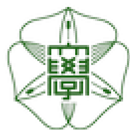
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1 Expression of vascular endothelial growth factor C in human pterygium.

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1 **Abstract**

2 **Purpose:** Vascular endothelial growth factor C (VEGF-C) and its receptor
3 VEGFR-3 mediate lymphangiogenesis. In this study, we analyzed the
4 expression of VEGF-C and VEGFR-3 as well as lymphatic vessels in the
5 pterygium and normal conjunctiva of humans.

6 **Methods:** Fifteen primary nasal pterygia and three normal bulbar
7 conjunctivas, surgically removed, were examined in this study. The
8 lymphatic vessel density (LVD) and blood vessel density (BVD) were
9 determined by the immunolabeling of D2-40 and CD31, markers for
10 lymphatic and blood vessels, respectively. VEGF-C and VEGFR-3
11 expression in pterygial and conjunctival tissue proteins was detected by
12 Western blotting. Expressions of VEGF-C and VEGFR-3 were evaluated
13 using immunohistochemistry.

14 **Results:** The LVD was significantly higher in the pterygium than normal
15 conjunctiva ($p<0.05$). Western blot demonstrated high-level expression of
16 VEGF-C and VEGFR-3 in the pterygium compared with normal
17 conjunctiva. VEGF-C immunoreactivity was detected in the cytoplasm of
18 pterygial and normal conjunctival epithelial cells. The number of
19 VEGF-C-immunopositive cells in pterygial epithelial cells was
20 significantly higher than in normal conjunctival cells ($p<0.05$). VEGFR-3
21 immunoreactivity was localized in the D2-40-positive lymphatic
22 endothelial cells.

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Conclusions: The present findings suggest the potential role of VEGF-C in the pathogenesis and development of a pterygium through lymphangiogenesis and the VEGF-C/VEGFR-3 pathway as a novel therapeutic target for the human pterygium.

Key words: VEGF-C, VEGFR-3, lymphangiogenesis, lymphatic vessel density, pterygium.

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1 **Introduction**

2 A pterygium is an elevated, superficial, external ocular mass that usually
3 forms over the perilimbal conjunctiva, and extends onto the corneal surface.
4 Pathologically, a pterygium is a proliferative, invasive, and highly
5 vascularized tissue.(Gebhardt et al., 2005) Kase *et al.* demonstrated that
6 proliferation activity was high in the pterygial epithelium compared to that
7 in the normal conjunctiva.(Kase et al., 2007a; Kase et al., 2007b) Moreover,
8 biological tissue growth and invasion are of importance in the pathology of
9 pterygium . (Bai et al., 2010) Indeed, there are transformed cells in
10 pterygial tissue, which is one of the characteristics of a tumor
11 phenotype.(Spandidos et al., 1997) Recently, it has been demonstrated that
12 significant preneoplastic lesions may be associated with the pterygium
13 (Chui et al., 2011), indicating that pterygia display tumor-like features.
14

15 The vascular endothelial growth factor (VEGF) family is a group of ligands
16 for the endothelial cell-specific VEGF tyrosine kinase receptors. These
17 growth factors play pivotal roles in the regulation of vascular and
18 lymphatic growth. Among the VEGF family, VEGF-C and VEGF-D can
19 stimulate the growth of lymphatic vessels, a process called
20 lymphangiogenesis, which contributes to the pathology of various human
21 disorders such as tumors and inflammation.(Achen et al., 1998; Joukov et
22 al., 1996) VEGF-C is exclusively essential for the initial sprouting, and for

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the subsequent survival of lymphatic endothelial cells.(Karkkainen et al., 2004) VEGF-C is known to be a ligand for the endothelial cell–specific tyrosine kinase receptor, VEGFR-3. In normal adult tissues, VEGFR-3 is expressed predominantly in the lymphatic endothelial cells,(Partanen et al., 2000) while, in pathological situations like chronic wound healing, VEGFR-3 can be abnormally expressed on not only lymphatic but also blood vessel endothelium.(Paavonen et al., 2000; Witmer et al., 2001)

Most studies have supported the hypothesis that VEGF-C is of prognostic value, and promotes lymphatic tumor progression in various human cancers, including gastric, breast, colon, lung, head and neck, and ovarian carcinomas.(Herrmann et al., 2007; Jüttner et al., 2006; Miyata et al., 2006; Trojan et al., 2006) In the ophthalmology field, VEGF-C and its receptor, VEGFR-3, mediate human corneal lymphangiogenesis following corneal transplantation.(Cursiefen et al., 2002) Moreover, the blockade of VEGFR-3 reportedly led to the inhibition of corneal inflammatory lymphangiogenesis,(Yuen et al., 2011) suggesting that the VEGF-C/VEGFR-3 pathway may contribute to the discovery of a novel therapeutic target in ocular surface disorders. However, to the best of our knowledge, VEGF-C and VEGFR-3 expression has yet to be examined in the human pterygium. The aim of this study was to analyze the expression of VEGF-C and VEGFR-3 as well as lymphatic vessels in the pterygium

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and normal conjunctiva of humans.

Material and Methods

Preparation of human tissues

Fifteen patients with primary nasal pterygia, surgically removed, were enrolled in this study. Normal bulbar conjunctival tissues were obtained from three patients during cataract surgery. The tissues were then fixed in 4% paraformaldehyde. After fixation, slides were washed in phosphate-buffered saline (PBS), and processed for paraffin sectioning. Written informed consent was obtained according to the Declaration of Helsinki. All human experiments conformed with the requirements of the ethics committee of Hokkaido University Graduate School of Medicine.

Conjunctival and lymphatic endothelial cell lines

Cultured human conjunctival cells were purchased from American Culture Collections (ATCC). Human Dermal Lymphatic Endothelial cell line were purchased from Promo Cell. These cell line were maintained in complete medium (Endothelial cell Growth medium 2; Promo Cell) under a humidified atmosphere containing 5% CO2 at 37°C.

