of Hsp27 in fibroblasts stimulated with IL-4; Hsp40 in epithelial cells stimulated with IL-4 and TNF-α and in fibroblasts stimulated with IL-4, TNF-α, and IL-1β; Hsp70 in epithelial cells stimulated with histamine and IL-4; and Hsp90 in fibroblasts stimulated with IL-1β, TNF-α, and IL-4. UV-B did not induce changes.

Conclusions: VKC conjunctiva displays distinctive quantitative patterns of Hsps as compared with healthy controls. Cultured conjunctival cells respond to cytokines and inflammatory stimuli with changes in the Hsps quantitative patterns. The data suggest that interaction between the chaperoning and the immune systems drives disease progression.

Vernal keratoconjunctivitis (VKC) is a severe, rare, chronic ocular allergy affecting mostly children and adolescents living in warm climates (1). Even though it tends to resolve at puberty, VKC affects the quality of life of patients and their parents. It is an IgE- and T cell-mediated allergic reaction with additional, ill-defined, nonspecific hypersensitivity

responses (2). Only 50% of VKC patients are positive to skin prick test and serum specific IgE. As in other atopic diseases, such as atopic dermatitis and asthma, the specific trigger remains elusive in most cases. A variety of nonspecific factors, including hot climate, sun light, dry wind, ultraviolet (UV)-B exposure, oxidative stress, and possible panallergens, exacerbate or perpetuate the ocular inflammation. In addition to typical Th2-derived cytokines, Th1-type cytokines, chemokines, growth factors, and enzymes are augmented in VKC patients (3, 4).

Conjunctival fibroblasts and epithelial cells activities and phenotypes *in vitro* can be modulated by diverse mediators involved in the ocular allergic reaction, such as histamine, IL-4, TGF-β1, and TNF-α (4–8), and by UV-B (9). Tissue remodeling reactions, stem cell deficiency, and various degrees of superficial corneal ulcers and scars are further consequences of chronic inflammation. Also, possible complications such as cataract and glaucoma due to prolonged topical treatment with corticosteroids can occur. Therefore, the risk of visual impairment in VKC is a real threat (10). Despite the fact that nowadays there is considerable evidence of participation of Hsp chaperones in inflammatory and autoimmune conditions and of interaction of the immune and chaperoning systems (11, 12), no studies have addressed these issues pertaining VKC.

Hsp chaperones designate a large group of molecules of which many are the products of heat shock genes (i.e., genes inducible by stressors), play various roles in protein homeostasis, and form part of the chaperoning system (11–14). Chaperones have been implicated in the pathogenesis of various diseases such as some types of cancer and inflammatory and autoimmune disorders (11, 12, 15, 16). Key questions still unanswered are: Do Hsps change quantitatively in VKC, suggesting their participation in pathogenesis and/or cytoprotection? Are Hsp levels affected by agents known to initiate or aggravate VKC, such as histamine and UV-B? The aims of this study were to determine the levels and distribution of Hsps in VKC-affected tissue and to assess the effect of cytokines and other factors known to be involved in VKC pathogenesis on the levels of Hsps in cultured conjunctiva cells.

Materials and methods

Conjunctiva biopsies

Conjunctival tissues from nine active VKC patients (7 males and 2 females; mean age 10.4 ± 3.6) and nine healthy age-matched normal subjects (controls) were studied. The control group included six males and three females (mean age 12.0 ± 5) who underwent eye surgery. None of these control subjects used contact lenses or had inflammatory signs and symptoms or history of allergy. Diagnosis of VKC was based on the typical clinical history and evaluation of signs and symptoms. All VKC patients were free of topical antiallergic drugs for at least 3 days and topical corticosteroids for at least 7 days before sample collection. Six VKC patients were positive to serum specific IgE for at least one pollen. In all VKC patients, a clinical activity score (0–4) was given considering the overall severity of the disease at the time of sample collection (1, 2).

Upper tarsal conjunctival biopsies were snap-frozen with or without optimal cutting temperature (OCT) compound (Histo-Line Laboratories, Pantigliate, MI, Italy) in liquid nitrogen and maintained at −80°C. This research was approved by the Institutional Review Board and adhered to the tenets of the Declaration of Helsinki. A written informed consent was obtained from all subjects or their parents before obtaining tissue specimens.

