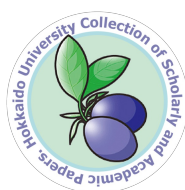




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1 Expression of vascular endothelial growth factor C in human pterygium.

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4 Junichi Fukuhara¹, Satoru Kase¹, Tsutomu Ohashi², Ryo Ando¹, Zhenyu
5 Dong¹, Kousuke Noda¹, Takeshi Ohguchi¹, Atsuhiko Kanda¹, Susumu
6 Ishida¹

7

8 ¹Department of Ophthalmology, Laboratory of Ocular Cell Biology and
9 Visual Science, Hokkaido University Graduate School of Medicine, Nishi
10 7, Kita 15, Kita-ku, Sapporo 060-8638, Japan

11 ²Ohashi Eye Center, Sapporo, Japan

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18 Address correspondence to: Satoru Kase
19 Department of Ophthalmology
20 Laboratory of Ocular Cell Biology & Visual Science
21 Hokkaido University Graduate School of Medicine
22 Nishi 7, Kita 15, Kita-ku, Sapporo 060-8638, Japan

23 Tel.: + 81 11 706 5944

24 Fax: + 81 11 706 5948

25 e-mail: kaseron@med.hokudai.ac.jp

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Abstract

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

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

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

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.

For Peer Review

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

1 the subsequent survival of lymphatic endothelial cells.(Karkkainen et al.,
2 2004) VEGF-C is known to be a ligand for the endothelial cell-specific
3 tyrosine kinase receptor, VEGFR-3. In normal adult tissues, VEGFR-3 is
4 expressed predominantly in the lymphatic endothelial cells,(Partanen et al.,
5 2000) while, in pathological situations like chronic wound healing,
6 VEGFR-3 can be abnormally expressed on not only lymphatic but also
7 blood vessel endothelium.(Paavonen et al., 2000; Witmer et al., 2001)
8
9 Most studies have supported the hypothesis that VEGF-C is of prognostic
10 value, and promotes lymphatic tumor progression in various human cancers,
11 including gastric, breast, colon, lung, head and neck, and ovarian
12 carcinomas.(Herrmann et al., 2007; Jüttner et al., 2006; Miyata et al., 2006;
13 Trojan et al., 2006) In the ophthalmology field, VEGF-C and its receptor,
14 VEGFR-3, mediate human corneal lymphangiogenesis following corneal
15 transplantation.(Cursiefen et al., 2002) Moreover, the blockade of
16 VEGFR-3 reportedly led to the inhibition of corneal inflammatory
17 lymphangiogenesis,(Yuen et al., 2011) suggesting that the
18 VEGF-C/VEGFR-3 pathway may contribute to the discovery of a novel
19 therapeutic target in ocular surface disorders. However, to the best of our
20 knowledge, VEGF-C and VEGFR-3 expression has yet to be examined in
21 the human pterygium. The aim of this study was to analyze the expression
22 of VEGF-C and VEGFR-3 as well as lymphatic vessels in the pterygium

1 and normal conjunctiva of humans.

2

3 **Material and Methods**

4

5 **Preparation of human tissues**

6 Fifteen patients with primary nasal pterygia, surgically removed, were
7 enrolled in this study. Normal bulbar conjunctival tissues were obtained
8 from three patients during cataract surgery. The tissues were then fixed in
9 4% paraformaldehyde. After fixation, slides were washed in
10 phosphate-buffered saline (PBS), and processed for paraffin sectioning.

11 Written informed consent was obtained according to the Declaration of
12 Helsinki. All human experiments conformed with the requirements of the
13 ethics committee of Hokkaido University Graduate School of Medicine.

14

15 **Conjunctival and lymphatic endothelial cell lines**

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

21

Immunofluorescence microscopic assay

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

Gold antifade reagent with DAPI; Invitrogen, Eugene, OR, USA).

To investigate the correlation of VEGF-A or MUC5AC expression with VEGF-C in pterygial tissues, immunohistochemistry was performed using serial sections, with the Alexa Fluor® 546 goat anti-mouse secondary antibody (1:500 dilution; Invitrogen). All slides were examined using a Keyence BZ-9000 (Keyence, Osaka, Japan) microscope.