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1 **Immunofluorescence microscopic assay**

2 Dewaxed paraffin sections were immunostained using the complex method.
3 Formalin-fixed, paraffin-embedded serial tissue sections were cut at a 4 μm
4 thickness and endogenous peroxidase activity was inhibited by immersing
5 the slides in 3% hydrogen peroxide in methanol for 10 min. As a
6 pretreatment, microwave-based antigen retrieval was performed in PBS.
7 Then, non-specific binding of the primary antibody was blocked by
8 incubating the slides in blocking bovine serum for 30 min. The slides were
9 incubated with primary antibodies at room temperature for 2 h. The
10 antibodies used in this study were VEGF-C (AF752) and VEGFR-3
11 (AF349; R&D Systems, Abingdon, UK) at concentrations of 10 and 15
12 μg/ml, respectively, D2-40 (1:100; Dako, Carpinteria, CA, USA), VEGF-A
13 (1:100; abcam, Tokyo, Japan), and MUC5AC (1:200; abcam, Tokyo,
14 Japan). Positive signals of D2-40 were visualized using diaminobendizine
15 as a substrate. In double staining immunohistochemistry, the sections were
16 incubated with the above-mentioned first antibody, followed by the Alexa
17 Fluor® 546 goat anti-rabbit antibody for 30 min, and FITC- conjugated
18 anti-CD31 monoclonal antibody (a marker for blood endothelial cells)
19 (1:200 Abcam, Tokyo, Japan), or FITC- conjugated anti-CD68 monoclonal
20 antibody (a marker for macrophages) (1:200; abcam, Tokyo, Japan) for 30
21 min at room temperature. After washing, sections were mounted using
22 mounting media with 4’,6- diamino-2-phenylindole (DAPI; SlowFade®

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1 Gold antifade reagent with DAPI; Invitrogen, Eugene, OR, USA).
2 To investigate the correlation of VEGF-A or MUC5AC expression with
3 VEGF-C in pterygial tissues, immunohistochemistry was performed using
4 serial sections, with the Alexa Fluor® 546 goat anti-mouse secondary
5 antibody (1:500 dilution; Invitrogen). All slides were examined using a
6 Keyenece BZ-9000 (Keyence, Osaka, Japan) microscope.
7
8 **Evaluation of VEGF-C-positive cells**
9 In microscopic observation, we counted the total number of epithelial cells
10 and VEGF-C-positive cells of the pterygium and normal conjunctiva in
11 three fields under high power (objective lens: 40×). To calculate the
12 positive rate of VEGF-C expressed in the cytoplasm of epithelial cells, the
13 number of DAPI-positive nuclei (blue) in the epithelium was calculated in
14 the field. And then the number of cells showing cytoplasmic
15 immunoreactivity (red) was calculated under merged images with VEGF-C
16 and DAPI. Cells positively stained for anti-VEGF-C antibody were noted
17 based on the labeling index as a percentage (%) in each specimen, and the
18 measurements were averaged.
19 In six out of 15 pterygium samples where pterygial head and body could be
20 clearly separated during excision and fixation of the tissues, VEGF-C
21 immunohistochemistry is evaluated to determine the difference of the
22 immunolocalization. The results regarding VEGF-C in pterygial and

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normal conjunctival tissues are presented as the mean. In addition, VEGF-A -positive epithelial cells of the pterygium are also evaluated. The masked evaluation of histology was performed by J.F, S. K, and A. K.

Quantification of lymphatic and blood vessel densities

The lymphatic vessel density (LVD), detected by immunostaining for D2-40, was quantitatively analyzed as previously described.(Aishima et al., 2008) Briefly, areas with highly D2-40-positive vessels (hot spots) were identified by scanning the sections at low magnification (objective lens 10×); then, the number of D2-40-positive vessels was counted in three fields under high power (objective lens: 40×) for each case. The mean value for the three fields was calculated as the LVD for each pterygium or normal conjunctiva. Blood vessels were identified by immunostaining for CD31, in which the blood vessel density (BVD) was quantitatively analyzed as determined in the LVD. The LVD/BVD ratio was subsequently calculated in each case.

Western blot analysis

Four different subjects of pterygium and normal conjunctiva were surgically removed, and then were sonicated in lysis buffer (1× RIPA buffer; Cell Signaling Technology, Danvers, MA, USA) with protease inhibitor (Roche, Basel, Switzerland) on ice, and centrifuged at 13,500 rpm

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for 20 min at 4 °C. These were stored at −80 °C until being assayed. These samples were electrophoretically separated on SDS–PAGE using a 4% stacking and 10% separating gels. Proteins in gels were electro-transferred (80 V, 90 min, 4 °C) to Hybond-P polyvinylidene difluoride transfer membranes (GE Healthcare, Buckinghamshire, UK). After transfer, the membranes were incubated for 1 h in a blocking solution which consisted of 5% skim milk powder in PBS containing 1% tween (PBST), washed briefly in PBST, and then probed with anti-VEGF-C polyclonal antibody (1:500; described above) and anti-VEGFR-3 polyclonal antibody (1:500; described above) diluted in 5% BSA/TBST. The membrane was extensively washed in PBST for 30 min and incubated with a 1:1,000 dilution of the appropriate horseradish peroxidase-conjugated donkey anti-goat IgG at room temperature for 60 min. Then, it was placed in chemiluminescent reagent (ECL plus, GE Healthcare, Buckinghamshire, UK) and exposed to a luminescent image analyzer (Fujifilm, Tokyo, Japan). Quantification of protein expression was determined by densitometric analysis using Image J software.

