Regulation of vascular endothelial growth factor-C by tumor necrosis factor-α in the conjunctiva and pterygium

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Abstract. Vascular endothelial growth factor C (VEGF-C) plays an important role in the development of a pterygium through lymphangiogenesis. We examined the association between VEGF-C and tumor necrosis factor-α (TNF-α) in the pathogenesis of pterygia. Cultured conjunctival epithelial cells were treated with TNF-α, and the gene expression levels of *VEGFC* were evaluated by quantitative polymerase chain reaction (qPCR) and VEGF-C protein expression levels were measured using an enzyme-linked immunosorbent assay (ELISA). In addition, using ELISA, we evaluated the VEGF-C protein expression in the supernatants of cultured conjunctival epithelial cells, in which we neutralized TNF-α using anti-TNF-α antibody. The gene expression of tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1A*), known as TNF receptor 1 (TNFR1), was confirmed using reverse transcription PCR in cultured conjunctival epithelial cells. Immunofluorescence microscopy was used to examine the localization of VEGF-C and TNFR1 in pterygium tissues and TNFR1 expression in cultured conjunctival epithelial cells. Immunohistochemistry was used to examine the localization of TNFR1 in pterygia and normal conjunctival tissues. *VEGFC* gene expression increased in cultured conjunctival epithelial cells 24 h after the addition of TNF-α. The secretion of VEGF-C protein was significantly increased 48 h after the stimulation of cultured conjunctival epithelial cells with TNF-α. Increased VEGF-C protein secretion stimulated by TNF-α was significantly reduced by anti-TNF-α neutralizing antibody treatment. In cultured conjunctival epithelial cells, *TNFRSF1A* and TNFR1 were expressed. TNFR1 was immunolocalized in normal conjunctival tissues and in human pterygium tissues as well as in VEGF-C-positive epithelial cells from human pterygia. Our data demonstrate that TNF-α mediates VEGF-C expression, which plays a critical role in the pathogenesis of pterygia.

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Introduction

A pterygium is an ocular mass that forms over the perilimbal conjunctiva and extends onto the corneal surface. There are no specific eye drops available to prevent a pterygium from invading the cornea, which may cause subsequent visual impairment. Pathologically, pterygium tissues are characterized by a proliferative epithelium, an invasive nature and are highly vascularized tissues (1). We previously demonstrated that there was a high level of proliferation in the pterygium epithelium compared with that in normal conjunctiva (2,3). Vascular endothelial growth factor (VEGF)-A, basic fibroblast growth factor (FGF2) (4) and erythropoietin receptor (5) have been reported to exist at higher levels in pterygium tissues and lead to a pterygium extension through angiogenesis. Previous findings have shown that not only blood vessels but also lymphatic vessels may be clearly observed in human pterygium tissues; histological analyses proved that the lymphatic microvessel density (LMVD) was significantly higher in pterygia compared with that in normal conjunctiva (6,7). Lin *et al* demonstrated that the LMVD was an independent risk factor and a valuable predictive factor for the recurrence of pterygia (8). We recently demonstrated that VEGF-C and the VEGF receptor-3-signaling pathway led to lymphangiogenesis, which was associated with the pathogenesis and development of pterygia (7). However, the molecular mechanisms underlying elevated VEGF-C expression have yet to be elucidated in the conjunctiva and pterygium.

According to previous findings, chronic stimulation by the inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β) may be responsible for the increased expression of matrix metalloproteinases (MMPs) in cultured primary pterygium body fibroblasts (9). These data have clinical implications for the progression of pterygia and recurrence associated with the incomplete excision of primary fibroblasts under the influence of ocular surface inflammation (9). On

the other hand, there was a highly significant correlation between VEGF-C and the levels of inflammatory cytokines such as TNF-α and IL-1β in the synovial fluid of patients with rheumatoid arthritis (RA). As a result, VEGF-C and the cytokines cause synovial inflammation and hyperplasia in RA by contributing to local lymphangiogenesis (10). The high expression of VEGF-C stimulated by TNF-α induces many human diseases such as chronic progressive kidney diseases (11), gallbladder carcinoma (12), melanoma lymph node metastasis (13) and breast cancer (14). Based on previous findings, we hypothesized that such inflammatory cytokines provoke VEGF-C expression in the human conjunctiva and pterygium.