Evaluation of VEGF-C-positive cells

In microscopic observation, we counted the total number of epithelial cells and VEGF-C-positive cells of the pterygium and normal conjunctiva in three fields under high power (objective lens: 40×). To calculate the positive rate of VEGF-C expressed in the cytoplasm of epithelial cells, the number of DAPI-positive nuclei (blue) in the epithelium was calculated in the field. And then the number of cells showing cytoplasmic immunoreactivity (red) was calculated under merged images with VEGF-C and DAPI. Cells positively stained for anti-VEGF-C antibody were noted based on the labeling index as a percentage (%) in each specimen, and the measurements were averaged.

In six out of 15 pterygium samples where pterygial head and body could be clearly separated during excision and fixation of the tissues, VEGF-C immunohistochemistry is evaluated to determine the difference of the immunolocalization. The results regarding VEGF-C in pterygial and

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

4
5 **Quantification of lymphatic and blood vessel densities**

6 The lymphatic vessel density (LVD), detected by immunostaining for
7 D2-40, was quantitatively analyzed as previously described.(Aishima et al.,
8 2008) Briefly, areas with highly D2-40-positive vessels (hot spots) were
9 identified by scanning the sections at low magnification (objective lens
10 10×); then, the number of D2-40-positive vessels was counted in three
11 fields under high power (objective lens: 40×) for each case. The mean
12 value for the three fields was calculated as the LVD for each pterygium or
13 normal conjunctiva. Blood vessels were identified by immunostaining for
14 CD31, in which the blood vessel density (BVD) was quantitatively
15 analyzed as determined in the LVD. The LVD/BVD ratio was subsequently
16 calculated in each case.

17
18 **Western blot analysis**

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

1 for 20 min at 4 °C. These were stored at –80 °C until being assayed. These
2 samples were electrophoretically separated on SDS–PAGE using a 4%
3 stacking and 10% separating gels. Proteins in gels were electro-transferred
4 (80 V, 90 min, 4 °C) to Hybond-P polyvinylidene difluoride transfer
5 membranes (GE Healthcare, Buckinghamshire, UK). After transfer, the
6 membranes were incubated for 1 h in a blocking solution which consisted
7 of 5% skim milk powder in PBS containing 1% tween (PBST), washed
8 briefly in PBST, and then probed with anti-VEGF-C polyclonal antibody
9 (1:500; described above) and anti-VEGFR-3 polyclonal antibody (1:500;
10 described above) diluted in 5% BSA/TBST. The membrane was
11 extensively washed in PBST for 30 min and incubated with a 1:1,000
12 dilution of the appropriate horseradish peroxidase-conjugated donkey
13 anti-goat IgG at room temperature for 60 min. Then, it was placed in
14 chemiluminescent reagent (ECL plus, GE Healthcare, Buckinghamshire,
15 UK) and exposed to a luminescent image analyzer (Fujifilm, Tokyo, Japan).

16 Quantification of protein expression was determined by densitometric
17 analysis using Image J software.

19 **VEGF-C production of cultured conjunctival cells**

20 The cultured conjunctival epithelial cells were plated in fresh medium
21 (2×10^4 cells/ml) per 60mm petri dish in serum-supplemented medium and
22 were incubated. In confluent condition, serum-containing medium was

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

5

6 **In vitro cell proliferation assay**

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

15

16 **Statistical analysis**

17 Student's t-test was used for statistical comparison of the number of
18 VEGF-C-positive cells, correlation with VEGF-A, LVD, and LVD/BVD
19 ratio between pterygium and normal control groups. Differences between
20 the means were considered significant when the probability values were
21 <0.05 .

1

2 **Results**

3 **LVD is high in pterygium**

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

11

12 **VEGF-C is expressed in pterygium**

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

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

13 **VEGFR-3 is expressed in lymphatic vessels in pterygium**

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

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

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

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

7
8 It has been demonstrated that VEGF-C promotes tumor progression.(Liu et
9 al., 2011) In fact, the pterygium displays tumor-like features.(Chui et al.,
10 2011; Spandidos et al., 1997) VEGF-A is the most important member of
11 VEGF family, and is known to strongly express in pterygial tissues with
12 significant mitogenesis and cell migration. We also examined
13 immunoreactivity for VEGF-C together with VEGF-A as a comparative
14 study. Indeed, VEGF-A-positive rate in pterygium epithelia was
15 significantly higher than VEGF-C. Therefore, it is indisputable that
16 VEGF-A plays a critical role in the various pathologies. On the other hand,
17 the results on relatively low number of VEGF-C-immunopositive pterygial
18 epithelial cells may reflect on more specific roles of VEGF-C in the
19 pathology like lymphangiogenesis.