VEGF-C production of cultured conjunctival cells

The cultured conjunctival epithelial cells were plated in fresh medium (2×10^4 cells/ml) per 60mm petri dish in serum-supplemented medium and were incubated. In confluent condition, serum-containing medium was

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removed and serum-free medium was added. The culture media were then harvested 0, 24 and 48 hours later, in which the concentration of VFGF-C was measured by using human VEGF-C ELISA kit (R&D Systems) according to the manufacturer's instructions.

In vitro cell proliferation assay

Human dermal lymphatic endothelial cells were seeded in 96-well plates at 100µl(2×10^4 cells/well), and 24 h later, 10µl of supernatant fluid containing VEGF-C described above was added to each well. The supernatant derived from cultured conjunctival epithelial cells, treated with serum-free condition decribed above, was harvested. Cell viability was assessed by a modified MTT assay using a Cell Counting Kit-8 (Dojin Laboratories, Japan) 24 and 48 hours later. Results were calculated as the percentage of viability according to the manufacturer's instructions.

Statistical analysis

Student's t-test was used for statistical comparison of the number of VEGF-C-positive cells, correlation with VEGF-A, LVD, and LVD/BVD ratio between pterygium and normal control groups. Differences between the means were considered significant when the probability values were <0.05.

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Results

LVD is high in pterygium

D2-40-positive lymphatic vessels were clearly seen in the stroma of the pterygium and normal conjunctiva (Figure 1, arrows). The LVD of the pterygium and normal conjunctiva was 8.2±2.8 and 4.8±0.3, respectively. The LVD was significantly higher in the pterygium than in the normal conjunctiva ($P < 0.001$, Table 1). In contrast, the LVD/BVD ratio of the pterygium (0.86±0.26) was not significant difference compared to that of the normal conjunctiva (0.71±0.05) ($P > 0.05$, Table 1).

VEGF-C is expressed in pterygium

The expression of VEGF-C in the pterygium and normal conjunctiva was evaluated by Western blot analysis and immunohistochemistry. Western blot demonstrated that VEGF-C protein expression was high in total proteins extracted from the pterygial tissues, whilst VEGF-C expression was less marked in normal conjunctival tissues (Figure 2A). Table 2 summarizes the immunohistochemical results of VEGF-C in human pterygial and normal conjunctival tissues. Immunoreactivity for VEGF-C was detected in all pterygial tissues and normal conjunctiva examined. VEGF-C immunoreactivity was detected in the cytoplasm of epithelial cells in the pterygium and normal conjunctiva (Figure 3A). A dot graph

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showed all data on VEGF-C-positive rates in pterygial and normal conjunctival tissues (Figure 3B), indicating that this histological difference of LVD might appear gradually. In contrast, immunolocalization of VEGF-C-positive cells between pterygial head (46%) and body (45%) epithelia was not statistically significant difference. Immunoreactivity for VEGF-A was also detected in all pterygial tissues (Figure 4A, B). The number of VEGF-A-immunopositive cells (54%) was significantly higher than that of VEGF-C (44%) (n=9, $P < 0.05$, Table 3). Immunoreactivity for VEGF-C was marginally detected in MUC5AC-positive goblet cells in pterygial epithelium (Figure 4E-H, arrows), and a few CD68-positive macrophages infiltrating the pterygial tissue stroma (Figure 4I-L, arrows).

VEGFR-3 is expressed in lymphatic vessels in pterygium

Expression of VEGFR-3 was evaluated by Western blot analysis and immunohistochemistry. As shown in Figure 2, Western blot showed that VEGFR-3 protein expression was strongly detected in pterygial tissues compared to normal conjunctival tissues ($p<0.05$). VEGFR-3 immunoreactivity (Figure 5, red) was localized in the D2-40-positive lymphatic endothelial cells (Figure 5, green), whereas immunoreactivity for VEGFR-3 was not detected in blood vessel endothelial cells.

VEGF-C is secreted in serum-starved cultured conjunctival epithelial

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cells

To investigate whether conjunctival epithelial cells produce VEGF-C, we measured the levels of the VEGF-C protein concentration in the culture medium at 0, 24, and 48 hours after replacement with serum-free medium. Protein concentrations of VEGF-C in the medium of 48 hours (188.0 pg/mg) were significantly higher than those of 0 hour (75.2pg/mg , n=3, $p<0.05$, Figure 6A).

VEGF-C promotes lymphatic endothelial cell proliferation

To investigate the role of VEGF-C in pterygium pathogenesis, we studied the effects of VEGF-C derived from conjunctival epithelial cells on cell proliferation of cultured lymphatic endothelial cells in vitro. After incubating with the supernatants 24 and 48 hours after replacement of serum-free medium described above, cell viability was then assessed. The former and latter supernatants were considered low and high VEGF-C concentrations, respectively. Addition of the supernatant obtained from low VEGF-C concentration did not have any significant effects on lymphatic cell viability examined. However, addition of supernatant obtained from high VEGF-C concentration had an increase in lymphatic cell proliferation at 24 hours (n=4, $p<0.05$, Figure 6B).

Discussion

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In this study, we demonstrated that VEGF-C was expressed in pterygial tissues using Western blot and immunohistochemistry. Moreover, immunoreactivity for VEGF-C was clearly detected in pterygial epithelial cells. As shown in Table 2, the present study demonstrated that the number of VEGF-C-positive cells in pterygial epithelium was significantly higher than those in normal conjunctival epithelium.

It has been demonstrated that VEGF-C promotes tumor progression.(Liu et al., 2011) In fact, the pterygium displays tumor-like features.(Chui et al., 2011; Spandidos et al., 1997) VEGF-A is the most important member of VEGF family, and is known to strongly express in pterygial tissues with significant mitogenesis and cell migration. We also examined immunoreactivity for VEGF-C together with VEGF-A as a comparative study. Indeed, VEGF-A-positive rate in pterygium epithelia was significantly higher than VEGF-C. Therefore, it is indisputable that VEGF-A plays a critical role in the various pathologies. On the other hand, the results on relatively low number of VEGF-C-immunopositive pterygial epithelial cells may reflect on more specific roles of VEGF-C in the pathology like lymphangiogenesis.

