Conjunctival expression of thymosin-β4 in vernal keratoconjunctivitis

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Purpose: Thymosin-β4 (Tβ4) is a small actin-sequestrating peptide that modulates inflammation and healing in different tissues. The aim of this study was to investigate the molecular and biochemical expression of Tβ4 in the healthy conjunctiva and in the conjunctiva of patients with vernal keratoconjunctivitis (VKC), a severe allergic eye disease characterized by chronic inflammation and marked tissue remodeling.

Methods: Conjunctival tissues, obtained from seven VKC patients and five sex/age-matched healthy subjects, were evaluated for Tβ4 expression by relative real-time PCR and light/confocal microscopy. The distribution patterns of Tβ4 in conjunctival sections as well as Tβ4 expression by mast cells (AA1), eosinophils (EG2), and fibroblast/myofibroblasts (Thy1/α-SMA) in VKC tissues were also evaluated by double immunofluorescence.

Results: Compared to healthy specimens, Tβ4 mRNA appeared upregulated in VKC conjunctival biopsies as detected by real-time PCR. In the healthy conjunctiva, Tβ4 protein expression was confined to the conjunctival epithelium, while a weak staining was observed in the stroma. In VKC conjunctival sections, Tβ4 immunoreactivity was selectively increased in the stroma where, by confocal analysis of VKC papillary formations, Tβ4 appeared to be mostly localized in eosinophils and activated fibroblasts/myofibroblasts.

Conclusions: Our data provides the first evidence of Tβ4 expression in the conjunctival tissues. The upregulation of both Tβ4 mRNA and protein in the conjunctival stroma, and its peculiar localization in eosinophils and myofibroblasts populating VKC lesions, suggest a possible role for Tβ4 in tissue inflammation and remodeling occurring in VKC.

Thymosin-β4 (Tβ4), a highly conserved water soluble 4.9 kDa peptide (43 amino acid), is the major G-actin sequestering peptide present in many tissues (blood, skin, and cornea) and cell types such as platelets, macrophages, lymphocytes, and neutrophils [1-5]. The biological effects of Tβ4 include cell migration, proliferation and survival, or apoptosis depending on the physiopathological conditions [6-8]. In experimental and in vitro studies, Tβ4 shows antiinflammatory properties and promotes angiogenesis and wound-healing/repair processes [9-12].

In a number of different models Tβ4 favors both ocular and skin wound-healing [13]. In vivo, following corneal debridement, topical Tβ4 plays an active function in promoting corneal re-epithelialization and downregulating inflammatory mediators (chemokines/cytokines) [14,15]. In vitro, Tβ4 increases transforming growth factor-β1 (TGF-β1) expression, as well as regulates laminin-5 expression in conjunctival and corneal epithelial cells, by a TGF-β independent mechanism [16]. Finally, Tβ4 modulates corneal matrix metalloproteinase levels and polymorphonuclear cell infiltration [17,18].

Our study aims at evaluating Tβ4 expression in conjunctival biopsies obtained from healthy subjects as well as from patients with vernal keratoconjunctivitis (VKC), a chronic severe allergic eye disease characterized by eosinophil infiltration and tissue remodeling/fibrosis [19,20].

METHODS

Patients and tissue handling: The study was performed in keeping with the guidelines of the Declaration of Helsinki for research involving human subjects as well as the Intramural Committee Guidelines. Informed consent was signed by each patient or their parents as required. Tarsal conjunctival biopsy specimens were obtained from seven patients with VKC (five males, two females; 11-25 years of age) and five age/sex matched-healthy subjects (controls), with no history of other ocular diseases at the time of surgery. The diagnosis of VKC was based on history, clinical diagnosis, and the presence of eosinophils in conjunctival scraping. Clinical scores (0-3: 0, absent; 1, weak; 2, mild; 3, severe) for each ocular symptom (itching, tearing, photophobia, and foreign body sensation) and each sign (conjunctival hyperemia, mucous discharge, papillae, and corneal epithelial erosion) were assigned at the time of examination. Total symptoms and signs scores were calculated and listed in Table 1.

Each conjunctival biopsy was cut into two pieces: One piece was frozen at -80 °C for molecular analysis, while the other was 10% formalin fixed and processed for light or confocal microscopy.

All analytical grade reagents and plasticware were, respectively, from SERVA (Weidelberg, Germany) and NUNC (Roskilde, Denmark), unless specified differently in the text.
Relative real-time-PCR: Total RNA was extracted from biopsies by the Qiagen Micro RNeasy kit (Qiagen, Milan, Italy), spectrophotometrically evaluated (O.D. 260/280 > 1.8), size-fractionated in a 1% agarose gel containing ethidium bromide (0.5 µg/ml), and photographed by a Kodak imager station (Kodak 550, Eastman Kodak Company, Sci. Imaging Systems, Rochester, NY) to verify the absence of any RNA degradation.

