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ORIGINAL ARTICLE

Transforming growth factor-β/Smad – signalling pathway and conjunctival remodelling in vernal keratoconjunctivitis

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Summary

Background Vernal keratoconjunctivitis (VKC) is a chronic ocular allergic inflammation characterized by corneal complications and the formation of giant papillae. Sma- and Mad-related proteins (Smad) modulate extracellular matrix gene expression during wound healing, inflammation and tissue remodelling.

Objective To investigate the relationship between allergic inflammation and TGF-β/Smad signalling pathway, expression in VKC patients and in primary cultured conjunctival fibroblasts exposed to mediators found previously over-expressed in VKC.

Methods Smad-2, -3, -7, phospho-(p)Smads, TGF-β1 and -β2 were evaluated in the conjunctiva of normal subjects (CT) and VKC patients by immunohistochemistry. The expression of Smads, pro-collagen I (PIP), TGF-β1, -β2, mitogen-activated protein kinase (p38/MAPK), c-Jun *N*-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK1/2) were also determined in conjunctival fibroblast cultures exposed to histamine, IL-4, -13, TGF-β1, IFN-γ and TNF-α using immunostaining or RT-PCR.

Results Immunostaining for Smad-2, -3, pSmad-2, -3, TGF-β1, -β2 and PIP was significantly increased in VKC stroma compared with CT. In conjunctival fibroblast cultures, Smad-3 and PIP were stimulated by histamine, IL-4, -13 and TGF-β1 exposure, while PIP was reduced by IFN-γ, and TNF-α mRNA expression of Smad-3 was increased by histamine, while Smad-7 was reduced by IL-4. In addition, histamine, IL-4 and TNF-α increased JNK and ERK1/2 expression.

Conclusion and Clinical Relevance The TGF-β/Smad signalling pathway is over-expressed in VKC tissues and modulated in conjunctival fibroblasts by histamine, IL-4, TGF-β1 and TNF-α. These mechanisms may be involved in fibrillar collagen production, giant papillae formation and tissue remodelling typical of VKC and might provide new therapeutic targets for its treatment.

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Introduction

Vernal keratoconjunctivitis (VKC) is a chronic allergic disease characterized by severe inflammation, corneal complications and tissue remodelling that leads to the formation of giant papillae. The chronic inflammatory response in VKC is characterized by the presence of increased numbers of Th2 lymphocytes, eosinophils and activated mast cells [1]. The disease is accompanied by a gamut of alterations involving structural cells and tissues such as conjunctival thickening, subepithelial fibrosis, mucous metaplasia, neovascularization and scarring [1]. Many elements contribute to this dramatic response, including epithelial changes, connective tissue deposition, inflammatory cell infiltration and glandular hypertrophy. Although it is well recognized that ocular surface inflammation is a prominent feature of VKC, the relationship between individual components and the progression of inflammation to remodelling of the conjunctiva in ocular allergic diseases is not well understood. Tissue fibrosis in various pathologies is mediated by growth factors through the signal of transcriptional factors. In VKC, several

growth factors, cytokines and enzymes have been found increased and over-expressed in tears and tissues, suggesting that remodelling processes and fibrosis are regulated by the interactions of different pathways [2]. An increased expression of several growth factors, including TGF-β1, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), has been shown previously in VKC conjunctival tissues and tears [3, 4].

Histamine, one of the main mediators of ocular allergy, has a stimulating effect on conjunctival fibroblasts and epithelial cells, inciting the production and expression of pro-inflammatory cytokines, IL-1, -6, -8, adhesion molecules and pro-collagens, highlighting once again the ever-broadening role of histamine in the pathogenesis of allergic conjunctival remodelling [5–8].

TGF-β is involved in both the regulation of allergic inflammation and tissue remodelling. Intracellular signalling by TGF-β members is mediated through binding to a heteromeric receptor complex [9, 10], leading to subsequent phosphorylation of its intracellular downstream effectors, the members of the Sma- and Mad-related protein (Smad) family of signal transducers [10, 11]. Eight Smad family members have been identified that, based on structural and functional differences, have been subdivided into three groups [10]: (1) receptor-associated Smads (R-Smads) are direct substrates of TGF-β family receptor kinases (Smad-1, -2, -3, -5, -8); (2) co-Smads that associate during signalling with these receptor-regulated Smads (Smad-4); and (3) inhibitory Smads (Smad-6 and -7) that antagonize the signalling function of the former groups. Once phosphorylated, Smads-2/3 associate as heterodimeric complexes with Smad-4 and move into the nucleus where they bind DNA and initiate target gene transactivation, either alone or in association with other transcriptional partners [10, 11]. In addition, TGF-β signals through multiple, Smad-independent, intracellular pathways [12] may activate members of the stress-activated mitogen-activated protein kinase (MAPK) cascade in various cell types [12–14]. The relevance of these signalling pathways in ocular surface diseases associated with tissue remodelling is still not fully understood.

