Expression of Vascular Endothelial Growth Factor in Human Ocular Adnexal Lymphoma

Satoshi Kinoshita, Satoru Kase, Ryo Ando, Zhenyu Dong, Junichi Fukuhara, Yoko Dong, Saori Inafuku, Kousuke Noda, Mika Noda, Atsuhiro Kanda, and Susumu Ishida

Laboratory of Ocular Cell Biology and Visual Science, Department of Ophthalmology, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan

Correspondence: Satoru Kase, Department of Ophthalmology, Hokkaido University Graduate School of Medicine, N7, W15, Kita-ku, Sapporo, Hokkaido 060-8638, Japan; kaseron@med.hokudai.ac.jp.

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Purpose. To examine the expression of VEGF in extranodal marginal zone B-cell lymphoma (EMZL) and reactive lymphoid hyperplasia (RLH) of human ocular adnexa, and analyze the correlation with the intratumoral microvessel density (MVD).

Methods. Twenty-two EMZL and 16 RLH tissues were examined in this study. Paraformaldehyde-fixed, paraffin-embedded tissue sections were processed for immunohistochemistry with antibodies against VEGF and CD20. Vascular endothelial growth factor expression was analyzed using the ELISA and RT-PCR in the EMZL tissues. Microvessel density was determined based on the immunoreactivity for anti-CD34 antibody.

Results. Vascular endothelial growth factor immunoreactivity was detected in the cytoplasm of lymphoid cells in EMZL and RLH. ELISA and RT-PCR confirmed VEGF protein and mRNA expressions in the EMZL tissue, respectively. Vascular endothelial growth factor–immunopositive rate in B-cells was significantly higher in 12 conjunctival EMZLs than four RLHs ($P < 0.01$) and 10 orbital EMZLs than 12 RLHs ($P < 0.05$). The MVD showed a significant positive correlation with the VEGF-immunopositive rate in conjunctival and orbital EMZLs.

Conclusions. This study demonstrated increased VEGF expression in human conjunctival and orbital EMZL compared with that in RLH, suggesting that VEGF plays a significant role in the pathogenesis and tumor angiogenesis of ocular adnexal lymphoma.

Keywords: marginal zone b-cell lymphoma, reactive lymphoid hyperplasia, conjunctival neoplasm, orbital neoplasm, angiogenesis, vascular endothelial growth factor

Extranodal marginal zone B-cell lymphoma (EMZL) is a common malignant tumor of the conjunctiva in humans; the frequency ranges from 55% to 75% in all ocular adnexal lymphomas.[1,2] The conjunctiva is the most common site of the ocular adnexa in which EMZL arises, followed by the orbit, lacrimal gland, lacrimal sac, and eyelid.[1] The clinical appearance of conjunctival EMZL typically reveals a "salmon-patch" lesion. Primary symptoms of orbital EMZL are proptosis, lid swelling, palpable mass, and ocular discomfort.[3] To make a definite diagnosis of these ocular adnexal EMZLs, pathological examination of the lesions is often used.[3] Extranodal marginal zone B-cell lymphoma develops as a result of prolonged antigen stimulation and disruption of B lymphocyte clonality, leading to monoclonal B-cell proliferation.[4,5] In contrast, reactive lymphoid hyperplasia (RLH) is a benign polyclonal lymphoproliferation, which can be difficult to differentiate morphologically from EMZL in some cases because the symptoms and appearance of RLH can be similar to that of EMZL.[6] We recently demonstrated that conjunctival EMZL could arise from RLH.[7,8]

Vascular endothelial growth factor (VEGF) is known to be not only an angiogenic factor, but also a tumor growth factor, which is expressed in various tumors including systemic lymphoma.[9] Xie et al.[10] demonstrated that VEGF was highly expressed in human primary gastric lymphoma, and the intratumoral microvessel density (MVD) was positively correlated with VEGF expression. These data suggest that VEGF expressed in lymphoma cells correlates with tumor angiogenesis. However, VEGF expression and MVD have yet to be evaluated in conjunctival and orbital EMZL.

In this study, we examined the expression of VEGF in human ocular adnexal EMZL and RLH, and analyzed the correlation with MVD.

Methods

Preparation of Human Tissues

Twenty-two ocular adnexal EMZLs and 16 RLHs, consisting of 12 conjunctival and 10 orbital EMZLs, and 4 conjunctival and 12 orbital RLHs, respectively, were studied. All these tumors were partially removed in each case. After fixation in 4% paraformaldehyde, the slides were washed in PBS and processed for paraffin sectioning. Informed consent was obtained according to the Declaration of Helsinki. All human samples conformed to the requirements of the ethics committee of Hokkaido University Graduate School of Medicine (Sapporo, Hokkaido, Japan).

Immunohistochemistry

Dewaxed paraffin sections were blocked with bovine serum albumin (BSA) and incubated with mouse monoclonal antibody against human VEGF (1:50 dilution; Abcam, Cam-

bridge, MA, USA) at room temperature for 2 hours, followed by Alexa Fluor 488 or 546 anti-mouse antibody (1:200 dilution; Life Technologies, Carlsbad, CA, USA) at room temperature for 30 minutes, and FITC-conjugated mouse monoclonal antibody against human CD20 (1:50 dilution; Exbio, Prague, Czech Republic) at room temperature for 1 hour. Finally, sections were mounted with mounting media with 4′, 6-diamino-2-phenylindole (DAPI; Life Technologies). To evaluate VEGF-positive lymphoid cells, the VEGF immunoreactivity was classified as negative, weak, and strong. Strong immunoreactivity was evaluated as VEGF-positive in this study. We examined all fields of specimens and determined high VEGF-positive fields at low power magnification (objective lens: 10×), and counted VEGF-immunopositive cells at high power magnification (objective lens: 40×) in two or three fields. The VEGF-positive rate was determined as the number of VEGF-positive cells colocalized with CD20-positive in the total number of CD20-positive cells in each specimen, as reported previously.[11] The VEGF-positive rate was averaged by two calculations blindly conducted by two authors (Satoshi Kinoshita and Satoru Kase).