The aim of this study was to analyze the changes in VEGF-C expression induced by TNF-α or IL-1β stimulation and to examine the relationship between VEGF-C and TNF receptor 1 (TNFR1) in the pterygium and normal conjunctival tissues of humans.

Materials and methods

Cell culture and chemicals. Cultured human conjunctival epithelial cells (clone 1-5c-4 HeLa derivative) were purchased from the European Collection of Authenticated Cell Cultures (Salisbury, UK). The cell line was cultured under a humidified atmosphere containing 5% CO_2 at 37˚C in Medium 199 (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum.

After serum starvation, human conjunctival epithelial cells were treated with recombinant human TNF-α (0, 4 and 20 ng/ml; R&D Systems, Minneapolis, MN, USA) or recombinant human IL-1β (0, 0.2, 2.0 and 20 ng/ml; Thermo Scientific Biomarkers, Hennigsdorf, Germany) for 24 and/or 48 h and processed for analysis to detect mRNA and protein expression levels. Phosphate-buffered saline (PBS) was added to the serum-free medium for the controls.

For the TNF-α neutralization bioassay, recombinant human TNF-α at 20 ng/ml (R&D Systems) was pre-incubated with 200 ng/ml of rabbit anti-TNF-α neutralizing antibody (D1B4; Cell Signaling Technology, Danvers, MA, USA) for 1 h at 37˚C. After pre-incubation, the cells were treated for 48 h and processed for analysis to detect VEGF-C protein expression levels. Normal rabbit IgG (R&D Systems) was used as the control for the anti-TNF-α neutralizing antibody.

Quantitative PCR (qPCR) and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cells using the TRIzol method (Life Technologies, Carlsbad, CA, USA). Reverse transcription to synthesize 1.0 μg of total RNA to cDNA was performed using GoScript Reverse Transcriptase (Promega, Madison, WI, USA) according to the manufacturer's instructions.

qPCR was performed using 0.1 μl cDNA from each sample, GoTaq qPCR Master Mix (Promega), 0.25 μM primers and 0.2 μl CXR reference dye (Promega), in a final volume of 20 μl for the SYBR assay. For the TaqMan assay, we used 0.1 μl cDNA from each sample, THUNDERBIRD Probe qPCR Mix, 0.2 μl ROX reference dye (both from Toyobo, Tokyo, Japan), 0.9 μM of primers and 0.25 μM of FAM-dye labeled TaqMan MGB probe in a final volume of 20 μl. After an initial denaturation at 95˚C for 2 min, the samples were subjected to 40 cycles of amplification comprised of denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 1 min. All data were calculated using the ΔΔCq method (15) using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* for normalization.

RT-PCR was performed using 0.1 μl cDNA from each sample, 0.5 units of GoTaq DNA polymerase (Promega), 0.4 μM of primers, 0.2 mM of dNTP mix and 1X GoTaq reaction buffer in a final volume of 20 μl. After an initial denaturation at 95˚C for 2 min, the samples were subjected to 35 cycles of amplification consisting of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 15 sec. The products were electrophoresed on 2% agarose gels, stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA) and visualized using RAS-4000 image analyzer (FujiFilm, Tokyo, Japan).

The following primers for genes were used: human *GAPDH* (NM_002046.5) sense, 5'-CCT GGC CAA GGT CAT CCA TG-3' and antisense, 5'-GGA AGG CCA TGC CAG TGA GC-3' (224 bp); and TNF receptor superfamily, member 1A (*TNFRSF1A*; NM_001065.3) sense, 5'-CTG CCA GGA GA AAC AGA ACA C-3' and antisense, 5'-CTC AAT CTG GGG TAG GCA CAA-3' (130 bp). The TaqMan assay primer and probe set for *VEGFC* (NM_005429.3; TaqMan assay ID: Hs00153458_m1) was purchased from Life Technologies.