20
21 The present study demonstrated that the LVD in the pterygium was
22 significantly higher than that in the normal conjunctiva. These results are

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

12
13 As we showed above, VEGF-C was expressed in human pterygial
14 epithelium; however, the regulation of VEGF-C protein expression is not
15 well understood. Recently, it was demonstrated that the expression levels
16 of cyclooxygenase (COX)-2 were correlated with VEGF-C protein
17 expression, and lymphangiogenesis.(Iwata et al., 2007) On the other hand,
18 Chiang *et al.* previously stated that COX-2 was expressed in pterygial
19 epithelium, (Chiang et al., 2007) suggesting that it may be a candidate key
20 molecule for the regulation of VEGF-C expression. Further studies are
21 needed to clarify the mechanism underlying VEGF-C regulation in the
22 pterygium.

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8 2 In adults, VEGFR-3 expression is limited to the lymphatic
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10 3 vessels.(Kaipainen et al., 1995) Under pathological conditions like chronic
11
12 4 wound healing, VEGFR-3 is abnormally expressed on not only lymphatic
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14 5 but also blood vessel endothelium.(Paavonen et al., 2000; Witmer et al.,
15
16 6 2001) In this study, we confirmed that VEGFR-3 was exclusively
17
18 7 expressed in lymphatic vessel endothelium in human pterygial tissues.
19
20 8 VEGF-C is a known ligand for VEGFR-3, and the overexpression of
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22 9 VEGF-C increases intratumoral lymphangiogenesis in breast cancer
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24 10 cells.(Skobe et al., 2001) Therefore, the present data suggest that VEGF-C
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26 11 produced by pterygial epithelial cells led to lymphangiogenesis though
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28 12 binding to VEGFR-3.
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37 14 To investigate the role of VEGF-C in pterygium pathogenesis, we
38
39 15 performed in vitro study of cell proliferation in lymphatic endothelial cells.
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41 16 Indeed, VEGFR-3 expression in the lymphatic vessels is well known.
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43 17 (Kaipainen et al., 1995) We revealed that cultured conjunctival epithelial
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45 18 cells secreted VEGF-C protein 48 hours incubation after serum-starved
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47 19 condition. It is well known that the cellular stress such as exposure to
48
49 20 ultraviolet causes pterygium pathogenesis, suggesting that VEGF-C could
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51 21 be secreted from the conjunctival epithelium during the onset of pterygium.
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53 22 Addition of supernatant containing relatively high VEGF-C protein
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1 concentration driven from conjunctival epithelium had an increase in
2 lymphatic endothelial cell proliferation. These results indicate that
3 VEGF-C secreted from epithelial cells contributes to the
4 lymphangiogenesis in pterygium.
5
6 VEGF-C expression is associated with the tumor phenotype, possibly
7 making it an attractive therapeutic target. Silencing of VEGF-C suppressed
8 tumor cell growth, migration, and invasion in vitro; tumor growth and
9 lymphangiogenesis were suppressed by the venous injection of shRNA
10 against VEGF-C in vivo.(Feng et al., 2011) Moreover, Padera *et al.*
11 investigated the effects of the VEGFR-3 tyrosine kinase inhibitor
12 vandetanib on the suppression of tumor growth, and the agent significantly
13 delayed tumor growth.(Padera et al., 2008) The suppression of VEGFR-3
14 and its ligand VEGF-C may contribute to the regression of pterygium
15 progression and lymphangiogenesis.
16
17 In colorectal cancer study, VEGF-C and VEGF-D were identified as
18 biomarkers for the resistance of Avastin.(Hu et al., 2007) Enkvetchakul et
19 al. reported that intralesional Avastin had a therapeutic effect on reduction of
20 tissue size of primary pterygium.(Enkvetchakul et al., 2011) The refore,
21 VEGF-C/VEGFR-3 pathway may be an adjunct therapeutic target for
22 pterygia, especially, for Avastin-resistant pterygia.