The present study demonstrated that the LVD in the pterygium was significantly higher than that in the normal conjunctiva. These results are

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consistent with a recently published report indicating lymphangiogenesis in human pterygial tissues.(Cimpean et al., 2011) Moreover, we analyzed LVD/BVD ratio, which showed no significant difference in human pterygial and normal conjunctival tissues in this study. These data suggest that not only angiogenesis but also lymphangiogenesis plays a critical role in the pathology of pterygium. On the other hand, it has been demonstrated that the inhibition of VEGF-C expression reduces lymphangiogenesis.(Chen et al., 2005) In our investigation, the LVD, LVD/BVD ratio, and number of VEGF-C-positive epithelial cells were significantly higher in the pterygium than normal conjunctiva, suggesting that VEGF-C expression is correlated with the LVD in the pterygium.

As we showed above, VEGF-C was expressed in human pterygial epithelium; however, the regulation of VEGF-C protein expression is not well understood. Recently, it was demonstrated that the expression levels of cyclooxygenase (COX)-2 were correlated with VEGF-C protein expression, and lymphangiogenesis.(Iwata et al., 2007) On the other hand, Chiang *et al.* previously stated that COX-2 was expressed in pterygial epithelium, (Chiang et al., 2007) suggesting that it may be a candidate key molecule for the regulation of VEGF-C expression. Further studies are needed to clarify the mechanism underlying VEGF-C regulation in the pterygium.

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In adults, VEGFR-3 expression is limited to the lymphatic vessels.(Kaipainen et al., 1995) Under pathological conditions like chronic wound healing, VEGFR-3 is abnormally expressed on not only lymphatic but also blood vessel endothelium.(Paavonen et al., 2000; Witmer et al., 2001) In this study, we confirmed that VEGFR-3 was exclusively expressed in lymphatic vessel endothelium in human pterygial tissues. VEGF-C is a known ligand for VEGFR-3, and the overexpression of VEGF-C increases intratumoral lymphangiogenesis in breast cancer cells.(Skobe et al., 2001) Therefore, the present data suggest that VEGF-C produced by pterygial epithelial cells led to lymphangiogenesis though binding to VEGFR-3.

To investigate the role of VEGF-C in pterygium pathogenesis, we performed in vitro study of cell proliferation in lymphatic endothelial cells. Indeed, VEGFR-3 expression in the lymphatic vessels is well known. (Kaipainen et al., 1995) We revealed that cultured conjunctival epithelial cells secreted VEGF-C protein 48 hours incubation after serum-starved condition. It is well known that the cellular stress such as exposure to ultraviolet causes pterygium pathogenesis, suggesting that VEGF-C could be secreted from the conjuntival epithelium during the onset of pterygium. Addition of supernatant containing relatively high VEGF-C protein

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concentration drived from conjunctival epithelium had an increase in lymphatic endothelial cell proliferation. These results indicate that VEGF-C secreted from epithelial cells contributes to the lymphangiogenesis in pterygium.

VEGF-C expression is associated with the tumor phenotype, possibly making it an attractive therapeutic target. Silencing of VEGF-C suppressed tumor cell growth, migration, and invasion in vitro; tumor growth and lymphangiogenesis were suppressed by the venous injection of shRNA against VEGF-C in vivo.(Feng et al., 2011) Moreover, Padera *et al*. investigated the effects of the VEGFR-3 tyrosine kinase inhibitor vandetanib on the suppression of tumor growth, and the agent significantly delayed tumor growth.(Padera et al., 2008) The suppression of VEGFR-3 and its ligand VEGF-C may contribute to the regression of pterygium progression and lymphangiogenesis.

In colorectal cancer study, VEGF-C and VEGF-D were identified as biomarkers for the resistance of Avastin.(Hu et al., 2007) Enkvetchakul et al. reported that intralesional Avastin had a therapeutic effect on reduction of tissue size of primary pterygium.(Enkvetchakul et al., 2011) The refore, VEGF-C/VEGFR-3 pathway may be an adjunct therapeutic target for pterygia, especially, for Avastin-resistant pterygia.

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The limitation of this study is that this study was not available using human cultured pterygial epithelial cells and lymphatic vessels. Establishment of a definite isolation system in these cells should develop the study of lymphangiogenesis in pterygial pathology. In addition, mouse model of opthalmic pterygium has yet to be available. If it will be possible in the future, blockade of VEGF-C/VEGFR-3 pathway by chemical agants may prove inhibition of pterygium development in vivo.

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References

Achen MG, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor

For Peer Review

3 (Flt4). Proc Natl Acad Sci U S A 95:548-553

Aishima S, Nishihara Y, Iguchi T, Taguchi K, Taketomi A, Maehara Y, Tsuneyoshi M (2008) Lymphatic spread is related to VEGF-C expression and D2-40-positive myofibroblasts in intrahepatic cholangiocarcinoma. Mod Pathol 21:256-264

Bai H, Teng Y, Wong L, Jhanji V, Pang CP, Yam GH (2010) Proliferative and migratory aptitude in pterygium. Histochem Cell Biol 134:527-535

Chen Z, Varney ML, Backora MW, Cowan K, Solheim JC, Talmadge JE, Singh RK (2005) Down-regulation of vascular endothelial cell growth factor-C expression using small interfering RNA vectors in mammary tumors inhibits tumor lymphangiogenesis and spontaneous metastasis and enhances survival. Cancer Res 65:9004-9011