The mechanism through which these activated pathways are terminated is also a highly regulated process. Protein phosphatase 2C-α (PPM1A/PP2Cα) is a member of the PP2C family of serine/threonine protein phosphatases. PP2C family members are known to be negative regulators of cell stress response pathways [15]. Through dephosphorylation of Smad-2/3 and MAP kinases, the PP2Cα has been shown to play a critical role in terminating TGF-β signalling [16].

To better understand the mechanisms that induce conjunctival changes in VKC, we first evaluated the presence of Smads and phosphorylated (p) Smads, TGF-β, PP2Cα and collagen in conjunctival tissues of patients with VKC compared with normal subjects and then their presence in conjunctival fibroblast cultures stimulated by numerous factors that have been found previously increased or involved in the pathogenesis of VKC. In addition, the mRNA expression of Smads, TGF-β1, -β2 and MAPK was evaluated in fibroblast cultures exposed to histamine, IL-4 and TNF-α.

Materials and methods

Subjects and tissue specimens

Nine tarsal VKC patients (six males and three females; mean age 11.2±4.9 years), in an active disease phase, free of topical mast cell stabilizers and/or antihistamines for at least 3 days and free of topical corticosteroids for at least 7 days, were included in the study. Diagnosis of VKC was based on the typical clinical history and evaluation of signs and symptoms [1]. All VKC patients presented giant papillae on the upper tarsal conjunctiva. Of the VKC patients, six were positive to serum-specific IgE for at least one aeroallergen, such as blue grass, mites, *parietariae*, *compositae* and tree pollens.

The control group included six subjects (five males, one female, mean age 13±5 years) who underwent surgery for strabismus. None of these subjects used contact lenses or had any inflammatory signs and symptoms or history of allergy. Upper tarsal conjunctival biopsies were performed under local (VKC patients) or general (control subjects) anaesthesia. All tissue specimens were snap frozen with OCT in liquid nitrogen and maintained at −70 °C for immunohistochemistry. This research was approved by the Institutional Review Board. A written informed consent was obtained from all subjects or their parents before obtaining tissue specimens.

Immunohistochemistry (IHC)

Serial 5-μm-thick cryosections were cut, mounted on gelatin-covered slides, fixed in acetone and processed for IHC. The following anti-human antibodies were used: Smad-2 and -3 (Zymed Laboratories, San Francisco, CA, USA), Smad-7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pSmad-2 (Upstate, Waltham, MA, USA), pSmad-3 (Abcam, Cambridge, MA, USA), protein phosphatase 2C isoform alpha (PPM1A/PP2Cα) (Santa Cruz Biotechnology), SMA (Dako, Glostrup, Denmark) and anti-TGF-β1 and anti-TGF-β2 (Santa Cruz Biotechnology), pERK1/2 and pJNK1 (Santa Cruz Biotechnology). Anti-collagen type I (Sigma-Aldrich, St Louis, MO, USA) was used for the identification of collagen distribution in tissues.

Briefly, samples were incubated, subsequently treated with avidin- and biotin-blocking solutions (Vector, Burlingame, CA, USA), serum from the same animal species as the secondary antibody (Vector) and monoclonal or

polyclonal antibodies at the appropriate dilution in Tris-buffered saline (TBS: 10 mM Tris, 150 mM NaCl, pH 7.4). Slides were then washed in TBS, incubated with biotinylated secondary antibodies (Vector), treated with the alkaline phosphatase ABC kit (Vectastain, Burlingame, CA, USA) and fast red (Sigma).

Light microscopic analysis was performed at a magnification of ×400. The positive red reaction was evaluated qualitatively in the epithelium and sub-epithelial stroma of conjunctival tissues. Owing to their location (just beneath the epithelial basement membrane and around the vessels) and shape (elongated fibroblasts or rounded inflammatory cells), cells were considered to be either structural (i.e., fibroblasts and endothelial cells) or inflammatory. The reaction was classified as very intense (3+), intense (2+), slight (1+) or absent (0). In all samples, cells positive for the molecules involved in collagen synthesis, pSmad-2, -3, TGF-β1, -β2, pERK1/2 and pJNK1 and the pSmad-2/3 inhibitor, PP2Cα, were evaluated quantitatively in five consecutive non-overlapping high-power fields. The final result, expressed as the number of positive cells per square millimetre, was calculated as the mean of all cell counts performed in each tissue specimen.