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8 2 The limitation of this study is that this study was not available using human
9
10 3 cultured pterygial epithelial cells and lymphatic vessels. Establishment of a
11
12 4 definite isolation system in these cells should develop the study of
13
14 5 lymphangiogenesis in pterygial pathology. In addition, mouse model of
15
16 6 opthalmic pterygium has yet to be available. If it will be possible in the
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18 7 future, blockade of VEGF-C/VEGFR-3 pathway by chemical agants may
19
20 8 prove inhibition of pterygium development in vivo.
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49 19 **References**

50
51 20 Achen MG, Jeltsch M, Kukk E, Mäkinen T, Vitali A, Wilks AF, Alitalo K,
52
53 21 Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a
54
55 22 ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor
56
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- 1 3 (Flt4). *Proc Natl Acad Sci U S A* 95:548-553
- 2 Aishima S, Nishihara Y, Iguchi T, Taguchi K, Taketomi A, Maehara Y,
3 Tsuneyoshi M (2008) Lymphatic spread is related to VEGF-C expression
4 and D2-40-positive myofibroblasts in intrahepatic cholangiocarcinoma.
5 *Mod Pathol* 21:256-264
- 6 Bai H, Teng Y, Wong L, Jhanji V, Pang CP, Yam GH (2010) Proliferative
7 and migratory aptitude in pterygium. *Histochem Cell Biol* 134:527-535
- 8 Chen Z, Varney ML, Backora MW, Cowan K, Solheim JC, Talmadge JE,
9 Singh RK (2005) Down-regulation of vascular endothelial cell growth
10 factor-C expression using small interfering RNA vectors in mammary
11 tumors inhibits tumor lymphangiogenesis and spontaneous metastasis and
12 enhances survival. *Cancer Res* 65:9004-9011
- 13 Chiang CC, Cheng YW, Lin CL, Lee H, Tsai FJ, Tseng SH, Tsai YY (2007)
14 Cyclooxygenase 2 expression in pterygium. *Mol Vis* 13:635-638
- 15 Chui J, Coroneo MT, Tat LT, Crouch R, Wakefield D, Di Girolamo N
16 (2011) Ophthalmic pterygium: a stem cell disorder with premalignant
17 features. *Am J Pathol* 178:817-827
- 18 Cimpean AM, Poenaru Sava M, Raica M, Ribatti D (2011) Preliminary
19 evidence of the presence of lymphatic vessels immunoreactive for D2-40
20 and Prox-1 in human pterygium. *Oncol Rep*
- 21 Cursiefen C, Schlötzer-Schrehardt U, Küchle M, Sorokin L,
22 Breiteneder-Geleff S, Alitalo K, Jackson D (2002) Lymphatic vessels in

1 vascularized human corneas: immunohistochemical investigation using
2 LYVE-1 and podoplanin. Invest Ophthalmol Vis Sci 43:2127-2135
3 Enkvetchakul O, Thanathanee O, Rangsin R, Lekhanont K,
4 Suwan-Apichon O (2011) A randomized controlled trial of intralesional
5 bevacizumab injection on primary pterygium: preliminary results. Cornea
6 30:1213-1218
7 Feng Y, Hu J, Ma J, Feng K, Zhang X, Yang S, Wang W, Zhang J, Zhang Y
8 (2011) RNAi-mediated silencing of VEGF-C inhibits non-small cell lung
9 cancer progression by simultaneously down-regulating the CXCR4, CCR7,
10 VEGFR-2 and VEGFR-3-dependent axes-induced ERK, p38 and AKT
11 signalling pathways. Eur J Cancer
12 Gebhardt M, Mentlein R, Schaudig U, Pufe T, Recker K, Nölle B, Al-Samir
13 K, Geerling G, Paulsen FP (2005) Differential expression of vascular
14 endothelial growth factor implies the limbal origin of pterygia.
15 Ophthalmology 112:1023-1030
16 Herrmann E, Eltze E, Bierer S, Köpke T, Gorge T, Neumann J, Hertle L,
17 Wülfing C (2007) VEGF-C, VEGF-D and Flt-4 in transitional bladder
18 cancer: relationships to clinicopathological parameters and long-term
19 survival. Anticancer Res 27:3127-3133
20 Hu WG, Li JW, Feng B, Beveridge M, Yue F, Lu AG, Ma JJ, Wang ML,
21 Guo Y, Jin XL, Zheng MH (2007) Vascular endothelial growth factors C
22 and D represent novel prognostic markers in colorectal carcinoma using