Immunohistochemistry (IHC)

Serial 5-μm-thick cryosections were fixed in 4% buffered formaldehyde and processed for IHC. Antibodies against the following human Hsps were used: Hsp10, Hsp27, Hsp40, Hsp60, Hsp70, Hsp90, Hsp105, and Hsp110. All antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), except anti-Hsp110 (BD Biosciences, San Jose, CA, USA). Primary antibodies were revealed as previously reported (4). Observation with a light microscope (Leica Axioplan, Wetzlar, Germany) was performed at a magnification of 400×. The positive reaction was evaluated in the epithelium and subepithelial stroma of conjunctival tissues and classified as very intense (3+), intense (2+), slight (1+), or absent (0). All the slides were evaluated by three investigators following a blind procedure.

Double immunofluorescence

Tissue sections were incubated with unmasking and with blocking solutions and subsequently incubated with the first primary antibody (mouse anti-human CD68 or mouse anti-human tryptase) for 1 h followed by the second primary antibody (anti-Hsp27, or anti-Hsp40, or anti-Hsp70, or anti-Hsp90) for another hour. The sections were then washed twice in phosphate-buffered saline (PBS) and incubated with goat-IgG antibody conjugated with fluorescein isothiocyanate and/or mouse-IgG antibody conjugated with Texas Red (Vector Laboratories, Burlingame, CA, USA) for 1 h at 23°C, and the nuclei were counterstained with Hoechst (Sigma, San Louis, MO, USA) for 15 min. The tissue was observed with a Zeiss fluorescence microscopy (Zeiss, Heidelberg, Germany) by two independent observers.

RNA isolation and quantitative PCR (qPCR) analysis

Total RNA was extracted from conjunctival tissues by TRIzol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA concentration of each sample was quantified using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Primers pairs (Table 1) were designed using the Primer3 Pick primers from a DNA sequence program and synthesized. Briefly, 1 μg of total RNA was retro-transcribed into cDNA using oligo$(dT)_{12\text{-}18}$, 25 ng primers and SuperScript II Reverse Transcriptase 200 U (Life Technologies). Quantitative

Table 1 Hsp chaperone genes investigated and primers used

Gene (accession number)	Name	Primer sequences
HSPB1 (NM_001540)	Heat shock 27-kDa protein 1	Fw: 5′-CAAGTTTCCTCCTCCCTGTCC-3′ Rv: 5′-AAGGTGACTGGGATGGTGA-3′
DNAJB1 (NM_006145.2)	DnaJ (Hsp40) homolog, subfamily B, member 1, transcript variant 1	Fw: 5′-GCCAATGGTACCTCTTTCAGCTAC-3′ Rv: 5′-CGCTGTAGATCTCTTCAAGGGA-3′
HSPA1A (NM_005345)	Heat shock 70-kDa protein 1A	Fw: 5′-AGGGAGGGTCTGGGTCA-3′ Rv: 5′-GGAAATGCAAAGTCTTGAAGCTCC-3′
HSPA1B (NM_005346.4)	Heat shock 70-kDa protein 1B	Fw: 5′-AGGGAGGGTCTGGGTCA-3′ Rv: 5′-ACAAAGAAGTGAAGCAGCAAAGAG-3′
HSP90AA1 (NM_001017963.2)	Heat shock protein 90-kDa alpha (cytosolic), class A member 1	Fw: 5′-GGTCTTCCCAGCATGTACTCAG-3′ Rv: 5′-ACAACGTGGACACTAAGAGAACA-3′
HSP90AB1 (NM_007355.2)	Heat shock protein 90-kDa alpha (cytosolic), class B member 1	Fw: 5′-ACCGAGCCCATTGACGA-3′ Rv: 5′-CCTTGCTCTCTTCCATCTTCTTCTTC-3′
PPIA (NM_021130.3)	Peptidylprolyl isomerase A (cyclophilin A)	Fw:-5′-GGGCTTTAGGCTGTAGGTCAA-3′ Rv: 5′-AACCAAAGCTAGGGAGAGGC-3′

Fw, forward; Rv, reverse; PPIA, peptidylprolyl isomerase A (housekeeping gene used as control).