Enzyme-linked immunosorbent assay (ELISA). The concentration of VEGF-C in cell cultured media was measured using the human VEGF-C ELISA kit (R&D Systems) according to the manufacturer's instructions.

Preparation of human tissues. Nasal pterygia were surgically removed from 10 patients (mean age, 73.6±5.6 years) who were enrolled in this study. Age-matched normal bulbar conjunctival tissues were obtained from patients (mean age, 62.3±4.2 years) during glaucoma surgery. The tissues were then fixed in 4% paraformaldehyde. After fixation, the slides were washed in PBS and processed for paraffin sectioning. This study was conducted in accordance with the World Medical Association Declaration of Helsinki. Written informed consent was obtained from all patients after receiving approval from the institutional review board of Hokkaido University Hospital (Sapporo, Japan) (IRB #014-0295).

Immunofluorescence microscopy. After hematoxylin and eosin staining (Sigma-Aldrich), formalin-fixed, paraffin-embedded serial tissue sections were deparaffinized and hydrated through exposure to xylene and graded alcohols followed by water. As a pre-treatment, microwave-based antigen retrieval was performed in a 10 mM citrate buffer (pH 6.0). The cultured human conjunctival epithelial cells were fixed in 4% paraformaldehyde and then washed in PBS. Ten percent serum depending on the secondary antibody source was used to block non-specific binding. The sections were incubated with the following primary antibodies: rabbit polyclonal anti-TNFR1 (ab19139; 1:100 dilution; Abcam, Cambridge, MA, USA) and goat polyclonal anti-VEGF-C (AF752; 1:10 dilution; R&D Systems). Secondary antibodies for fluorescence detection were Alexa Fluor 488 and 546 (Life Technologies). 4',6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining and sections were mounted with a fluorescent

mounting medium (Dako, Glostrup, Denmark). The sections were visualized under a Keyence BZ-9000 fluorescence microscope (Keyence, Tokyo, Japan).

Immunohistochemistry. Following the microwave-based antigen retrieval treatment, the sections were incubated in 0.3% hydrogen peroxide in order to block endogenous peroxidase activity and 10% normal goat serum (Life Technologies) to block non-specific binding. The sections were then incubated with rabbit polyclonal anti-TNFR1 antibody (ab19139; 1:100 dilution; Abcam). We replaced anti-TNFR1 antibody with PBS as a negative control. Color was developed using 3,3'-diaminobenzidine tetrahydrochloride (Dako) and counterstained with hematoxylin. The sections were examined under a Keyence BZ-9000 fluorescence microscope (Keyence).

Statistical analysis. All the results are expressed as the means ± SED as indicated. The student's t-test was used for statistical comparison of the concentration of VEGF-C protein. A p-value <0.05 was considered to indicate a statistically significant difference between the means.

Results

Increased expression and secretion of VEGF-C in cultured human conjunctival epithelial cells following TNF-α stimulation. Firstly, we examined the gene expression of *VEGFC* in cultured human conjunctival epithelial cells stimulated with TNF-α or IL-1β for 24 h. The *VEGFC* expression level increased (fold-change, 1.60±0.35, n=6, p<0.01) following stimulation with 20 ng/ml TNF-α (Fig. 1). By contrast, the gene expression of *VEGFC* mildly increased (fold-change, 1.23±0.17, n=6, p<0.05) with the addition of 0.2 ng/ml IL-1β (Fig. 2).