- 1 quantitative image analysis. *Eur Surg Res* 39:229-238
- 2 Iwata C, Kano MR, Komuro A, Oka M, Kiyono K, Johansson E, Morishita
- 3 Y, Yashiro M, Hirakawa K, Kaminishi M, Miyazono K (2007) Inhibition of
- 4 cyclooxygenase-2 suppresses lymph node metastasis via reduction of
- 5 lymphangiogenesis. *Cancer Res* 67:10181-10189
- 6 Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela
- 7 O, Kalkkinen N, Alitalo K (1996) A novel vascular endothelial growth
- 8 factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2)
- 9 receptor tyrosine kinases. *EMBO J* 15:1751
- 10 Jüttner S, Wissmann C, Jöns T, Vieth M, Hertel J, Gretscher S, Schlag PM,
- 11 Kemmner W, Höcker M (2006) Vascular endothelial growth factor-D and
- 12 its receptor VEGFR-3: two novel independent prognostic markers in gastric
- 13 adenocarcinoma. *J Clin Oncol* 24:228-240
- 14 Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH,
- 15 Dumont D, Breitman M, Alitalo K (1995) Expression of the fms-like
- 16 tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during
- 17 development. *Proc Natl Acad Sci U S A* 92:3566-3570
- 18 Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV,
- 19 Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K
- 20 (2004) Vascular endothelial growth factor C is required for sprouting of the
- 21 first lymphatic vessels from embryonic veins. *Nat Immunol* 5:74-80
- 22 Kase S, Osaki M, Sato I, Takahashi S, Nakanishi K, Yoshida K, Ito H,

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

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

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

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

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

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

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

- 1 vessels in human tissues. FASEB J 14:2087-2096
- 2 Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi
- 3 L, Alitalo K, Claffey K, Detmar M (2001) Induction of tumor
- 4 lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat
- 5 Med 7:192-198
- 6 Spandidos DA, Sourvinos G, Kiaris H, Tsampanlakis J (1997) Microsatellite
- 7 instability and loss of heterozygosity in human pterygia. Br J Ophthalmol
- 8 81:493-496
- 9 Trojan L, Rensch F, Voss M, Grobholz R, Weiss C, Jackson DG, Alken P,
- 10 Michel MS (2006) The role of the lymphatic system and its specific growth
- 11 factor, vascular endothelial growth factor C, for lymphogenic metastasis in
- 12 prostate cancer. BJU Int 98:903-906
- 13 Witmer AN, van Blijswijk BC, Dai J, Hofman P, Partanen TA, Vrensen GF,
- 14 Schlingemann RO (2001) VEGFR-3 in adult angiogenesis. J Pathol
- 15 195:490-497
- 16 Yuen D, Pytowski B, Chen L (2011) Combined Blockade of VEGFR-2 and
- 17 VEGFR-3 Inhibits Inflammatory Lymphangiogenesis in Early and Middle
- 18 Stages. Invest Ophthalmol Vis Sci
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10 3 Figure 1
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12 4 Immunohistochemistry for D2-40 in the human pterygium and normal
13 conjunctiva. (A,B) The left panel is a normal conjunctiva (A) and the right
14 panel is pterygium (B). Immunoreactivity for D2-40 proteins is found in
15 the cellular membrane of lymphatic endothelial cells (black arrows). In
16 contrast, immunoreactivity for D2-40 is not observed in blood vessels
17 containing red blood cells in the lumen (red arrows). Immunofluorescence
18 for D2-40 (*red*: E) and CD31 (*green*: D), and DAPI nuclear staining (*blue*:
19 C) in the pterygium. Immunoreactivity of D2-40 is observed at the site of
20 lymphatic vessels (E,F), but is not observed in CD31-positive blood vessel
21 endothelial cells (D,F). The scale bar represents 100 μ m.
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39 15 Figure 2
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41 16 Western blot analysis using anti-VEGF-C and anti-VEGFR-3 antibody.
42 Protein expression of VEGF-C as well as VEGFR-3 is clearly detected in
43 both pterygial and normal conjunctival tissue. Increased VEGF-C and
44 VEGFR-3 expressions are observed in the pterygium (A). Quantification
45 analysis reveals that VEGFR-3 expression is significantly higher in
46 pterygium than conjunctiva (B, *: P<0.05).
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1 Figure 3

2 A: Immunohistochemistry for VEGF-C (*red*) and DAPI nuclear staining
3 (*blue*) in the human pterygium and normal conjunctiva. VEGF-C
4 immunoreactivity is observed in the cytoplasm of pterygial and normal
5 conjunctival epithelia. The number of VEGF-C-positive cells is high in the
6 pterygium. In the normal conjunctiva, however, the number of
7 VEGF-C-positive cells is low. The scale bar represents 50 μ m.
8 B: Dot graph in VEGF-C-positive rates in all cases of pterygia and normal
9 conjunctivas examined. *: $P < 0.05$.