RT-PCR analysis was carried with Rotor Gene RG-3000A Real Time PCR system (Corbett Research, Sydney, Australia) using SYBR Green I dye (Roche, Mannheim, Germany) and combined sense and antisense primers at 300 nM final concentration. The following cycling conditions were used: initial denaturation step of 10 min at 95°C, 45 cycles of amplification consisting of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. cDNA samples were analyzed in triplicate. Gene expression was evaluated with the ΔCt method using peptidylprolyl isomerase A (PPIA) as reference gene (17).

Cell cultures

Wong–Kilbourne-derived Chang conjunctival (ChWK) epithelial cells were obtained from ATCC (clone 1-5c-4) and were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (GIBCO, New York, USA) and antibiotics. Fibroblasts were obtained from upper tarsal conjunctival biopsies of two of the normal subjects and cultivated in DMEM supplemented with 10% FBS. Third- to fifth-passage fibroblasts were used for the experiments.

Epithelial cells and fibroblasts grown on 6-cm-diameter cell plates (Falcon, Durham, NC) were maintained in serum-free conditions supplemented with 10 ng/ml of histamine (Sigma) or 1 ng/ml of human recombinant IL-1β, or 10 ng/ml IL-4, or TNF-α (Peprotech, London, UK), then detached with trypsin, washed with PBS, and frozen at −80°C until lysis. Preliminary data showed that 1 or 10 ng/ml cytokines yielded the best results for the purposes of this study. UV-B irradiation was performed after 18 h of cell starvation, and then, PBS was replaced with complete culture medium supplemented with 10% FBS for 4, 10, or 24 h and harvested for cell lysis as described above. Irradiation was carried out with a central wavelength of 302 nm for 30 min, using a interferential filter, had a full width half maximum (FWHM) bandwidth of 11 nm (from 296 nm up to 307 nm), obtaining a final dose of 11 mJ/cm^2. Three culture wells were used for each treatment. After 4, 10, or 24 h of exposure, Hsp27, Hsp40, Hsp70, and Hsp90 levels were determined by Western blotting.

Western blotting

Conjunctival fibroblasts and epithelial cell pellets were treated for 45 min at 4°C with RIPA lysis buffer (1% v/v Triton X-100, 0.5% w/v deoxycholic acid, 10 mM EDTA in PBS, Sigma) supplemented with protease inhibitor cocktail. After removing particulate material by centrifugation, supernatants were collected and protein concentration was determined using the Bradford Assay. Ten μg of lysates was separated on 10% polyacrylamide gels and transferred to nitrocellulose membrane. After blocking nonspecific binding sites, the nitrocellulose membrane was incubated with anti-Hsp40 (Novus Biologicals, Cambridge, UK) or anti-Hsp60, anti-Hsp70, anti-Hsp27, anti-Hsp90 (Santa Cruz) diluted in TBS, 0.1% Tween-20 (TBST; Tris-buffered saline and Tween 20, Sigma) for 2 h at 23°C. After three washes in TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse-IgG (GE Healthcare, Buckinghamshire, UK) for Hsp40, or anti-goat-IgG HRP-conjugated (R&D Systems, Minneapolis, MN, USA) for the other Hsp. The antibody reaction was revealed by chemiluminescence using ECL Plus (GE Healthcare). Blots were sequentially incubated with monoclonal anti-β-actin antibody (Sigma) diluted 1:4,000 and processed as described above. Densitometric values of Hsp-positive bands were normalized with the corresponding value of β-actin.

Statistical analysis

Western blotting was performed in triplicate, and the data are presented as mean ± standard error (SEM). Comparison between groups was made using Student's unpaired test. The comparison of Hsp levels in VKC and normal samples was

analyzed using the nonparametric Mann–Whitney *U*-test. Spearman's correlation analysis was performed to determine the relationship between Hsp tissue expression and clinical severity of the disease. For statistical significance, the threshold *P* value was $P \leq 0.05$.