IHC on conjunctival fibroblasts

Fibroblast cultures were obtained from upper tarsal conjunctival biopsies of three normal, non-atopic subjects who underwent eyelid surgery. Anaesthesia was induced by topical 2% lidocaine. Briefly, biopsies were washed, cut in small pieces, seeded in culture wells (NUNC, Roskilde, Denmark) containing 100 mL of DMEM medium (Sigma) supplemented with 10% fetal calf serum (Sigma), L-glutamine (2 mM) and antibiotics (penicillin 100 U/mL, streptomycin 100 mg/mL; Sigma). Samples were incubated at 37 °C in 5% CO_2 in a humidified air atmosphere. Fibroblasts were subcultured with 0.05% trypsin and replated into 24-well plates (>95% vitality). Cells were characterized morphologically and stained positively with vimentin and negatively with cytokeratines. Third- to fifth-passage fibroblasts were used for experiments. To evaluate the effects of mediators and cytokines on pro-collagen I (PIP) (anti-PIP, Santa Cruz Biotechnology), Smads and TGF-β1 expression, 5000 cells/well were seeded in 200 mL of culture medium on multi-well chamber slides (NUNC). After 24 h, the medium in each well was replaced with 200 mL of serum-free medium. After 24 h, the medium was replaced with fresh medium supplemented with 0, 1, 10 and 20 ng/mL of histamine, tryptase, elastase, eotaxin (all from Sigma), metalloprotease (MMP)-9 (Chemicon, Temecula, CA, USA) and human recombinant IL-4, -13, IFN-γ, TGF-β1 and TNF-α (Peprotech, London, UK). After 12, 24 and 48 h exposure time, the wells were washed using PBS and fixed with 4% formaldehyde for 20 min at room temperature. Preliminary data showed that the 24-h exposure time and 10 ng/mL concentration were the ideal experimental conditions for the purposes of this study; therefore, these conditions were considered for all subsequent experiments. Three culture wells were used for each treatment and experiments repeated twice. After washing and drying, slides were processed for indirect IHC for the expression of Smad-2, -3, -7, TGF-β1 and PIP using the same technique described above for the conjunctival tissues.

RNA isolation and real-time PCR

Conjunctival fibroblasts were exposed to 10 ng/mL of histamine, IL-4 or TNF-α for 24 h and total RNA was extracted from cultured cells using the mono-phasic solution of phenol and guanidine isothiocyanate method (TRIzol reagent, Life Technologies, GIBCO BRL, Carlsbad, CA, USA). Briefly, the cells were lysed by the addition of 1.0 mL of TRIzol reagent and total RNA was subsequently isolated according to the manufacturer's instructions. Concentrations of RNA were determined by measuring the absorbance at 260 nm in samples stored at −80 °C. For each target gene, primers and probe were selected using Primer3 software (Roche Molecular Diagnostics, Pleasanton, CA, USA): Smad-2: caggcctttacagcttctctg/gtggcaatccttttcgatg; Smad-3: gtctgcaagatcccaccag/agccctggttgaccgact; Smad-7: cgatggattttctcaaaccaa/attcgttccccctgtttca; TGF-β1:cag ccggttgctgaggta/agcagcacgtggagctgt; TGF-β2: ccaaagggt acaatgccaac/cagatgcctctggatttatggtatt; P38: atgccgaagatg aactttgc/tcttatctgagtccaatacaagcatc; JNK: gggcagccctctcc ttta/cattgacagacgacgatgatg; ERK1/2: caaagaactaatttttgaag agactgc/tcctctgagcccttgtcct.

Gene expression was measured using real-time quantitative PCR on a Rotor-gene TM5500 (Corbett Research, Sydney, Australia). PCR reactions were carried out using the primers at 300 nm and the SYBR Green (Invitrogen, Carlsbad, CA, USA) using 2 mm $MgCl_2$, with 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All cDNA samples were analysed in duplicate. Fluorescence thresholds (C_t) were determined automatically by software, with efficiencies of amplification for the studied genes ranging between 92 and 110%. For each cDNA sample, the C_t value of the reference gene β-actin was subtracted from the C_t value of the target sequence to obtain the DC_t. Experiments were repeated at least three times.

Statistical analysis

Differences in histological staining scores between VKC and normal samples were analysed using the non-parametric Mann–Whitney *U*-test, as is recommended for scored data. The non-parametric Kruskal–Wallis one-way ANOVA test by rank was used to analyse the multiple cytokine/mediator effects on cell cultures and thus the data are expressed as mean ranks. For statistical

significance, the assigned *P*-value was $\leqslant 0.05$. Analyses were performed using SAS® 9.1.3 software for personal computers and the GraphPad Prism version 4.00 for Mac (GraphPad Software, San Diego, CA, USA).