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11 Figure 4

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

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

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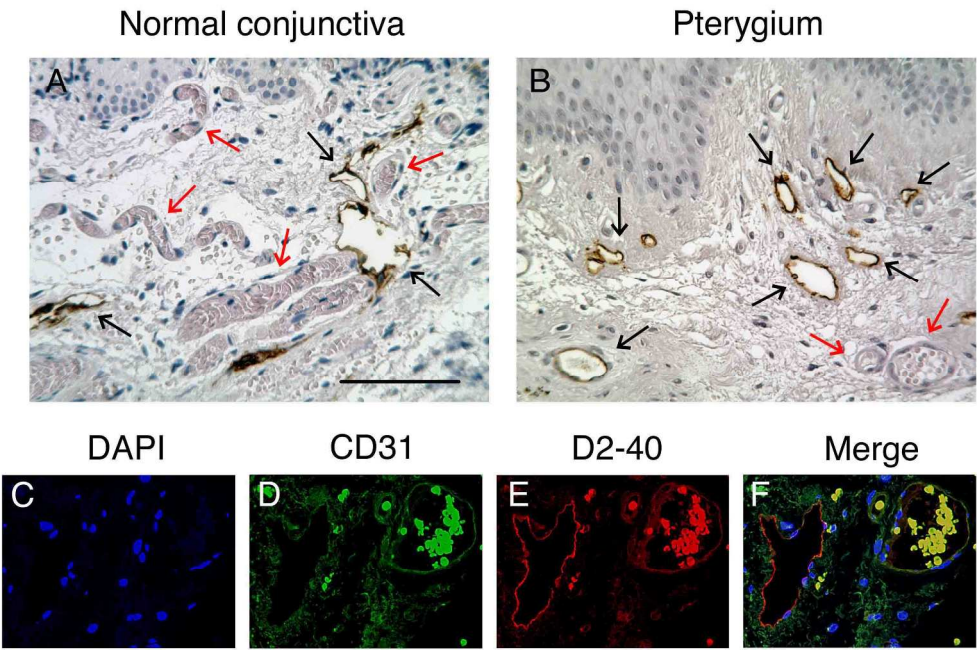
8 2 Double staining immunohistochemistry was performed for VEGFR-3 (red)
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10 3 and D2-40 (green) in pterygial tissue. VEGFR-3 immunoreactivity is
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12 4 colocalized with D2-40 –positive lymphatic vessels in the pterygium. The
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14 5 scale bar represents 50 μ m.
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22 8 A: Alteration of VEGF-C concentrations in the supernatants of cultured
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24 9 conjunctival epithelial cells. VEGF-C concentration was assessed by using
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26 10 VEGF-C ELISA kit 0, 24, and 48 hours after replacement of serum-free
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28 11 medium. Concentration of VEGF-C protein in the medium of 48 hour is
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30 12 significantly higher than that of 0 hour ($n=3$, *: $P < 0.05$).
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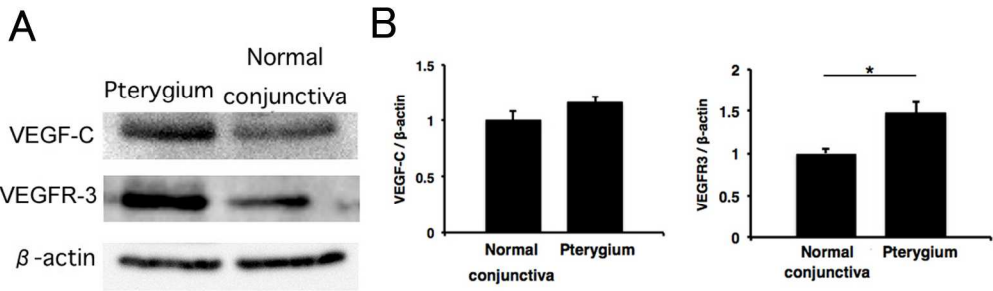
34 13 B: In vitro cell proliferation assay in cultured lymphatic endothelial cells,
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36 14 treated with the supernatants derived from cultured conjunctival epithelial
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38 15 cells. After incubating with the supernatants 24 and 48 hours after
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40 16 replacement of serum-free medium described above, cell viability was then
41
42 17 assessed. The former and latter supernatants are considered low and high
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44 18 VEGF-C concentrations (conc.), respectively. Cell viability of lymphatic
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46 19 endothelial cells treated with high VEGF-C conc. is significantly higher
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48 20 than that of low VEGF-C conc. at 24 hour. ($n=4$, *, $P < 0.05$)
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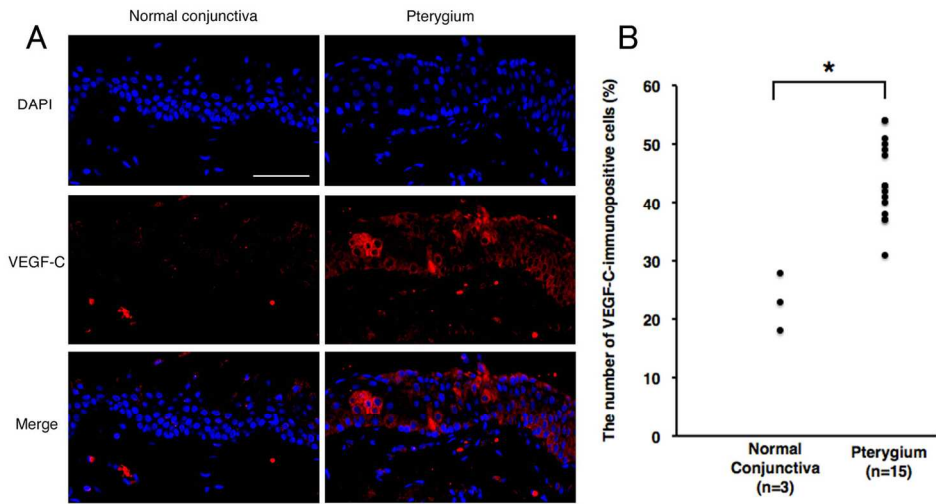