Results

Examination of biopsies from VKC patients

Tissue levels of the Hsp10, Hsp27, Hsp40, Hsp60, Hsp70, Hsp90, Hsp105, and Hsp110 were evaluated by immunohistochemistry (IHC; Table 2). In normal and pathological tissues, all proteins were present in the stroma, while Hsp27 and Hsp90 were detected also in the epithelium. VKC specimens showed increased levels of Hsp27, Hsp40, Hsp70, and Hsp90 in the stroma, compared to controls (Fig. 1). By contrast, in VKC epithelium, only Hsp27 levels were significantly higher than in controls; the levels of the others did not change significantly. The clinical activity score of the disease at the time of sample collection was significantly correlated with stromal expression of Hsp27 ($r = 0.66$; $P = 0.034$), Hsp40 ($r = 0.76$; $P = 0.01$), Hsp70 ($r = 0.73$; $P = 0.015$), and Hsp90 ($r = 0.88$; $P = 0.0006$).

Double immunofluorescence revealed colocalization of CD68-positive cells and of some Hsps only in the stroma of VKC tissues: about 20% of CD68-positive cells were also positive to Hsp27, 50% to Hsp40, 30% to Hsp70, and 70% to Hsp90 (Fig. 2A). Tryptase-positive cells colocalized also with Hsp40 (8% of cells), Hsp70 (10%), and Hsp90 (15%), but not with Hsp27 (Fig. 2B).

The qPCR analyses, performed only for those Hsps with increased levels as determined by IHC, did not show changes in their mRNA levels. This may be due to the fact that qPCR is performed on biopsy fragments that include the two mucosa layers (epithelium and stroma), while IHC can detect the Hsp molecules *in situ*, in both layers separately providing information on tissue localization and a measure of Hsp levels distinctive of each layer.

Table 2 Hsp levels in VKC and controls determined by immunohistochemistry

	Conjunctival stroma		Conjunctival epithelium	
Hsp chaperone	VKC	Control	VKC	Control
Hsp10	+*	+/–	–	–
Hsp27	++	+/–	+++	+
Hsp40	+++	+/–	–	–
Hsp60	+/–	+/–	–	–
Hsp70	++	+/–	–	–
Hsp90	++	+	++	+
Hsp105	+/–	+/–	–	–
Hsp110	+/–	+/–	–	–

*See text (Materials and Methods, Immunohistochemistry) for explanation of symbols (reaction intensity).

Effect of immune system products and inducers on conjunctival Hsps

Only levels of Hsp27, Hsp40, Hsp70, and Hsp90 that showed changes *in vivo* were analyzed in conjunctival cell cultures. Hsp27 levels significantly increased only in fibroblasts after IL-4 or TNF-α stimulation (Fig. 3A). Hsp40 levels significantly increased in fibroblasts after IL-1β, or IL-4, or TNF-α and in epithelial cells after IL-4 stimulation (Fig. 3B). Hsp70 levels significantly increased only in epithelial cells after histamine, or IL-4 stimulation (Fig. 3C). Hsp90 levels significantly increased in fibroblasts after TNF-α, or IL-1β, or IL-4 stimulation (Fig. 3D). UV-B irradiation did not increase the levels of any Hsp in neither conjunctival cell type tested.

Discussion

In the last two decades, it has been established that chaperones, the main components of the physiological chaperoning system, interact with the immune system at various levels and are involved in inflammation and autoimmunity in ways not yet completely elucidated. Therefore, it is pertinent to investigate the levels and distribution of Hsps in tissues affected by inflammatory and allergic-autoimmune diseases with no satisfactory treatments, such as VKC, to explore new roads that might lead to the development of novel therapies using chaperones for cytoprotection and as anti-inflammatory agents. It would be worthwhile to develop chaperonotherapies, positive, that is, promote chaperone function, or negative, that is, block chaperone effects (12, 18). Before these studies can be undertaken in VKC, it is necessary to unveil the quantitative patterns of Hsps in affected tissues and characterize the effects of immune system products on the Hsps in the conjunctiva.