To determine whether stimulation with TNF-α or IL-1β increases VEGF-C secretion from cultured human conjunctival epithelial cells, we measured the VEGF-C protein concentrations in the supernatants 24 and 48 h after the addition of TNF-α or IL-1β. Forty-eight hours after the addition of 20 ng/ml TNF-α, the protein concentration of VEGF-C increased (195.92±33.41 pg/mg, n=6, p<0.05), which was significantly higher when compared with the controls (Fig. 3). By contrast, there was no significant difference in VEGF-C concentrations with or without IL-1β stimulation (data not shown).

Neutralization of TNF-α in VEGF-C secretion from cultured human conjunctival epithelial cells. To verify that TNF-α stimulation increases VEGF-C secretion from cultured human conjunctival epithelial cells, we treated the cultured cells with TNF-α and anti-TNF-α neutralizing antibody, and examined the secretion levels of VEGF-C. As shown in Fig. 4, increased VEGF-C protein secretion following TNF-α addition was significantly decreased by anti-TNF-α neutralizing antibody treatment (140.44±1.92 to 119.33±4.41 pg/ml, n=3, p<0.05), whereas there were no specific changes following treatment with normal rabbit IgG (200 ng/ml) (Fig. 4).

Expression of TNFR1 in cultured human conjunctival cells and pterygium tissues. TNF-α signaling occurs through two types of receptor, TNFR1 and TNFR2. TNFR1 is expressed

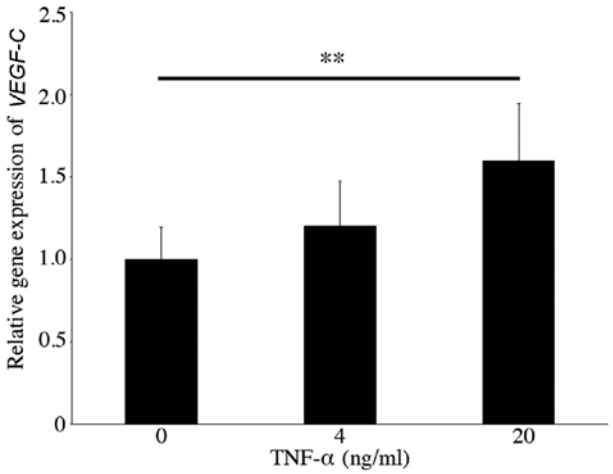

Figure 1. Upregulation of relative expression level of vascular endothelial growth factor C (*VEGF-C*) 24 h after the stimulation of cultured human conjunctival epithelial cells with tumor necrosis factor-α (TNF-α) (n=6, each). **p<0.01.

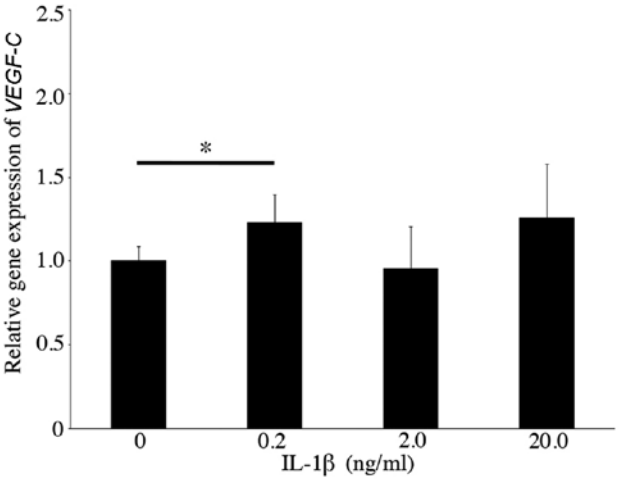

Figure 2. Relative expression level of vascular endothelial growth factor C (*VEGF-C*) 24 h after the stimulation of cultured human conjunctival epithelial cells with interleukin-1β (IL-1β) (n=6, each). *p<0.05.