Results

IHC expression of PIP, Smads, pSmads and TGF-β in conjunctival tissues

Pro-collagen I staining was high in all VKC samples and faint in normal conjunctiva. The immunostaining scores of Smad-2, -3, TGF-β1 and -β2 in the stroma of VKC tissues were significantly greater than normal controls, while in VKC conjunctival epithelium, only TGF-β1 was significantly greater (Table 1) (Fig. 1).

Table 1. Mean immunostaining scores for Smads, pSmads, TGF-β1, –β2 and transcription factors in the conjunctival epithelium and stroma of VKC patients and normal subjects (CT)

		VKC	CT	*P*
PIP	Stroma	3.0±0	1.2±0.2	<0.01
Smad-2	Epithelium	0.6±0.4	0.5±0.7	NS
	Stroma	1.9±0.6	0.5±0.4	<0.005
pSmad-2	Epithelium	0.1±0.1	0.2±0.1	NS
	Stroma	0.8±0.2	0.2±0.1	NS
Smad-3	Epithelium	0.5±0.5	0.8±0.9	NS
	Stroma	1.8±0.8	1.0±0.4	<0.05
pSmad-3	Epithelium	0.2±0.1	0.3±0.1	NS
	Stroma	0.7±0.1	0.6±0.2	NS
Smad-7	Epithelium	0.4±0.3	0.6±0.5	NS
	Stroma	1.5±0.8	0.9±0.7	NS
TGF-β1	Epithelium	3.0±0	1.4±1.0	<0.05
	Stroma	2.9±0.2	1.1±0.8	<0.005
TGF-β2	Epithelium	1.5±0.2	1.8±0.4	NS
	Stroma	1.6±0.1	0.8±0.2	<0.05
PPC2α	Epithelium	0.2±0.1	0.2±0.1	NS
	Stroma	0.8±0.2	0.3±0.1	NS
pERK1/2	Epithelium	0.9±0.2	0.3±0.2	NS
	Stroma	0.8±0.2	0.6±0.1	NS
pJNK	Epithelium	1.1±0.2	0.4±0.1	NS
	Stroma	1.1±0.2	0.4±0.1	NS

NS, non-significant; VKC, vernal keratoconjunctivitis.

Immunostaining for PPC2α, pSmad-2, -3, pERK1/2 and pJNK1 in pathological tissues was less evident than the Smads. The pSmad-3 was particularly localized in the fibrous, subepithelial region of VKC tissues and in endothelial cells (Fig. 1). The molecules mostly involved in collagen synthesis were also quantified by counting the number of positive cells per square millimetre in conjunctival subepithelial stroma. A significantly higher cellular expression in VKC tissues compared with controls was found for pSmad-2 and -3, pERK1/2, pJNK1, TGF-β1, -β2 and PPC2α (Table 2). Cells positive for these factors were considered to be both structural and inflammatory cells.

IHC expression of Smad-2, -3, -7, TGF-β and PIP in conjunctival fibroblast cultures

In conjunctival fibroblast cultures, Smad-2 and -7 were not modified compared with non-stimulated cells (data not shown), while Smad-3 cell staining was significantly increased by histamine, IL-4, -13 and TGF-β1 exposure for 24 h ($P<0.05$) (Figs 2 and 3). The intranuclear staining of Smad-3 in fibroblasts showed a nuclear translocation of this protein (Fig. 3). TGF-β1 expression was increased by histamine, IL-4, -13 and eotaxin ($P<0.05$). PIP expression was stimulated by histamine, IL-4, -13 and TGF-β1 ($P<0.05$), and reduced by IFN-γ and TNF-α ($P<0.05$) (Figs 2 and 3).

Smads and TGF-β mRNA expression in conjunctival fibroblast cultures

In conjunctival fibroblast cultures, Smad-3 mRNA expression was stimulated by histamine, IL-4 and TNF-α, while Smad-7 mRNA was stimulated by histamine and TNF-α but reduced by IL-4. Similarly, TGF-β1 mRNA expression was stimulated by IL-4, while TGF-β2 mRNA expression was stimulated by both histamine and TNF-α (Fig. 4).

p38/MAPK, JNK and ERK1/2 expression in conjunctival fibroblast cultures

Under our experimental conditions, p38/MAPK mRNA expression was not detectable in conjunctival fibroblast

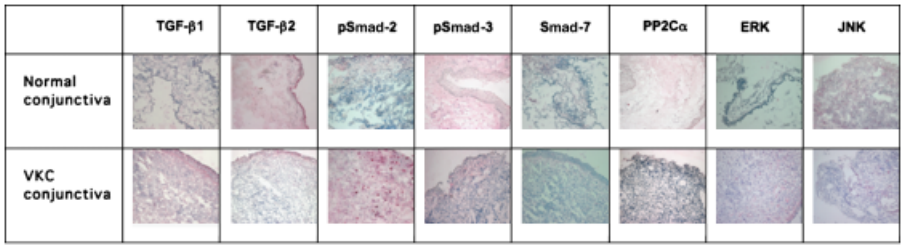

Fig. 1. Immunohistochemical images from VKC and normal conjunctiva. TGF-β1 and -2 were expressed to a greater degree in pathological tissues. pSmad-2, -3, ERK and JNK cell expression was also greater in VKC tissues. The inhibitory factor, Smad-7, was expressed in VKC to a slight degree, while PPC2α cellular expression was greater in VKC than normal tissues (original magnification ×200).