Total RNA (3 µg) samples were reverse transcribed to a final volume of 20 µl using 50 µl oligo dT21 primer, 1 mM dNTP mix, and 200 U reverse transcriptase (SuperScript II; Gibco Invitrogen, Carlsbad, CA), in a programmable PTC100 thermocycler (MJ Research, Watertown, MA).

Real-time PCR was performed on a volume of 20 µl containing 3 µl cDNA (for Tβ4) or 1 µl cDNA (for GAPDH) and 17 µl master mix containing 10 µl of 10X SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.5 µl of each primer (10 µM), and 6 µl DEPC-treated water. Specific one intron-spanning primer for Tβ4 (forward: 5'-TTA CGA TTC GCC TGC TTG C-3'; reverse: 5'-ATT CGA TAA GTC GAA ACT GAA GAA G-3'; 120 bp amplifying product, GenBank U38967) was generated using the Primer3 software [21] and prepared by MWG Biotech (Ebersberg, Germany). GenBank software was used to select the complete mRNA sequence of this small gene. The program was set at 95 °C/15 min, followed by 44 cycles of denaturation at 94 °C/30 s, annealing at 60 °C/25 s, and extension at 75 °C/30 s, completed by a further extension at 75 °C/5 min, all carried out in an Opticon2 MJ thermocycler (MJ Research). The melting curve was analyzed by elevating the temperature from 60 °C to 95 °C while monitoring fluorescence. SYBR green fluorescence was monitored after each elongation period. GAPDH was used as a housekeeping gene, because of its constant expression in healthy/fibrotic conjunctiva. Samples were amplified with GAPDH primers (forward: 5'-GAA GGG GTC ATT GAT GCC AAC-3'; reverse: 5'-GGG AAG GTG AAG GTC GGA GTC-3'; 137 bp amplifying product; 53 °C Ta; GenBank BC013310) for determination of the initial relative quantity of cDNA in each sample, and then all PCR products were normalized to that amount. Negative controls (without template or with total RNA) were produced for each run. Tissue samples were amplified in triplicate. The threshold value of detection (Ct) is the cycle at which PCR product rises above our arbitrary number, and it was set at the exponential phase of amplification. PCR products were of the correct size, as determined by agarose gel electrophoresis. Averages were calculated and differences in Ct data were evaluated by REST® software [22]. Data are expressed in the graphics as fold-expression ratio of normalized target gene (±SEM), according to the software results.

Light and confocal microscopy: Paraffin-embedded conjunctival biopsies were serially sliced into 5 µm sections, attached to pretreated slides (Biopita), and processed according to a standard procedure. Briefly, endogenous peroxidase signal was quenched by incubation in 3% H2O2 for 5 min, and antigen was retrieved by enzymatic pretreatment with hyaluronidase (1 mg/ml, in sodium acetic acid; ICN, Costa Mesa, CA). A brief blocking/permeabilizing step was carried out for 20 min (0.8% bovine serum albumin, BSA-0.3% Triton X-100 (TX) in 10 mM phosphate-buffered saline, PBS) before addition of the specific rabbit antihuman Tβ4 antibodies (1 µg/ml; Immunodiagnosticostick AG., Wiesenstr, Bensheim, Germany) or an irrelevant isotype-matched IgG antibodies (Vector Laboratories, Inc., Burlingame, CA). Specific binding was detected using an indirect Avidin Biotin Peroxidase technique (Vectastain Elite II ABC kit, Vector Laboratories) with DAB as specific substrate (Dako Corp., Carpinteria, CA). Mounted slides were examined at a magnitude of 40X or 60X oil immersion, using an Eclipse E400 microscope (Nikon, Tokyo, Japan) equipped with a light acquisition system (Lucia Software, Nikon). The acquired digital images were brightness/contrast processed with the Adobe Photoshop 7.0 program (Adobe Systems Inc., San Jose, CA). Only one observer estimated independently, and in a blind fashion, the intensity of Tβ4 staining without taking into consideration the staining in the vessel walls. The evaluation of the staining was graded, according to Bochaton-Piallat and coworkers [23], as follows: -, no staining (absent); +, slight (focal labeling); ++, intense (approximately 50% of the tissue); and +++, very intense (extensive labeling).