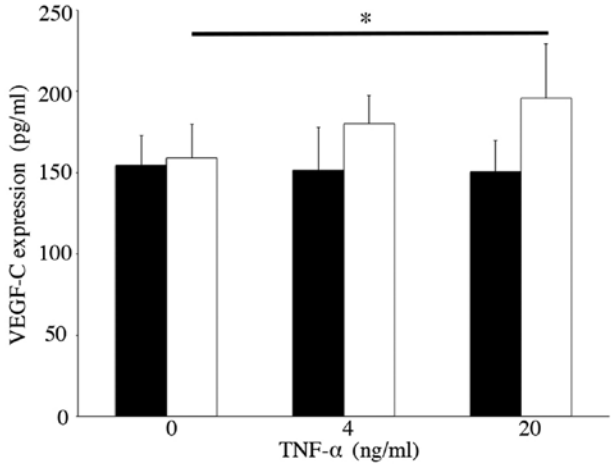

Figure 3. Alterations in vascular endothelial growth factor C (VEGF-C) concentrations in the supernatants of cultured human conjunctival epithelial cells. The VEGF-C concentration was assessed using the VEGF-C ELISA kit 24 and 48 h after tumor necrosis factor-α (TNF-α) (0, 4 and 20 ng/ml) stimulation (n=6, each). *p<0.05. Black bars, 24 h. White bars, 48 h.

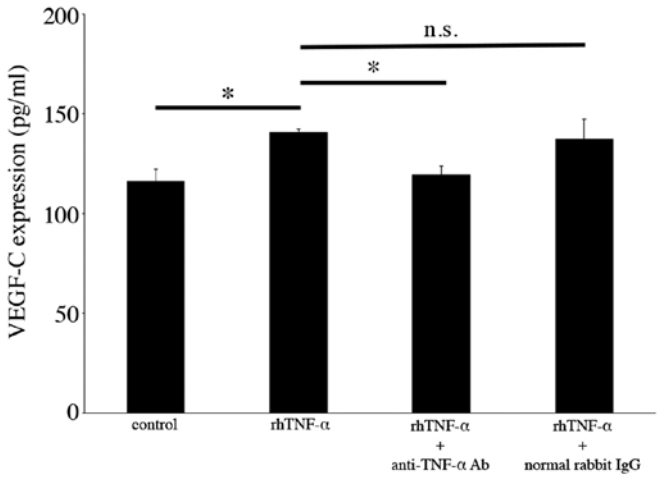

Figure 4. Alterations in vascular endothelial growth factor C (VEGF-C) concentrations in the supernatants of cultured human conjunctival epithelial cells by anti-tumor necrosis factor-α (TNF-α) neutralization antibody (anti-TNF-α Ab). VEGF-C concentrations were assessed using the VEGF-C ELISA kit 48 h after TNF-α 20 ng/ml stimulation (n=3, each). *p<0.05. n.s., not significant. rhTNF-α, recombinatn human TNF-α.

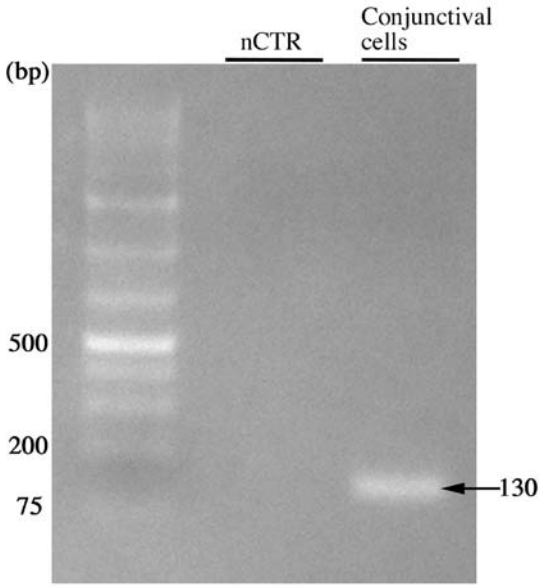

Figure 5. Gene expression of tumor necrosis factor receptor superfamily, member 1A (*TNFRSF1A*) in cultured human conjunctival epithelial cells. *TNFRSF1A* was detected in cultured human conjunctival epithelial cells by RT-PCR. H_2O was applied as a negative control (nCTR).