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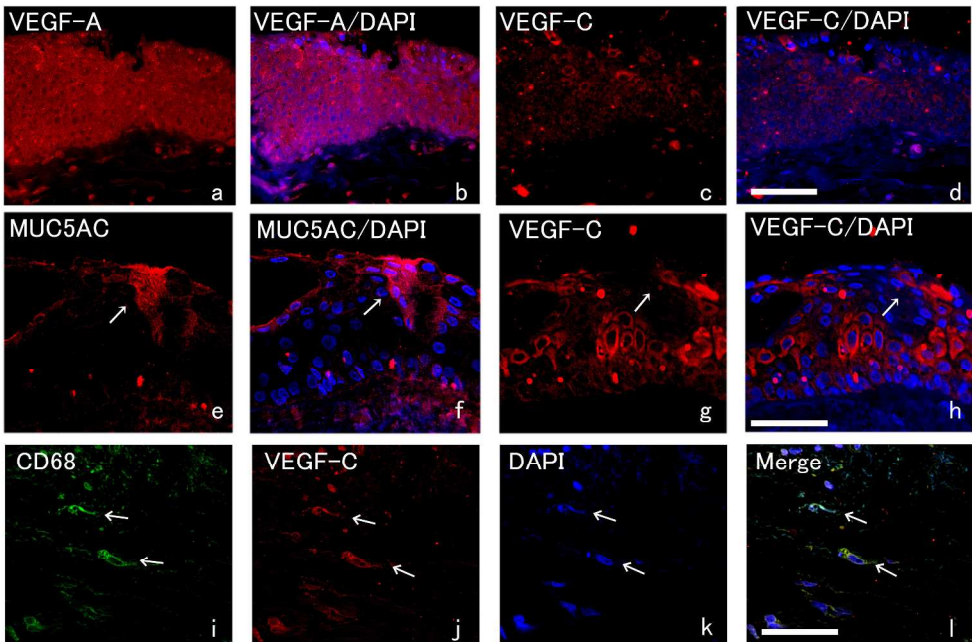
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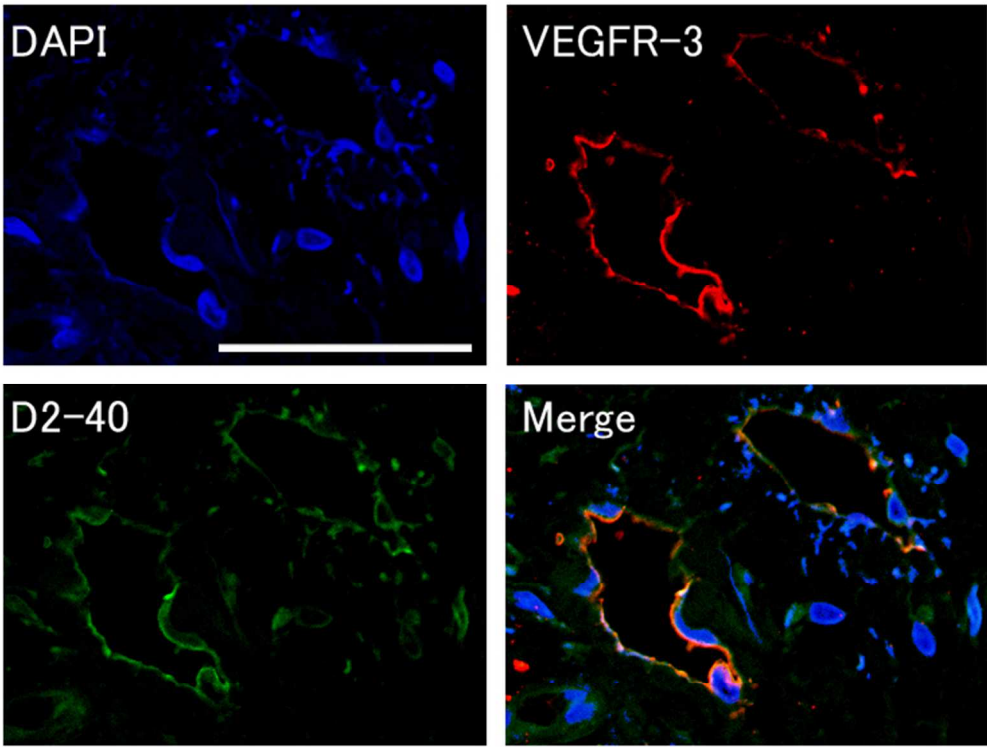
Western blot analysis using anti-VEGF-C and anti-VEGFR-3 antibody.
254x81mm (300 x 300 DPI)