Chiang CC, Cheng YW, Lin CL, Lee H, Tsai FJ, Tseng SH, Tsai YY (2007) Cyclooxygenase 2 expression in pterygium. Mol Vis 13:635-638

Chui J, Coroneo MT, Tat LT, Crouch R, Wakefield D, Di Girolamo N (2011) Ophthalmic pterygium: a stem cell disorder with premalignant features. Am J Pathol 178:817-827

Cimpean AM, Poenaru Sava M, Raica M, Ribatti D (2011) Preliminary evidence of the presence of lymphatic vessels immunoreactive for D2-40 and Prox-1 in human pterygium. Oncol Rep

Cursiefen C, Schlötzer-Schrehardt U, Küchle M, Sorokin L, Breiteneder-Geleff S, Alitalo K, Jackson D (2002) Lymphatic vessels in

For Peer Review

vascularized human corneas: immunohistochemical investigation using LYVE-1 and podoplanin. Invest Ophthalmol Vis Sci 43:2127-2135

Enkvetchakul O, Thanathanee O, Rangsin R, Lekhanont K, Suwan-Apichon O (2011) A randomized controlled trial of intralesional bevacizumab injection on primary pterygium: preliminary results. Cornea 30:1213-1218

Feng Y, Hu J, Ma J, Feng K, Zhang X, Yang S, Wang W, Zhang J, Zhang Y (2011) RNAi-mediated silencing of VEGF-C inhibits non-small cell lung cancer progression by simultaneously down-regulating the CXCR4, CCR7, VEGFR-2 and VEGFR-3-dependent axes-induced ERK, p38 and AKT signalling pathways. Eur J Cancer

Gebhardt M, Mentlein R, Schaudig U, Pufe T, Recker K, Nölle B, Al-Samir K, Geerling G, Paulsen FP (2005) Differential expression of vascular endothelial growth factor implies the limbal origin of pterygia. Ophthalmology 112:1023-1030

Herrmann E, Eltze E, Bierer S, Köpke T, Görge T, Neumann J, Hertle L, Wülfing C (2007) VEGF-C, VEGF-D and Flt-4 in transitional bladder cancer: relationships to clinicopathological parameters and long-term survival. Anticancer Res 27:3127-3133

Hu WG, Li JW, Feng B, Beveridge M, Yue F, Lu AG, Ma JJ, Wang ML, Guo Y, Jin XL, Zheng MH (2007) Vascular endothelial growth factors C and D represent novel prognostic markers in colorectal carcinoma using

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quantitative image analysis. Eur Surg Res 39:229-238

Iwata C, Kano MR, Komuro A, Oka M, Kiyono K, Johansson E, Morishita Y, Yashiro M, Hirakawa K, Kaminishi M, Miyazono K (2007) Inhibition of cyclooxygenase-2 suppresses lymph node metastasis via reduction of lymphangiogenesis. Cancer Res 67:10181-10189

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 15:1751

Jüttner S, Wissmann C, Jöns T, Vieth M, Hertel J, Gretschel S, Schlag PM, Kemmner W, Höcker M (2006) Vascular endothelial growth factor-D and its receptor VEGFR-3: two novel independent prognostic markers in gastric adenocarcinoma. J Clin Oncol 24:228-240

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K (1995) Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. Proc Natl Acad Sci U S A 92:3566-3570

Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K (2004) Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. Nat Immunol 5:74-80

Kase S, Osaki M, Sato I, Takahashi S, Nakanishi K, Yoshida K, Ito H,

For Peer Review

Ohno S (2007a) Immunolocalisation of E-cadherin and beta-catenin in human pterygium. Br J Ophthalmol 91:1209-1212

Kase S, Takahashi S, Sato I, Nakanishi K, Yoshida K, Ohno S (2007b) Expression of p27(KIP1) and cyclin D1, and cell proliferation in human pterygium. Br J Ophthalmol 91:958-961

Liu P, Zhou J, Zhu H, Xie L, Wang F, Liu B, Shen W, Ye W, Xiang B, Zhu X, Shi R, Zhang S (2011) VEGF-C promotes the development of esophageal cancer via regulating CNTN-1 expression. Cytokine 55:8-17

Miyata Y, Kanda S, Ohba K, Nomata K, Eguchi J, Hayashida Y, Kanetake H (2006) Tumor lymphangiogenesis in transitional cell carcinoma of the upper urinary tract: association with clinicopathological features and prognosis. J Urol 176:348-353

Paavonen K, Puolakkainen P, Jussila L, Jahkola T, Alitalo K (2000) Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. Am J Pathol 156:1499-1504

Padera TP, Kuo AH, Hoshida T, Liao S, Lobo J, Kozak KR, Fukumura D, Jain RK (2008) Differential response of primary tumor versus lymphatic metastasis to VEGFR-2 and VEGFR-3 kinase inhibitors cediranib and vandetanib. Mol Cancer Ther 7:2272-2279

Partanen TA, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M, Stacker SA, Achen MG, Alitalo K (2000) VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood

For Peer Review

vessels in human tissues. FASEB J 14:2087-2096

Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M (2001) Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med 7:192-198

Spandidos DA, Sourvinos G, Kiaris H, Tsamparlakis J (1997) Microsatellite instability and loss of heterozygosity in human pterygia. Br J Ophthalmol 81:493-496

Trojan L, Rensch F, Voss M, Grobholz R, Weiss C, Jackson DG, Alken P, Michel MS (2006) The role of the lymphatic system and its specific growth factor, vascular endothelial growth factor C, for lymphogenic metastasis in prostate cancer. BJU Int 98:903-906

Witmer AN, van Blijswijk BC, Dai J, Hofman P, Partanen TA, Vrensen GF, Schlingemann RO (2001) VEGFR-3 in adult angiogenesis. J Pathol 195:490-497