in almost all mammalian cell types whereas TNFR2 is typically found in immune endothelial cells. Among numerous cell types, TNFR1 exists as the key mediator of TNF signaling except in the lymphoid system (16,17), as well as in other human conjunctival epithelial cell lines (18). In this study, we explored the existence of TNFR1 and the gene expression of *TNFR1* (also known as *TNFRSF1A*) in cultured human conjunctival epithelial cells and pterygium tissues. *TNFRSF1A* was detected in cultured human conjunctival epithelial cells (Fig. 5). TNFR1 was also immunolocalized in cultured human conjunctival cells (Fig. 6); the TNFR1 signal was similar to that observed in the cultured dorsal root ganglion cell body (19). We then immunohistochemically examined TNFR1 expression in human tissues, in either pterygia or normal conjunctiva (Fig. 7). TNFR1 immunoreactivity was detected in the pterygium epithelial cells (Fig. 7B arrows), whereas the reactivity was less marked in normal conjunctival epithelia (Fig. 7E). Moreover, TNFR1 signals (Fig. 8D and F) were observed in VEGF-C-positive epithelial cells from human pterygia (Fig. 8A and C) using serial sections.

Discussion

In this study we demonstrated for the first time to the best of our knowledge, that stimulation by TNF-α, but not IL-1β, enhanced both the gene expression and the protein secretion of VEGF-C in cultured human conjunctival epithelial cells. It is known that TNF-α leads to cell proliferation in the pterygium by increasing the expression of MMPs (9). In addition, high levels of VEGF-C secretion from epithelial cells induces lymphangiogenesis, which play a critical role in the pathogenesis and development of pterygia (7). It has also been reported that immunoreactivity for TNF-α was marked in pterygium tissues (20). Therefore, our data suggest that TNF-α-mediated VEGF-C expression may be an important trigger of lymphangiogenesis during the development of pterygia.

It has been shown that TNFR1 is expressed by a variety of cell types (16,18,21). In this study, *TNFRSF1A* and TNFR1 were detected in cultured human conjunctival epithelial cells. Moreover, TNFR1 immunoreactivity was strongly detected in the pterygium tissues. In addition, we further demonstrated that TNFR1 immunoreactivity was detected in VEGF-C-positive epithelial cells from human pterygia. According to the immunoreactivity results, we hypothesized that VEGF-C was secreted from the TNFR1-positive cells. These results suggest that TNF-α possibly binds to TNFR1, which results in increased VEGF-C secretion and its gene expression.

TNFR1 immunoreactivity was found not only in epithelial cells from human pterygia but also in normal conjunctival epithelia. This result suggests that the increased expression of TNF-α rather than the constitutive presence of TNFR1 may stimulate the ligand-receptor system, which causes VEGF-C induction leading subsequently to a higher lymphatic vessel number in the pterygium than in the normal conjunctiva. According to previous findings, acute ultraviolet irradiation exposure results in the induction of cornea-derived proinflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α (22). It is generally known that pterygia are closely associated with ultraviolet light (23). Indeed, ultraviolet exposure may lead to an increase in the production of TNF-α in stromal cells of the human cornea (22). Exposure to ultraviolet light may be one of the regulators of TNF-α expression. Further studies are needed to clarify the regulation of TNF-α in the ocular surface.