Table 2. Expression of PPC2α, pSmad and TGF-β, ERC and JNK in conjunctival cells of the subepithelial stroma (mean number of cells/mm^2) in VKC patients and normal tissues (CT)

	PPC2α	pSmad-2	pSmad-3	TGF-β1	TGF-β2	pERK1/2	pJNK
VKC	23.3±5.7	36.1±0.2	29.6±4.3	186.5±19.5	137.1±13.5	30.1±9.5	49.5±10
CT	4.0±2.4	0.98±0.6	5.6±2.8	44.6±11.8	22.8±12.3	7.3±1.6	12.1±3.3
P	<0.05	<0.005	<0.001	<0.001	<0.001	<0.01	<0.05

TGF, transforming growth factor; JNK, c-Jun *N*-terminal kinase; VKC, vernal keratoconjunctivitis.

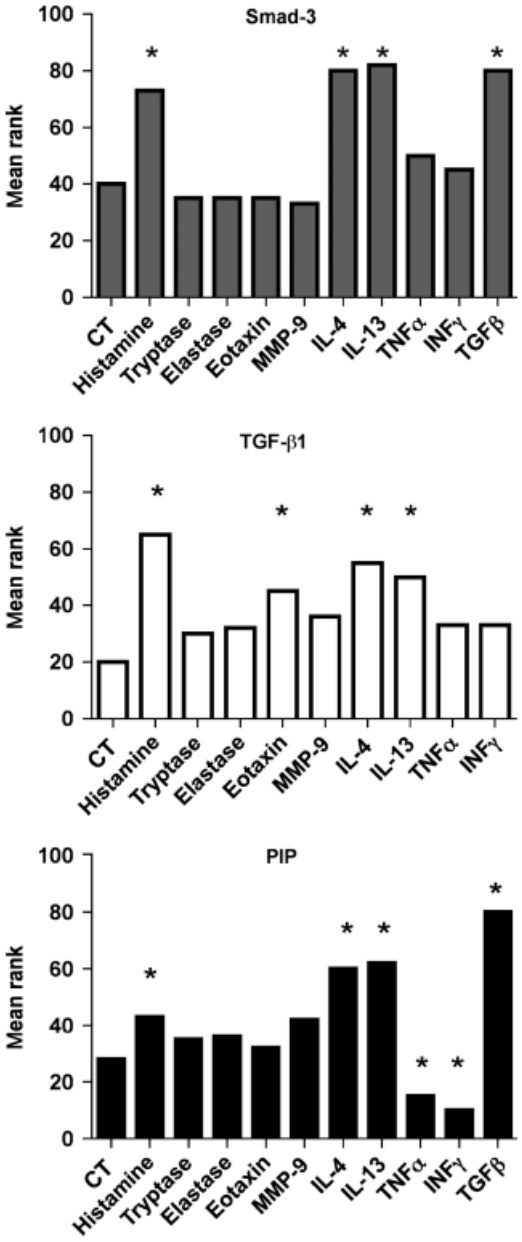

Fig. 2. Fibroblast immunostaining after stimulation for 24 h with different mediators involved in the allergic response. Smad-3 cytological expression was significantly stimulated by exposure to 10 ng/mL histamine, IL-14, -13 or TGF-β1. TGF-β1 expression was stimulated by histamine, IL-4, -13 and eotaxin. Pro-collagen I (PIP) was significantly increased by histamine, IL-4, -13 and TGF-β1, and reduced by IFN-γ and TNF-α (*$P<0.05$). Data were analysed by the Kruskal–Wallis test and expressed as mean rank.

cultures either at baseline or after cytokine exposure (data not shown). However, histamine, IL-4 and TNF-α increased JNK and ERK1/2 mRNA expression by two or three-fold (Fig. 4).

Discussion

TGF-β is a crucial regulator of extracellular matrix (ECM) deposition, as it controls both the expression of components of the ECM network, such as the fibrillar collagens and the expression of protease inhibitors [9, 14]. In VKC giant papillae, besides an increased deposition of ECM components, an increased expression of MMP-1, -2, -3, -8, -9 and -10 [17, 18] has been shown. Conversely, both tissue inhibitor of MMP (TIMP)-1 and -2 showed a stronger signal in VKC, but were also highly expressed in all control samples [18].