Yuen D, Pytowski B, Chen L (2011) Combined Blockade of VEGFR-2 and VEGFR-3 Inhibits Inflammatory Lymphangiogenesis in Early and Middle Stages. Invest Ophthalmol Vis Sci

For Peer Review

Figure legends

Figure 1

Immunohistochemistry for D2-40 in the human pterygium and normal conjunctiva. (A,B) The left panel is a normal conjunctiva (A) and the right panel is pterygium (B). Immunoreactivity for D2-40 proteins is found in the cellular membrane of lymphatic endothelial cells (black arrows). In contrast, immunoreactivity for D2-40 is not observed in blood vessels containing red blood cells in the lumen (red arrows). Immunofluorescence for D2-40 (*red*: E) and CD31 (*green*: D), and DAPI nuclear staining (*blue*: C) in the pterygium. Immunoreactivity of D2-40 is observed at the site of lymphatic vessels (E,F), but is not observed in CD31-positive blood vessel endothelial cells (D,F). The scale bar represents 100 μm.

Figure 2

Western blot analysis using anti-VEGF-C and anti-VEGFR-3 antibody. Protein expression of VEGF-C as well as VEGFR-3 is clearly detected in both pterygial and normal conjunctival tissue. Increased VEGF-C and VEGFR-3 expressions are observed in the pterygium (A). Quantification analysis reveals that VEGFR-3 expression is significantly higher in pterygium than conjunctiva (B, *: $P<0.05$).

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Figure 3

A: Immunohistochemistry for VEGF-C (*red*) and DAPI nuclear staining (*blue*) in the human pterygium and normal conjunctiva. VEGF-C immunoreactivity is observed in the cytoplasm of pterygial and normal conjunctival epithelia. The number of VEGF-C-positive cells is high in the pterygium. In the normal conjunctiva, however, the number of VEGF-C-positive cells is low. The scale bar represents 50 µm.

B: Dot graph in VEGF-C-positive rates in all cases of pterygia and normal conjunctivas examined. *: $P < 0.05$.

Figure 4

Immunoreactivity for VEGF-A (red; a, b), VEGF-C (red; c, d, g, h), and MUC5AC (red: e, f) in serial sections, and double staining immunohistochemistry with CD68 (green: I, l), VEGF-C (j, l) and DAPI nuclear staining (blue; b, d, f, h, k, l) in pterygium.

Immunoreactivity for VEGF-A is strongly detected in pterygial epithelium , where VEGF-C is partially expressed (a-d, arrow). Immunoreactivity for VEGF-C is also detected in MUC5AC-positive goblet cells in pterygial epithelium (e-h, arrows). CD68, a marker for macrophage, reveals co-localization with VEGF-C in pterygial tissue stroma (i-l, arrows). The scale bar represents 50 µm.

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Figure 5

Double staining immunohistochemistry was performed for VEGFR-3 (red) and D2-40 (green) in pterygial tissue. VEGFR-3 immunoreactivity is colocalized with D2-40 –positive lymphatic vessels in the pterygium. The scale bar represents 50 μm.

Figure 6

A: Alteration of VEGF-C concentrations in the supernatants of cultured conjunctival epithelial cells. VEGF-C concentration was assessed by using VEGF-C ELISA kit 0, 24, and 48 hours after replacement of serum-free medium.Concentration of VEGF-C protein in the medium of 48 hour is significantly higher than that of 0 hour (*n*=3, *: $P < 0.05$).

B: In vitro cell proliferation assay in cultured lymphatic endothelial cells, treated with the supernatants derived from cultured conjunctival epithelial cells. After incubating with the supernatants 24 and 48 hours after replacement of serum-free medium described above, cell viability was then assessed. The former and latter supernatants are considered low and high VEGF-C concentrations (conc.), respectively. Cell viability of lymphatic endothelial cells treated with high VEGF-C conc. is significantly higher than that of low VEGF-C conc. at 24 hour. (*n*=4, *, $P < 0.05$)

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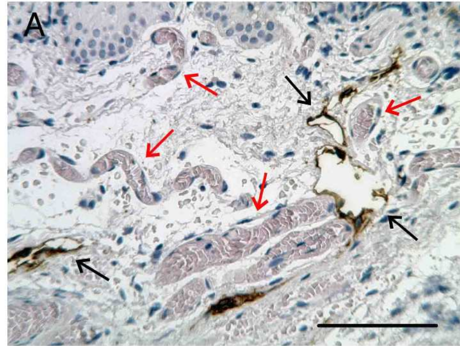

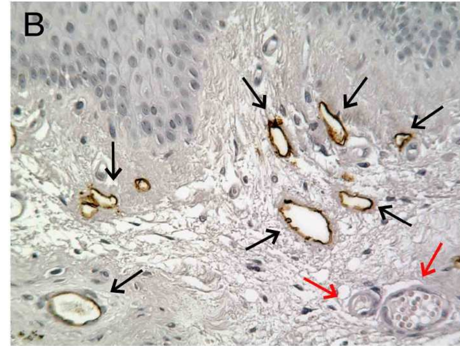

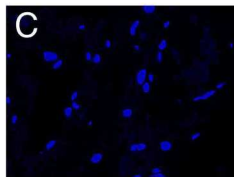

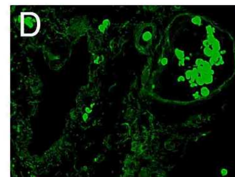

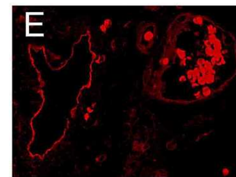

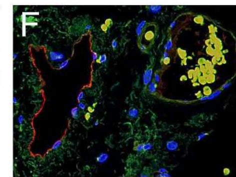

250x170mm (300 x 300 DPI)