Sections were processed for confocal analysis according to single/double immunofluorescence. Sections were 0.3% TX-PBS permeabilized and incubated overnight at 4 °C with a specific antibody mixture of rabbit antihuman Tβ4 antibodies (2 µg/ml, Immunodiagnostics AG.) and EG1 (1/50, Peninsula, Upsala, Sweden), AA1 (1/200, R&D Systems, Minneapolis, MN), or α-smooth muscle actin, (α-SMA, 1/70; Dako) antibodies [24], diluted in 0.1% BSA-PBS. Specific binding of the primary antibodies was detected using secondary cy5 conjugated donkey antimouse (for EG2, AA1, or α-SMA) and cy2 conjugated donkey antirabbit antibodies (for Tβ4), diluted 1/200 in 0.1% Tween20-PBS and incubated at room tempera-

<table>
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<tr>
<th>Patient</th>
<th>Gender/Age</th>
<th>Signs (score)</th>
<th>Symptoms (score)</th>
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<th>Tβ4 protein</th>
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Clinical, histopathological, and molecular data of the seven patients with vernal keratoconjunctivitis (VKC) enrolled in the study. The calculation of total symptoms (itching, tearing, photophobia, and foreign body sensation) and signs (hyperemia, mucous discharge, papillae, and corneal features) scores were as described in detail in Methods. The sharp (hash mark) indicates the presence of corneal ulcer. Healthy conjunctiva showed a C0 mean value of 30, while GAPDH C0 values ranged between 19-21. In the “Tβ4 protein E/S” column, the epithelial (E) and stromal (S) immunoreactivity scores are as follows: absent; +, slight; ++, intense; ++++, very intense. The asterisk indicates realtime PCR: Threshold cycles (Ct) mean values. The mRNA expression is inversely proportional to the C₀ at normalization.
ture for 45 min (Jackson ImmunoResearch Laboratories, West Grove, PA). Irrelevant isotype-matched IgG antibodies were also incubated and the sections analyzed as control for staining specificity (Vector Laboratories). Antifade/gel (Vector) mounted sections were examined at 60X/oil immersion using a E2000U three lasers confocal microscope equipped with C1 software (Nikon). Light and fluorescent pictures were made using the Adobe Photoshop 7.0 program.

Statistical analysis: Results are expressed as means±SEM. For PCR analysis, we evaluated C\text{t} data with REST® software (see relative real time-PCR section). All statistical data was elaborated using the ANOVA program followed by Tukey-Kramer post-hoc. The Kendall’s rank correlation coefficient (Tau) was also calculated to identify relationships between parameters. Analysis were performed by using the statistical package StatView II for PC (Abacus Con-

Figure 1. Tβ4 mRNA/protein is expressed by healthy conjunctival biopsies. A: Specific Tβ4 fluorescent amplification in healthy conjunctival specimens as detected by reverse-transcription of conjunctival total RNA and amplification with Tβ4 or GAPDH primers to generate PCR products by PCR analysis. B: Immunoreactivity for Tβ4 in healthy conjunctiva as detected by the avidin-biotin peroxidase technique. Nuclei were counterstained with hematoxylin (40X). D: Fluorescent Tβ4 immunoreactivity (green) tended to localize in the conjunctival epithelium, mainly in the basal membrane (white-arrows), and few Tβ4 positive cells localized in the stroma. Blue staining is referred to Toto-3 nuclear marker (60X/oil immersion). C,E: Irrelevant IgG immunoreactivity (isotype) for light and fluorescent staining, respectively (40X and 60X/oil immersion).
RESULTS

The characteristics of the patients enrolled in the study are reported in Table 1. In particular, all the patients showed active VKC and were all free from any topical/systemic therapy at the moment of tarsal conjunctival sampling.

Healthy and VKC conjunctival biopsies express Tβ4 mRNA and protein: Tβ4 mRNA was expressed in conjunctival biopsies of healthy subjects as demonstrated by real time-PCR (Figure 1A). Light microscopy showed an intense immunolocalization of Tβ4 protein in the conjunctival epithelium (score +++) of healthy tissues (Figure 1B), while only a weak staining (score +) was observed in the stroma. By using fluorescent analysis, we found Tβ4 (green/cy2) was specifically expressed at the basal epithelial layer (Figure 1D). No specific staining was observed in control sections (Figure 1C-E).

Relative real time-PCR showed that Tβ4 mRNA was upregulated in VKC conjunctival biopsies (Ct=21.20±2.49), when compared to healthy ones (Ct=31.13±0.62; 532.63 increase, p<0.05; REST-ANOVA; Figure 2A). The specificity of Tβ4 PCR products was confirmed by a single melting curve, measured from 72-95 °C during the amplification (Figure 2B). In VKC conjunctiva, Tβ4 protein expression was increased in both epithelium (score ++++) and stroma (score ++), as demonstrated by immunohistochemistry (Table 1; Figure 2C). In the stroma, Tβ4 was found in cells morphologically resembling inflammatory cells and activated fibroblasts. The semi-quantitative evaluation of the IHC-staining for each conjunctiva is reported in Table 1.