In the present study, both TGF-β1 and -2 were highly expressed in VKC conjunctiva in association with Smads. The number of cells positive for TGF-β, pSmad-2 and -3, pERK and pJNK was significantly higher in VKC compared with normal tissues, suggesting that the TGF-β/Smad/MAPK signal pathways are activated in this disease, working either together or simultaneously. Conjunctival fibroblasts showed an increased activity not only when stimulated by TGF-β but also after exposure of other mediators involved in conjunctival allergic inflammation. In fact, histamine, the main mediator of the allergic response, is particularly elevated in VKC because of a defect in histaminase enzyme activity and an increased release by mast cells [19], as well as IL-4, which is known to be fibrogenic and involved in the pathogenesis of VKC [3, 20], increased PIP production, TGF-β and Smad-3 mRNA expression. In contrast, the Th1 type cytokine, IFN-γ, decreased PIP production as shown by its reduced fibroblast immunostaining. The pro-inflammatory cytokine, TNF-α, stimulated both Smad-3 and the inhibitory Smad-7, partially increasing TGF-β expression but ultimately reducing PIP production, as shown previously in a similar *in vitro* model [20].

Conjunctival fibroblasts as well as those from other tissues express functionally active histamine receptors [21, 22]. Histamine was shown to enhance proliferation, migration and pro-collagen and cytokine production from conjunctival fibroblasts *in vitro*, effects that were partially inhibited by H1 and H2 antagonists [5, 7, 8]. Histamine

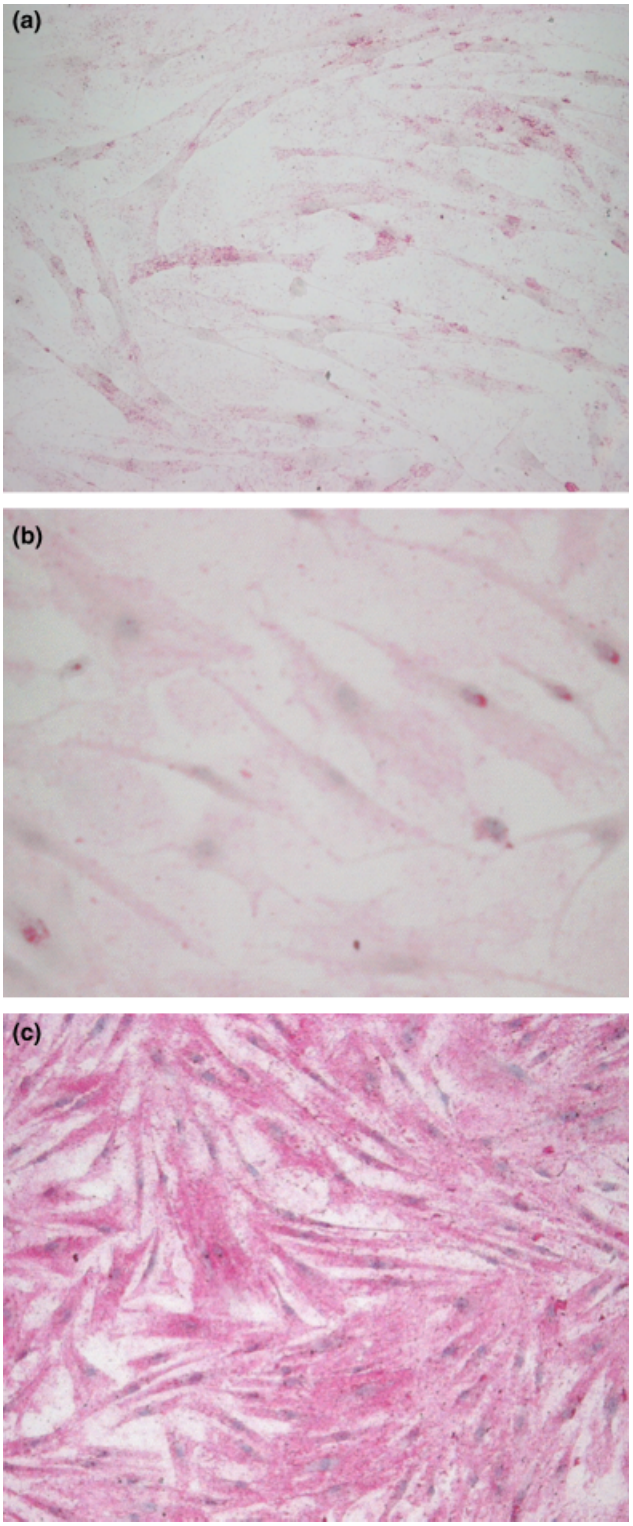

Fig. 3. Representative immunostaining of cells exposed to various mediators for 24 h. (a) TGF-β1 intracellular expression after 10 ng/mL histamine exposure. (b) Smad-3 nuclear expression (red) after 10 ng/mL IL-4 exposure. (c) intense cellular PIP staining induced by 10 ng/mL IL-4 exposure (original magnification ×400).