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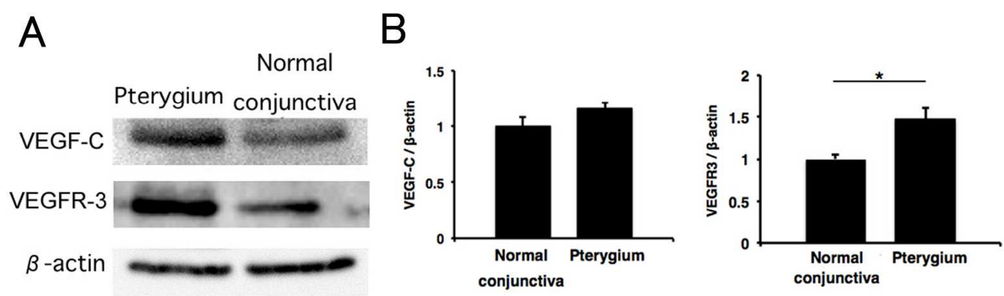

Western blot analysis using anti-VEGF-C and anti-VEGFR-3 antibody.
254x81mm (300 x 300 DPI)

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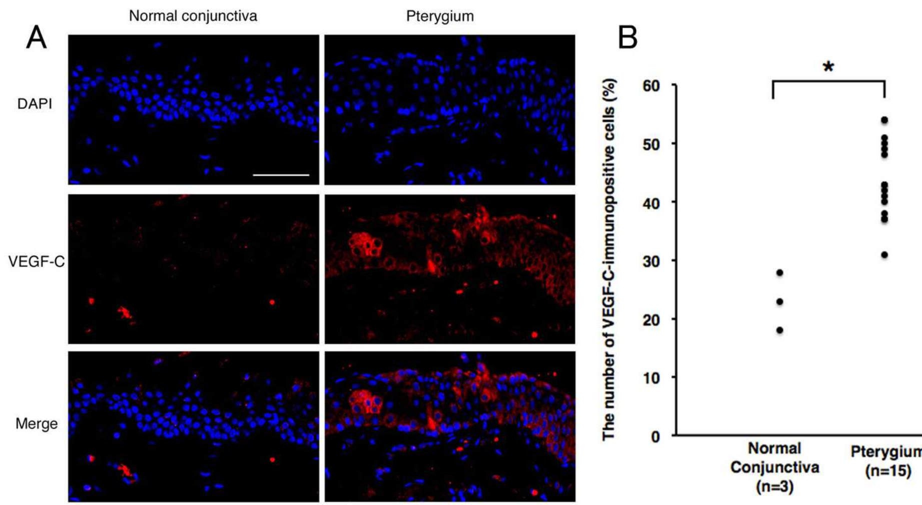

Immunohistochemistry for VEGF-C and DAPI nuclear staining in the human pterygium and normal conjunctiva.
143x74mm (300 x 300 DPI)

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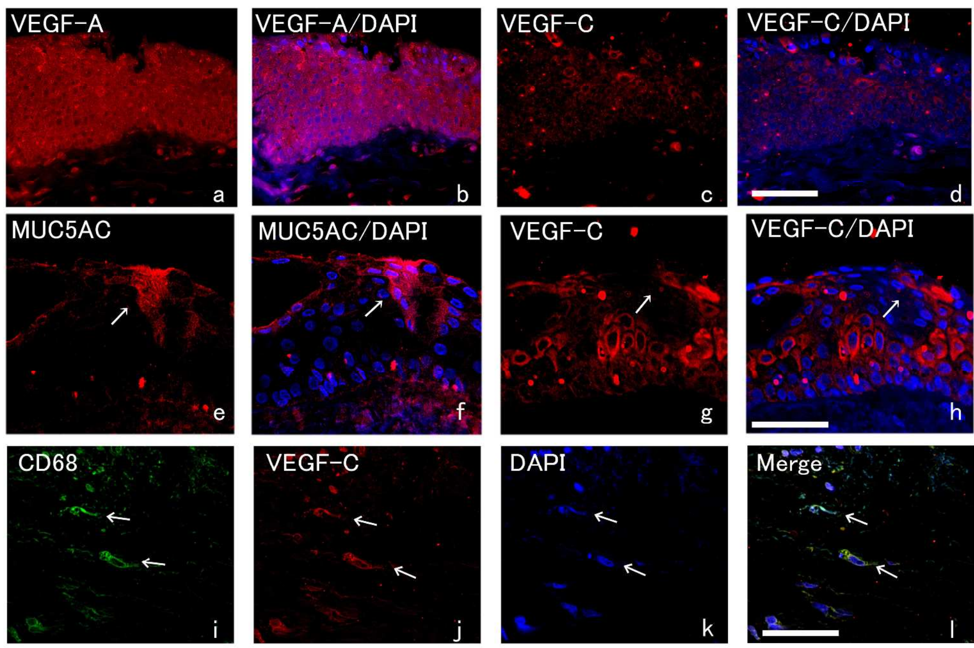

Immunoreactivity for VEGF-A (red; a, b), VEGF-C (red; c, d, g, h), and MUC5AC (red: e, f) in serial sections, and double staining immunohistochemistry with CD68 (green: I, l), VEGF-C (j, l) and DAPI nuclear staining (blue; b, d, f, h, k, l) in pterygium.
338x224mm (300 x 300 DPI)