Understanding VKC pathophysiology and treatment continues to be a challenging task for ophthalmologists, allergists, and pediatricians because the etiology is unclear and antiallergic therapies are often unsuccessful. Severe signs and symptoms in VKC are triggered most frequently by nonspecific stimuli such as exposure to sun light and high temperatures and partially explained by increased or reduced levels of proinflammatory (19) and of anti-inflammatory factors (20), respectively. However, the initial molecular events that induce the inflammation in VKC and the complexity of factors that orchestrate and maintain inflammation remain unclear.

It has been suggested that smaller Hsps (<56 kDa) are anti-inflammatory and larger ones (>65 kDa) are proinflammatory, while Hsp60 (range 56–65 kDa) can be both (21). In the present study, we found significantly increased levels of Hsp27, Hsp40, Hsp70, and Hsp90 in VKC conjunctiva compared to controls, mostly in conjunctival stroma and colocalized with CD68 and with tryptase. Both macrophages and mast cells are abundant in VKC and correlated with the inflammatory status (22). Interestingly, Hsps found significantly increased in VKC tissues were correlated with the clinical severity score of the disease. These results suggest that Hsps participate in the pathological process as cytoprotective

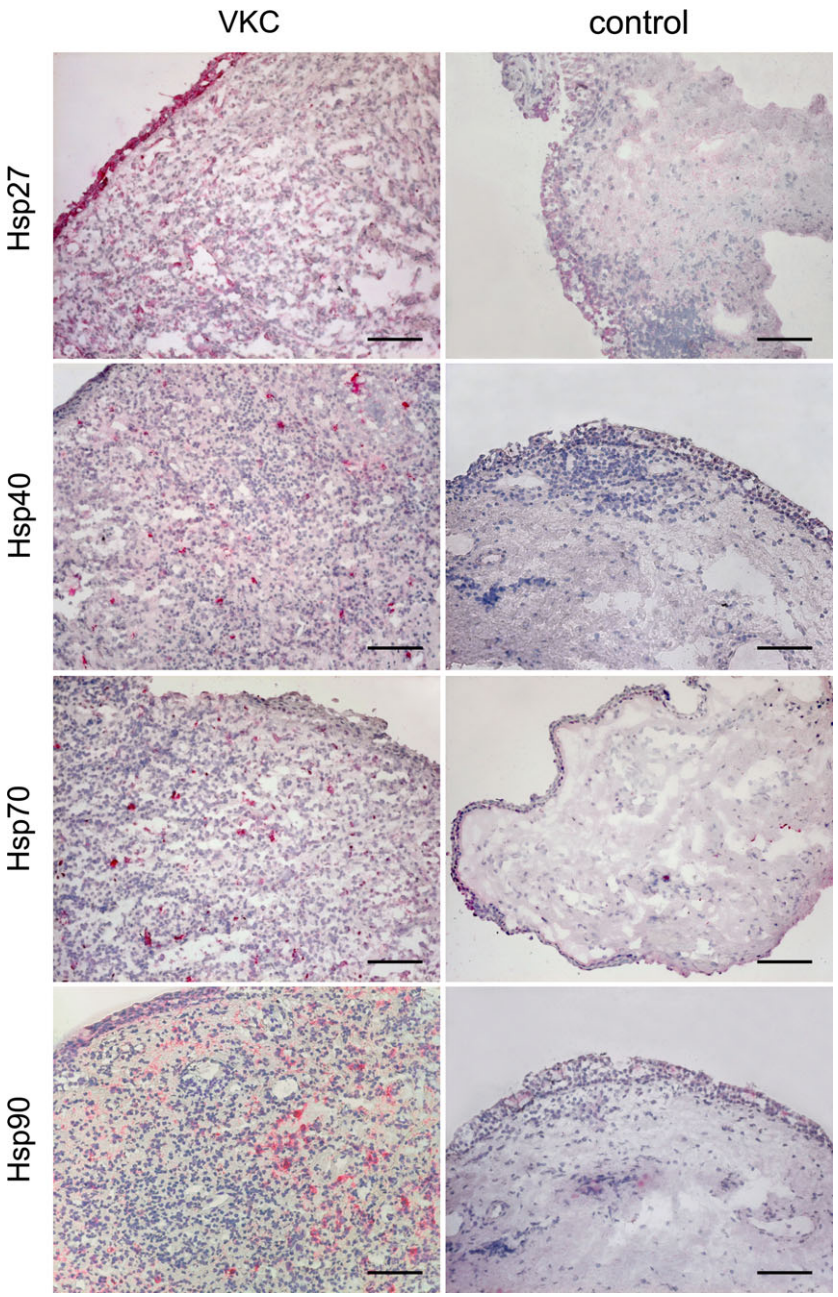

Figure 1 Hsp immunostaining (in red) in biopsies from VKC conjunctiva compared to those from healthy controls. Representative images show increased Hsp27, Hsp40, Hsp70, and Hsp90 levels in the conjunctiva from VKC patients. Negative controls were prepared by omitting the primary antibody. Bar: 200 μm.

or pathogenic, as proposed for other pathologies (23–27). This may also suggest that a modulation/inhibition of the overexpressed proinflammatory Hsp70 and Hsp90 may play a role in reducing the inflammatory burden in VKC. We did not study the quantitative patterns of Th2 or Th1 cytokines that might be associated with increased levels of Hsps; however, a prevalent Th2 pattern of cytokine production, associated with a Th1 pattern in more severe inflammatory states, has been reported in VKC (7).

To determine whether immune system products and stimuli induce Hsps in the conjunctiva and contribute to its inflammation, we exposed conjunctiva epithelial cells and fibroblasts to a panel of agents, including UV-B radiation, that have been implicated in VKC (3). Histamine, IL-1, IL-4, and TNF-α increased Hsp levels, while UV-B exposure did not under our experimental conditions. Noteworthy, the responses were distinctive of cell and Hsp type (Table 3). This suggests that the overall response of the two conjuncti-