and TGF-β1 have been shown previously to enhance the proliferation of lung fibroblasts [22] and the expression of connective tissue growth factor (CTGF) [23], suggesting a new link between histamine and airway remodelling. Interestingly, the histamine-induced CTGF expression in lung fibroblasts was completely abolished by TNF-α [23]. The role of TNF-α in the pathogenesis of severe ocular allergies is not yet clear although an up-regulation of TNF-α in VKC patients [18], similarly to what has been found in severe asthma [24], has been reported. It can be speculated that higher TNF-α levels might act as a natural protection factor against tissue remodelling or a possible defect in the activation of the TNF-α axis might lead to remodelling in VKC and asthma patients.

TGF-β is also implicated in the physiology of T cells, peripheral T cell homeostasis, tolerance to self-antigens, T cell differentiation during immune responses, and is typically expressed by regulatory T cells [25]. However, our knowledge of the role of TGF-β in allergic inflammatory disease is inconsistent. In active severe inflammation and tissue remodelling, such as in the case of VKC, it is more likely that over-expression of TGF-β is involved in tissue remodelling.

VKC is characterized by extensive eosinophilia of the conjunctiva and high levels of IL-5 [1, 18]. The mechanism by which eosinophils may contribute to tissue remodelling is likely a result of their expression of TGF-β [26]. The importance of IL-5 and eosinophils to tissue remodelling has been shown by reduced levels of TGF-β in the remodelled airways of IL-5-deficient mice [27]. Furthermore, reduced levels of TGF-β as well as TGF-β-positive eosinophils in anti-IL-5-treated asthmatic subjects suggested that eosinophil expression of TGF-β contributed to airway remodelling in asthma [28]. It can be speculated that similar mechanisms occurs in VKC.

TGF-β has been shown to activate members of the stress-activated MAP kinase cascade in various cell types and experimental conditions [13, 14]. The relevance of these signalling pathways in the tissue remodelling of ocular allergy is still not fully understood. In the present study, IL-4 and histamine increased the expression of JNK and ERK1/2 signals together with an increased expression of the TGF-β/Smad signal pathway, showing that the transduction signals of multiple intracellular mediators are activated in VKC fibroblasts after exposure to key inflammatory mediators.

Histamine can induce regulation and activation of MAPK, p38, ERK1/2 and JNK differently in different tissues and experimental conditions, such as vascular smooth muscle cells [29], gastric epithelial cells [30], gingival fibroblasts [31], human pancreatic carcinoma cells *in vitro* [32] and adrenal cromaffin cells [33]. Both MAPK and Smad signalling pathways have been shown to independently and additively regulate collagen gene expression by transcriptional activation [34], suggesting