Surgical resection of the proliferative tissues and subsequent reconstruction are the only conventional treatments for human pterygia. However, postoperative recurrence has been reported in >50% of cases (24). Therefore, the development of pharmacotherapy may contribute to the further prevention of pterygium invasion and subsequent recurrence. In this study, we demonstrated that TNF-α mediates VEGF-C expression,

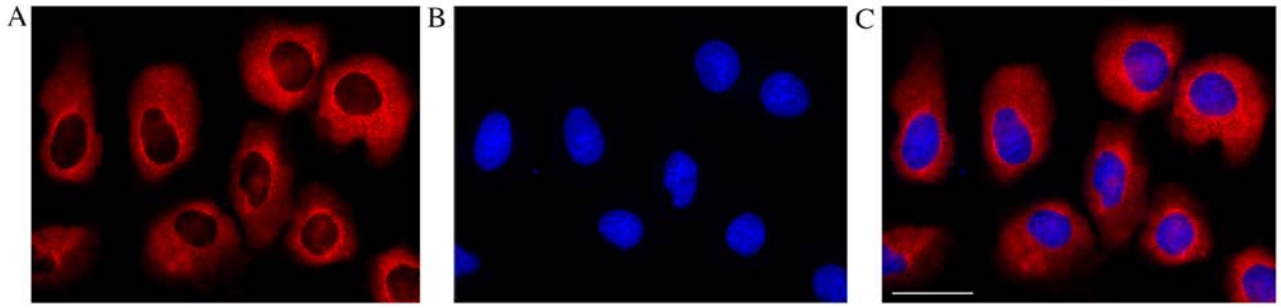

Figure 6. Immunofluorescence images of TNF receptor 1 (TNFR1) expression in cultured human conjunctival cells. (A) TNFR1 (red), (B) DAPI (blue) and (C) merge images. Scale bar, 10 μm.