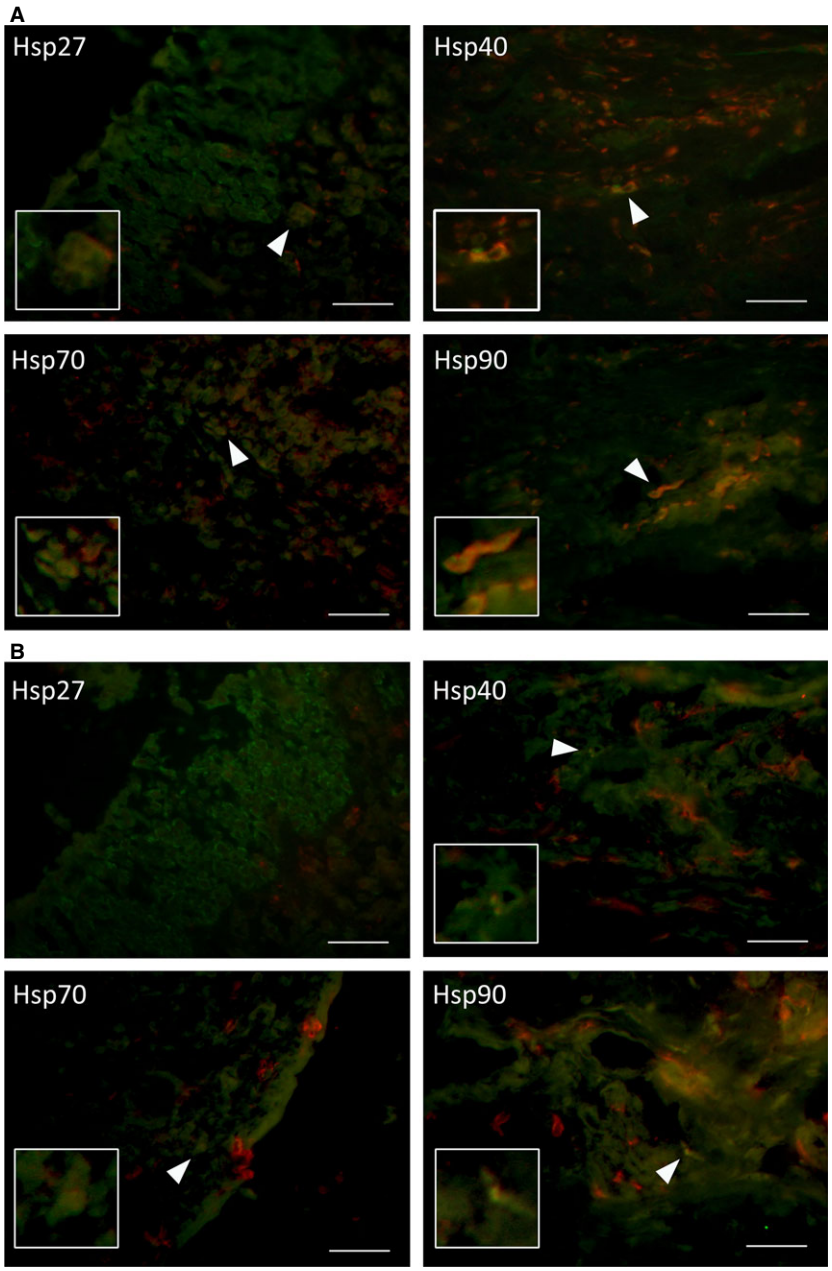

Figure 2 (A) Double immunofluorescence revealing colocalization of CD68 (red) with Hsps (green), as indicated, in the conjunctiva from VKC patients. (B) Double immunofluorescence revealing colocalization of tryptase (red) with various Hsp (green), as indicated, in the conjunctiva from VKC patients. Bar: 100 μm. In the boxes, high magnification of double-staining cells (arrowhead).

val layers to stimuli is delicately coordinated and orchestrated to produce well-defined sets of Hsps, whose imbalances may drive the course of the disease toward progression–aggravation or toward stabilization–remission. We also observed a prevalent induction of lower MW Hsps (Hsp27 and Hsp40) by IL-4. In contrast, a prevalent induction of higher MW Hsps (Hsp70 and Hsp90) was observed after proinflammatory challenges.

In conclusion, our study supports the hypothesis that Hsp27, Hsp40, Hsp70, and Hsp90 are actively involved in VKC, while other Hsps such as Hsp10, Hsp60, Hsp105, and Hsp110 are not, or if they are, they would do so in ways not detectable with the methods used in this study. Our data indicate active interaction between the chaperoning and the immune systems, with perhaps a mutual modulatory role between the two systems. A delicate balance in this modula-