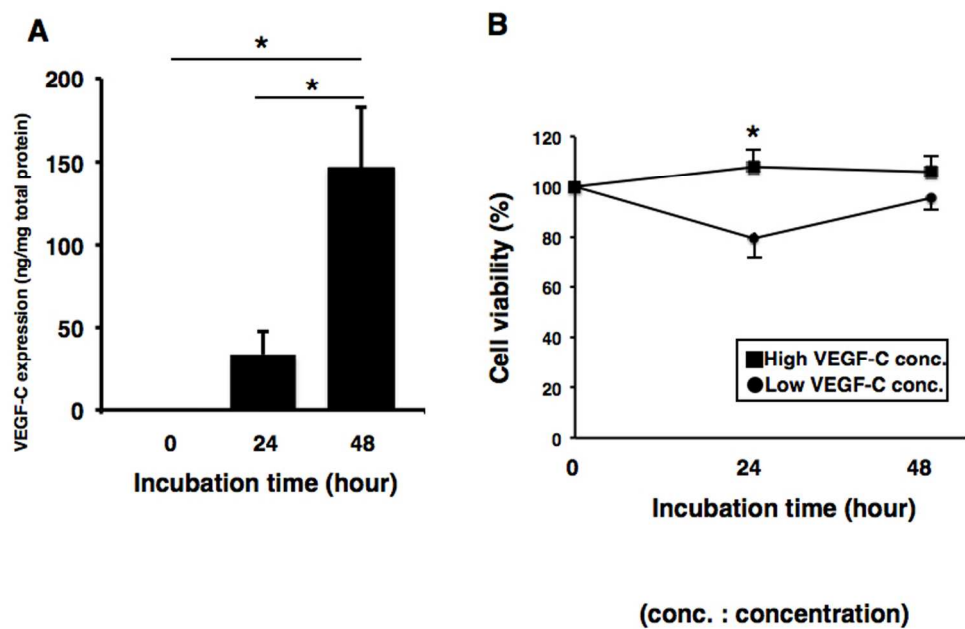
Immunohistochemistry for VEGF-C and DAPI nuclear staining in the human pterygium and normal conjunctiva.
143x74mm (300 x 300 DPI)



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)



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



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)

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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

For Peer Review

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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.

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.