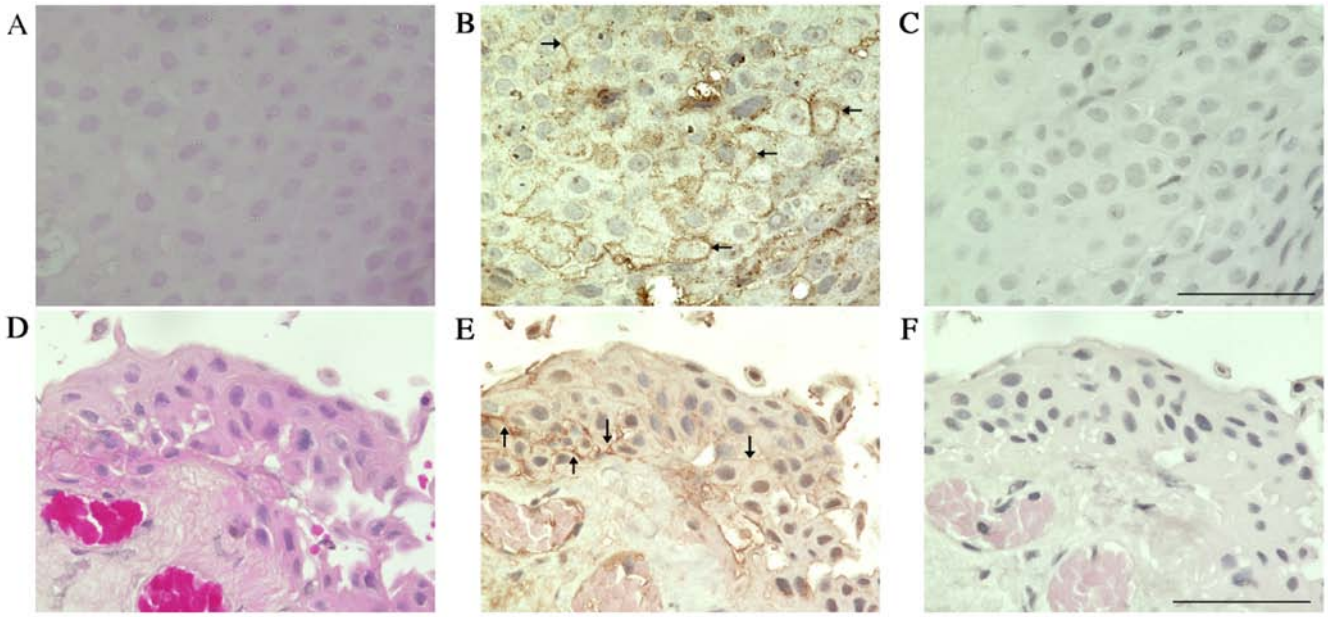

Figure 7. TNF receptor 1 (TNFR1) expression in human tissues, in either (A-C) the pterygium or (D-F) normal conjunctiva. (A and D) H&E staining of tissues. (B and E) Immunohistochemistry of TNF receptor 1 (TNFR1) expression. Black arrows indicate the expression of TNFR1 on the cell membranes of (B) pterygium epithelial cells and (E) in the normal conjunctiva. TNFR1 expression was not detected in the phosphate-buffered saline (PBS) controls (C and F). Scale bar, 50 μm.

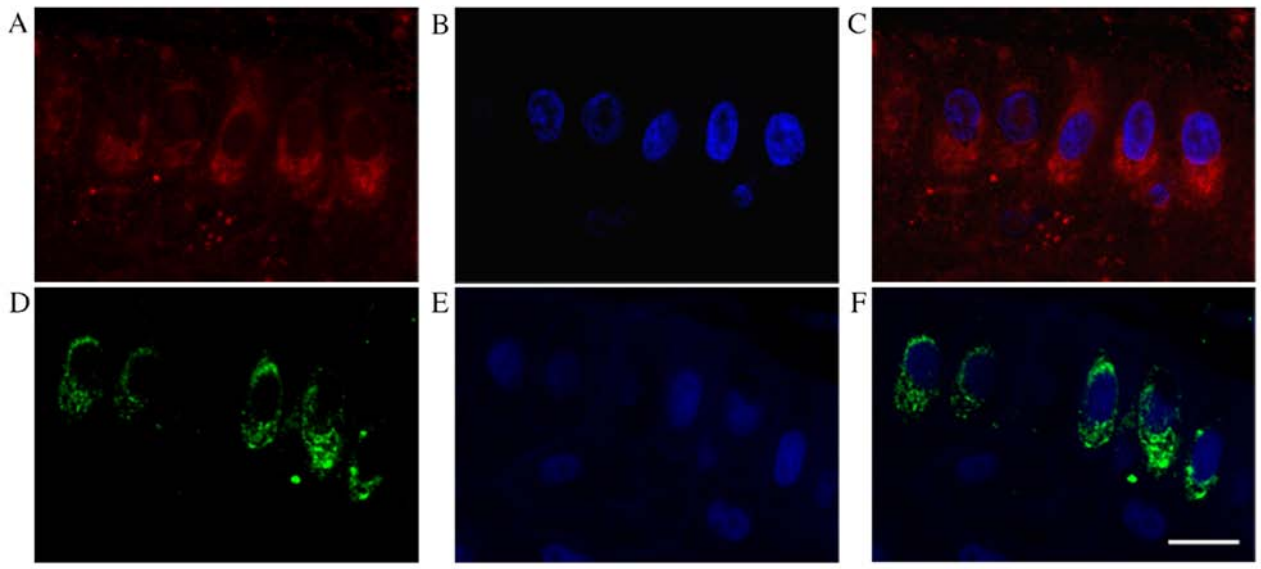

Figure 8. Immunofluorescence images showing (A and C) vascular endothelial growth factor C (VEGF-C) expression (red), (D and F) TNF receptor 1 (TNFR1) expression (green), and (B, C, E and F) DAPI nuclear staining (blue) in the pterygium. TNFR1 immunoreactivity (D and F; green) is detected in VEGF-C-positive epithelial cells from human pterygia (A and C; red) using serial sections. Scale bar, 10 μm.

suggesting that blockage of TNF-α may be a novel therapeutic target for the treatment of pterygia in the future.

In conclusion, TNF-α induced VEGF-C expression in cultured human conjunctival epithelial cells. This pathway may involve the upstream regulation of VEGF-C expression and secretion in the pathogenesis and development of lymphangiogenesis in pterygia.

Acknowledgements

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