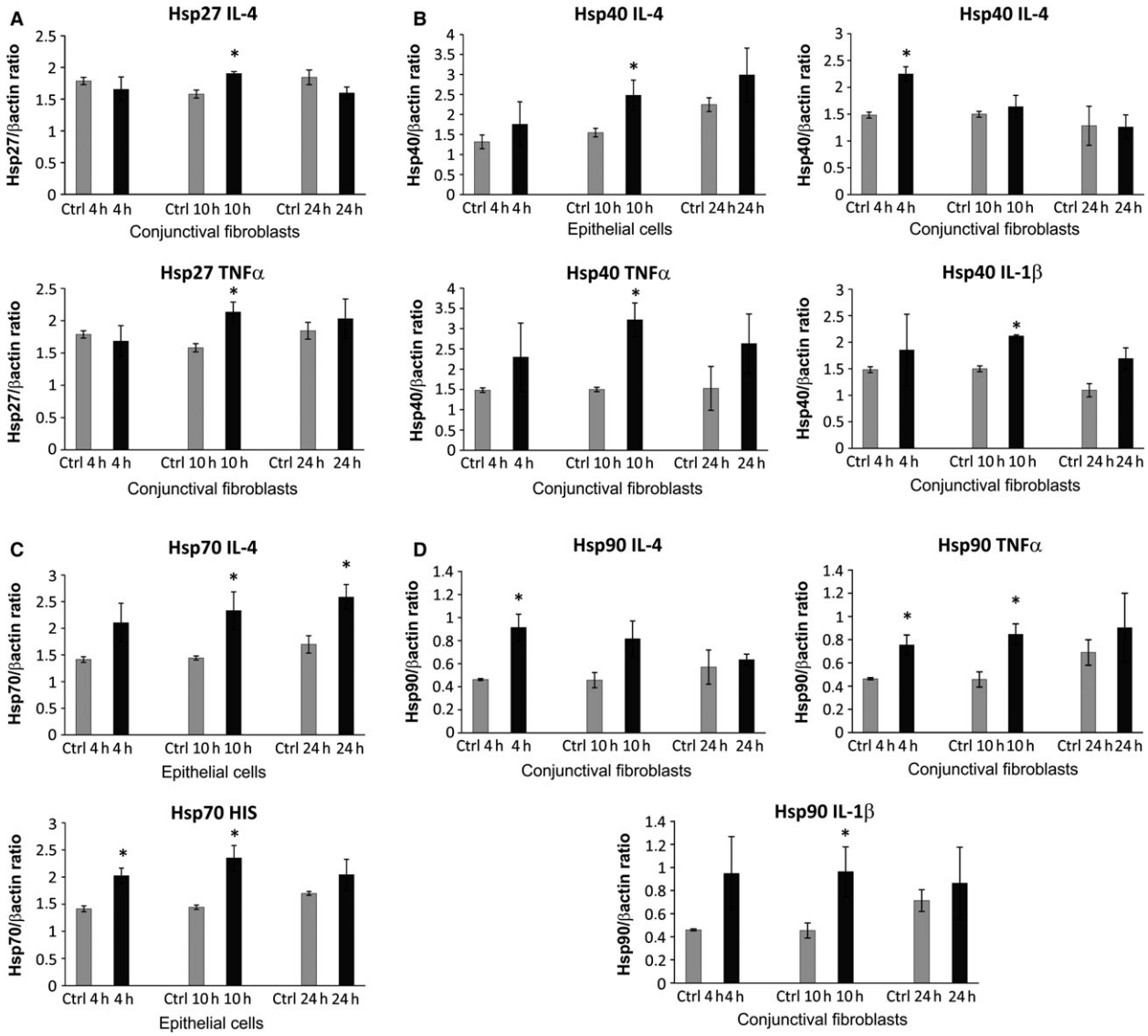

Figure 3 Increased levels of Hsp27 (panel A), Hsp40 (panel B), Hsp70 (panel C), Hsp90 (panel D) in cultured conjunctival epithelial cells or fibroblasts, as indicated, treated with either histamine (His), or IL-4, or IL-1β, or TNF-α, as indicated. Proteins were extracted at 4, 10, or 24 h post-treatment, analyzed by Western blotting and compared to the corresponding untreated controls (Ctrl).

tion can be envisioned that if altered in one direction can lead to rapid disease progression, or if unbalanced in the opposite direction can bring about disease stabilization or even remission. Gene expression and possible gene modification of the overexpressed Hsps will be further studied in a larger cohort of VKC patients. The understanding of the chaperones' roles in VKC conjunctiva will open new therapeutic scenarios leading, for example, to the use of specific topical inducers or inhibitors of Hsps (depending on whether the targeted Hsp is cytoprotective or etiopathogenic), as an effective chaperonotherapy (18), in turn preventing the severe complications of this eye disorder.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Table 3 The response to stimulation of cultured conjunctiva cells with cytokines and immune system inducers is distinctive of cell and Hsp type*

Cell types	Stimulus	Hsp
Fibroblast	IL-4 (Th2)	Hsp27, Hsp40, Hsp90
	TNF-α (inflammatory)	Hsp27, Hsp40, Hsp90
	IL-1β (inflammatory)	Hsp40, Hsp90
	Histamine	None
	UV-B	None
Epithelial	IL-4 (Th2)	Hsp40, Hsp70
	TNF-α (inflammatory)	None
	IL-1β (inflammatory)	None
	Histamine	Hsp70
	UV-B	None

*See Fig. 3a–d.

Author contribution

AL, PB, FC, and ADS involved in conception and design. AL, PB, FC, ADS, ET, SC, AJC, and BZ involved in analysis and interpretation. AL, PB, FC, AJLM, and ECdeM involved in writing the article. ECdeM, AJLM, PV, BZ, and MA critically revised the article. AL, DG, ECM, AJLM, and MA involved in final approval of the article. MB, AP, EAM, AM, and EMSM involved in data collection. AL, PB, ASD, BZ, and PV involved in provision of materials, patients, or resources. ET and SC involved in statistical expertise. None received funding. ET, EC, and FC involved in literature search. AL, PB, and AJLM involved in administrative, technical, or logistic support.

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