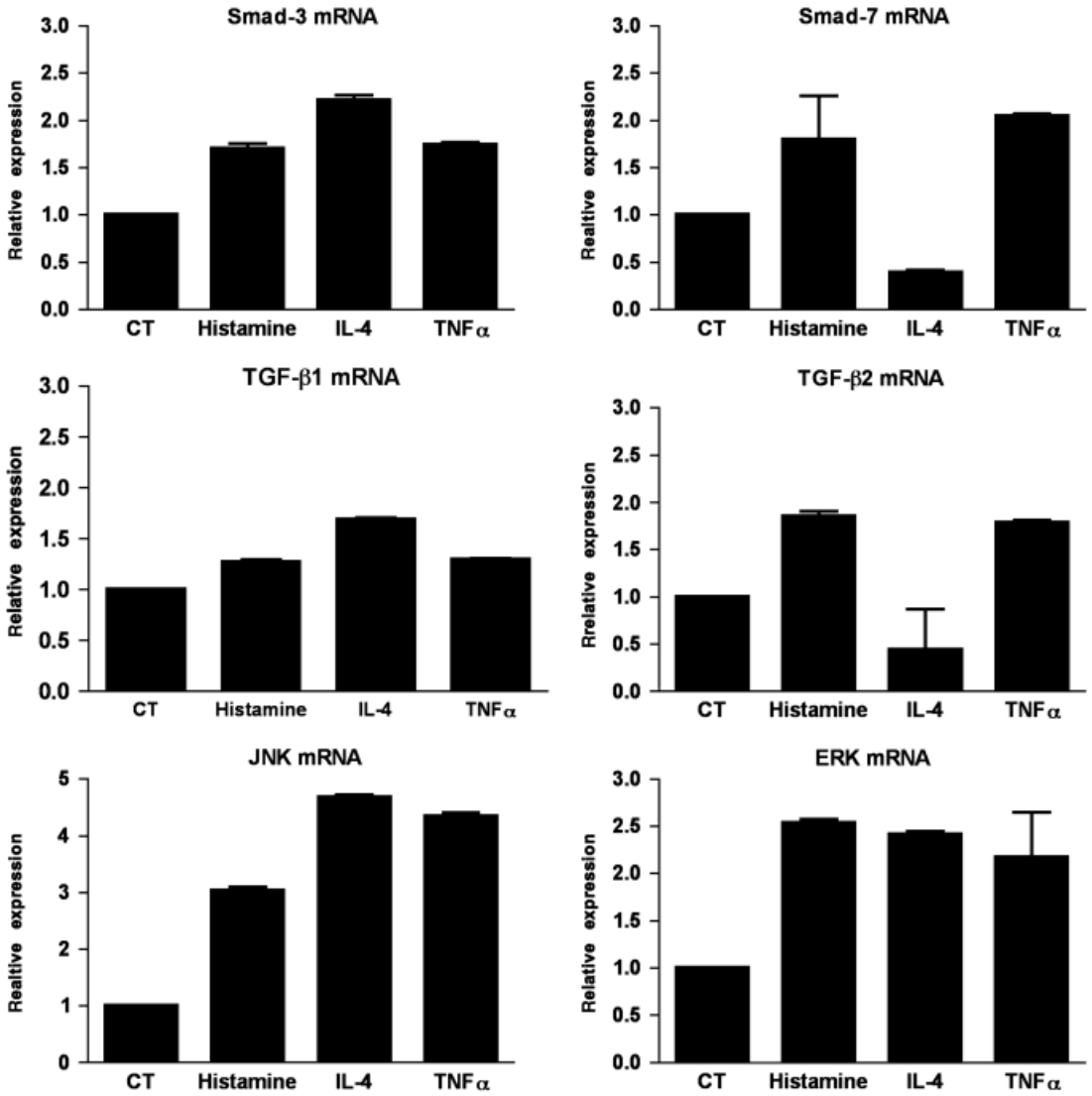

Fig. 4. RT-PCR-determined relative expression of Smad-3, -7, TGF-β1, -β2, JNK and ERK 1/2 by conjunctival fibroblasts exposed to 10 ng/mL of histamine, IL-4 and TNF-α. Histamine stimulated the expression of Smad-3 and -7, TGF-β2, JNK and ERK1 mRNA. IL-4 increased the expression of Smad-3, TGF-β1, JNK and ERK, while TNF-α increased the expression of Smad-3, -7, TGF-β2, JNK and ERK.

that different factors may induce fibrillar collagen production directly or indirectly through the TGF-β/Smad signal pathway.

The identification of a phosphatase, PPM1A/PPC2α, that directly dephosphorylates Smad-2 and -3, thus limiting their activation [35], is just an example of the complexity of signal pathways involved in physiological connective tissue biosynthesis and in pathological tissue fibrosis [36]. The increased number of PPC2α cells in VKC together with the increased expression of the inhibitory Smad-7 upon fibroblast stimulation apparently are not able to counteract the increasing PIP production and collagen deposition in VKC. These findings reinforce the concept that multiple activating and inhibitory pathways are over-expressed in VKC and that an ultimate imbalance among different signals may be responsible for the many clinical pathologies presented.

In conclusion, chronic conjunctival changes in VKC are characterized by a prevalent Th2-type inflammatory response and pro-fibrotic TGF-β and Smad proteins production, both of which can lead to the typical pathogenic remodelling response. Pro-inflammatory mediators found previously increased in active VKC patients, such as histamine, IL-4 and -13, stimulate TGF-β and procollagen production in conjunctival fibroblast cultures together with up-regulation of the intracellular mediators Smad-3, JNK and ERK1/2. These '*in vitro*' findings provide a link between the prevalent inflammatory response and the increased collagen production occurring in patients with VKC.

Endogenous regulatory mechanisms will be further investigated in order to elucidate why similar and redundant factors involved in fibrotic mechanisms evolve into different pathologies. Knowledge of the TGF-β-induced signalling pathways in various cell types and diseases such as chronic allergies, trachoma or pemphigoid, might provide us with therapeutic targets for the treatment of some of the most devastating ocular surface diseases. Restoring homeostasis to healing responses, or simply neutralizing the key pro-fibrotic mediators, may prevent or slow the progression of conjunctival remodelling and fibrosis.

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