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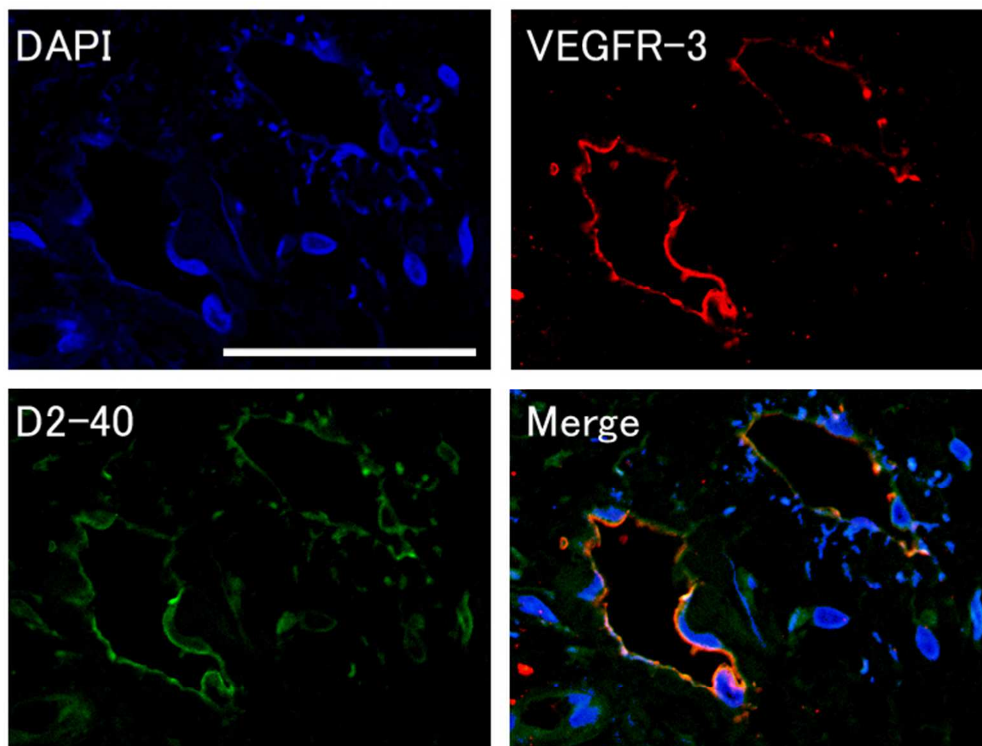

Double staining immunohistochemistry was performed for VEGFR-3 (red) and D2-40 (green) in pterygial tissue.
62x50mm (300 x 300 DPI)

view

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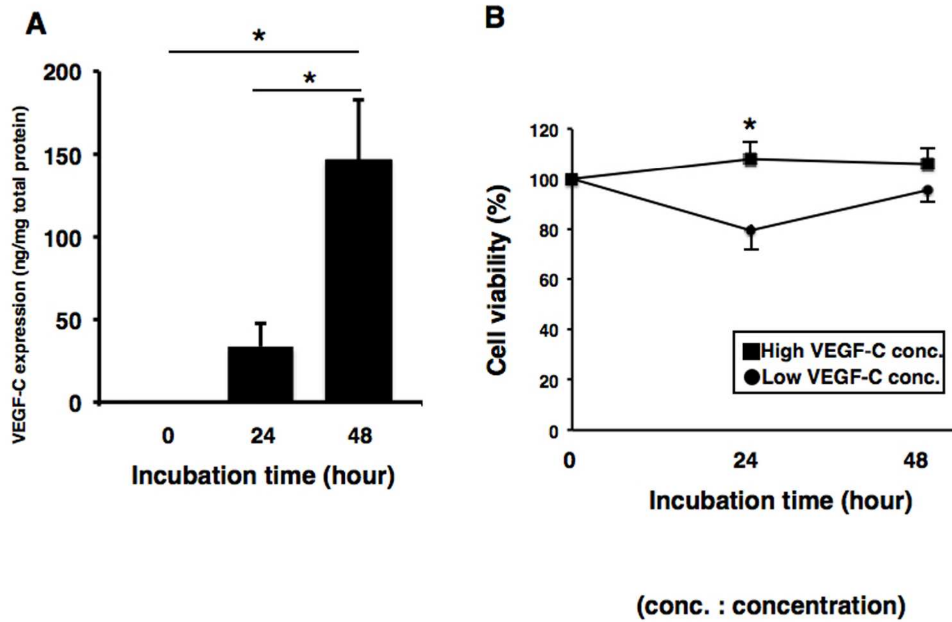

A: Alteration of VEGF-C concentrations in the supernatants of cultured conjunctival epithelial cells. B: In vitro cell proliferation assay in cultured lymphatic endothelial cells, treated with the supernatants derived from cultured conjunctival epithelial cells.
282x181mm (300 x 300 DPI)

Review

Table1. Lymphovascular density and blood vessel density in human pterygial and normal conjunctival tissues

		Lymphovascular density (LVD)	LDV/blood vessel density
Pterygium	(n=15)	8.2±2.8	0.86±0.26
Normal conjunctiva	(n=3)	4.8±0.3	0.71±0.05
	p-values	<0.001	n.s

Mean±SD

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Table2.The number of VEGF-C-immunopositive cells in pterygial and normal conjunctival cells.

Pterygium				Normal conjunctiva			
No.	Gender	Age (years)	VEGF-C(%)	No.	Gender	Age (years)	VEGF-C (%)
1	M	72	43	1	M	54	18
2	M	83	31	2	F	70	23
3	F	75	49	3	M	80	28
4	M	71	38				
5	M	68	37				
6	M	77	51				
7	M	69	37				
8	M	75	54				
9	F	84	43				
10	F	60	42				
11	F	66	41				
12	F	66	40				
13	M	73	48				
14	F	86	54				
15	M	75	50				
Mean			44				23

M, male; F, female.

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Table3. The number of VEGF-Cand VEGF−A-immunopositive cells in pterygial epithelial cells.

No.	Gender	Age (years)	VEGF-C(%)	VEGF-A(%)
1	M	72	43	53
2	M	83	31	39
3	F	75	49	59
4	M	71	38	63
5	M	68	37	48
6	M	77	51	58
7	M	69	37	51
8	M	75	54	66
9	F	84	43	48
Mean			44	54

M, male; F, female.

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