Kendall’s rank test showed no significant correlation between total symptom (Tau=-0.571; p=0.0715) and sign (Tau=-0.619; p=0.0509) scores and Tβ4 mRNA expression (Ct). Interestingly, the two patients having corneal ulcers (case 1 and 6) showed the highest Tβ4 mRNA values (see Table 1).

Tβ4 protein localizes mainly in eosinophils and myofibroblasts populating VKC conjunctiva: In view of the aforesaid finding and of the particular pathology of VKC, with marked infiltration of mast cells, eosinophils and activated fibroblasts, we studied VKC specimens by double-immunofluorescence with Tβ4 antibodies (green/cy2-labeled) and specific markers (red/Cy5-labeled) of mast cells (AA1), activated eosinophils (EG2) and myofibroblasts (α-SMA). Tβ4 was found in both eosinophils and myofibroblasts (Figure 3). No specific staining was observed in control sections (data not shown).

DISCUSSION

This is the first report of Tβ4 expression in the healthy human conjunctiva and of Tβ4 overexpression in VKC, a chronic allergic inflammatory disease of the ocular surface with intense tissue remodeling/fibrosis [19,20,26,27].

Tβ4 expression in the healthy human conjunctiva was confined to the epithelium, both at the molecular and biochemical level. Several data suggest a specific pathophysiological role for Tβ4 in corneal epithelial cells, both in vivo and in vitro [12-15,28]. Our data suggest that Tβ4 might have a similar role in regulating proliferation as well as differentiation/activation of the conjunctival epithelium, during pathological conditions.

It has been shown that Tβ4 plays a relevant role in healing/repair process and angiogenesis, representing a link between inflammatory mediators and extracellular matrix [13,14,17,18,28]. In particular, Tβ4 promotes corneal reepithelialization, downregulates inflammatory mediators (chemokines/cytokines and growth factors, such as IL1α/β, ...
IL6, IL8 and TGFβ1), and modulates both conjunctival and corneal epithelial cell matrix metalloproteinase and laminin-5 expression after experimental wounding [10,12-18]. In line with the suggested antiinflammatory function, Tβ4 decreases neutrophil tissue infiltration and migration in an experimental model of corneal alkali injury [15-18].

Our data showed that Tβ4 is specifically increased in the stroma of VKC conjunctivæ. VKC conjunctiva is characterized by increased mast cells and infiltrating eosinophils. In particular, eosinophils play a crucial role in the pathogenesis of VKC by releasing cytotoxic mediators and cytokines [20,29]. From confocal studies, we found mast cells did not appear to directly contribute to the stromal Tβ4 increase observed in VKC conjunctivæ. The expression of Tβ4 in the eosinophils infiltrating the conjunctivæ of VKC patients warrants further studies to evaluate Tβ4 potential role in allergic inflammation driven by eosinophils. Additionally, many activated fibroblasts (myofibroblasts) [24] showed Tβ4 ex-

Figure 3. Specific Tβ4 protein distribution in cells populating the VKC conjunctivæ. Double-fluorescent labeling for Tβ4 (green/cy2) and specific immune/structural markers (red/cy5) in VKC biopsies counterstained with Toto-3 (blue). Overlay (first column) showing Tβ4 (cy2) immunoreactivity in mast cells (AA1/cy5), eosinophils (EG2/cy5), and myofibroblasts (α-SMA/cy5). Second and third columns show the single staining for each specific overlays. (60X/oil immersion).
pression in VKC lesions. This finding was observed by specific staining with α-SMA antibodies, a specific marker characterizing both fibroblast activation and differentiation into myofibroblasts. In addition, a pilot study carried out on primary cultures of fibroblasts showed that VKC-derived fibroblasts (largely myofibroblasts) expressed high amounts of Tβ4 both at the molecular (26.59 increase in referring to healthy ones; p<0.05 REST-ANOVA) and biochemical level (data not shown), indicating their direct contribution to the Tβ4 overproduction observed in VKC stroma. Since VKC conjunctivitis is characterized by a profound tissue remodeling, reflected by the typical giant papillae formations, the expression of Tβ4 by conjunctival fibroblasts might suggest a role for Tβ4 in extracellular matrix remodeling and cell contraction during wound repair. The observation that the only two patients having corneal ulcers (case 1 and 6) showed the highest Tβ4 mRNA values suggests a potential role of this protein in corneal wound healing.

In conclusion, this study showed that Tβ4 is expressed by the healthy conjunctival epithelium and is overexpressed in both the epithelium and the inflamed stroma of VKC tissues. Further clinical and experimental studies should aim to elucidate the potential role of Tβ4 in the inflammatory and tissue remodeling processes of VKC.

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REFERENCES