To determine the MVD in EMZL tissues, the specimens were blocked with BSA, followed by peroxidase block solution. Mouse monoclonal antibody against human CD34 (1:100 dilution; Abcam), an endothelial cell marker, was incubated at room temperature for 2 hours. Visualization was performed using the Envision HRP kit (DAKO, Carpinteria, CA, USA). All slides were examined using a Keyence BZ-9000 (Keyence, Osaka, Japan) microscope. Microvessel density in tumor tissues was measured with the "hot spot" method.[12] Briefly, we examined all fields of specimens and determined high-density fields showing CD34-positive at low power magnification (objective lens: 10×), and then counted the number of microvessels of the two or three high-density fields under high power magnification (objective lens: 40×). Microvessel density (vessels/mm^2) was calculated as mean number of microvessels per area of observed fields.

Diagnosis of EMZL and RLH

Histologic evaluation by hematoxylin and eosin (H&E) staining was conducted in all tissues to ensure the presence of small- to medium-sized atypical lymphoid cells and lymphoepithelial lesions. Immunohistochemistry with anti-CD20, CD3, CD5, CD10 (DAKO), and cyclin D1, as well as the detection of κ/γ deviation were analyzed. Immunoglobulin heavy chain (IgH) gene rearrangement was determined by Southern blot analysis or PCR methods, and flow cytometry was applied to confirm the B-cell monoclonality in the tumor tissue, as described previously.[4] Systemic examinations included positron emission tomography–computed tomography, magnetic resonance imaging (MRI), and bone marrow puncture.

Enzyme-Linked Immunosorbent Assay (ELISA)

Four EMZL tissues, surgically removed, were washed in PBS to remove blood, and then sonicated in radio-immunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Danvers, MA, USA) with protease inhibitor (Roche, Basel, Switzerland) on ice, and centrifuged at 17,730*g* for 20 minutes at 4°C. The supernatant fluid was stored at −80°C until assayed. The VEGF level in the fluid was determined with ELISA kits for human VEGF (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols. The total protein concentration was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA)

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from nine formalin-fixed, paraffin-embedded (FFPE) tissue sections, consisting of five orbital EMZLs and four conjunctival EMZLs (High Pure FFPE RNA Micro Kit; Roche Applied Science, Indianapolis, IN, USA).

Reverse transcription was performed with GoScrip Reverse Transcriptase (Promega, Madison, WI, USA) and oligo dT(20) primers, as described previously.[13] To investigate gene expression of conjunctival and orbital EMZLs, RT-PCR analyses were performed with the following primers for human genes used: *VEGF* (forward 5′- AGT CCA ACA TCA CCA TGC AG –3′; reverse 5′- TTC CCT TTC CTC GAA CTG ATT T –3′), and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*; forward 5′- CCT GGC CAA GGT CAT CCA TG –3′; reverse 5′- GGA AGG CCA TGC CAG TGA GC –3′).

Statistical Analysis

All results are expressed as mean ± SD with n-numbers as indicated. Student's *t*-test was used for statistical comparison between the groups. Differences between the means were considered significant when the probability values were less than 0.05. Spearman's correlation coefficient was used to examine the correlation between VEGF expression and MVD.

Results

Morphological and Histopathologic Characteristics of EMZL and RLH

Slit-lamp examination revealed conjunctival tumors with a salmon-patch appearance both in EMZL (Fig. 1A) and RLH (Fig. 1B). In an MRI scan, T1-weighted images demonstrated large tumors located within the exrtaocular muscles in EMZL (Fig. 1C) and in the lacrimal grand in RLH (Fig. 1D).

Extranodal marginal zone B-cell lymphomas histologically demonstrated small to medium-sized atypical lymphoid cell infiltration in the stroma (Figs. 1E, 1G). Particularly, in conjunctival EMZL, atypical lymphoid cells invaded the conjunctival epithelium (lymphoepithelial lesions; Fig. 1E). The lymphoma cells were CD20-positive in the EMZL tissue and reactive CD3-positive small lymphocytes were intermingled. No EMZL tissues contained CD5-, CD10-, or cyclin D1-positive atypical lymphoid cells in this study. Together with histologic examinations, 13 cases demonstrated IgH gene rearrangement, one case showed monoclonal B-cell proliferation analyzed by flow cytometry, and four cases revealed marked κ/γ deviation with immunostaining. All patients with EMZL were classified as Stage 1E or 2E based on the Ann Arbor classification following systemic examination. Reactive lymphoid hyperplasia tissues histologically showed relatively loose cellularity made up of mononuclear cell infiltration without cellular atypia (Figs. 1F, 1H). Lymphoid follicles, fibrosis, and infiltration by eosionophils were observed in several cases of orbital RLH. Immunoreactivity for CD20 was detected in the infiltrated mononuclear cells, while RLH showed no lymphoepithelial lesions or IgH gene rearrangement in this study.

Expression of VEGF in EMZL and RLH

Vascular endothelial growth factor immunoreactivity was strongly detected in the cytoplasm of atypical lymphoid cells in EMZL (Fig. 2A). In ELISA, we also detected VEGF expression of ocular adnexal EMZL tissues: the concentration

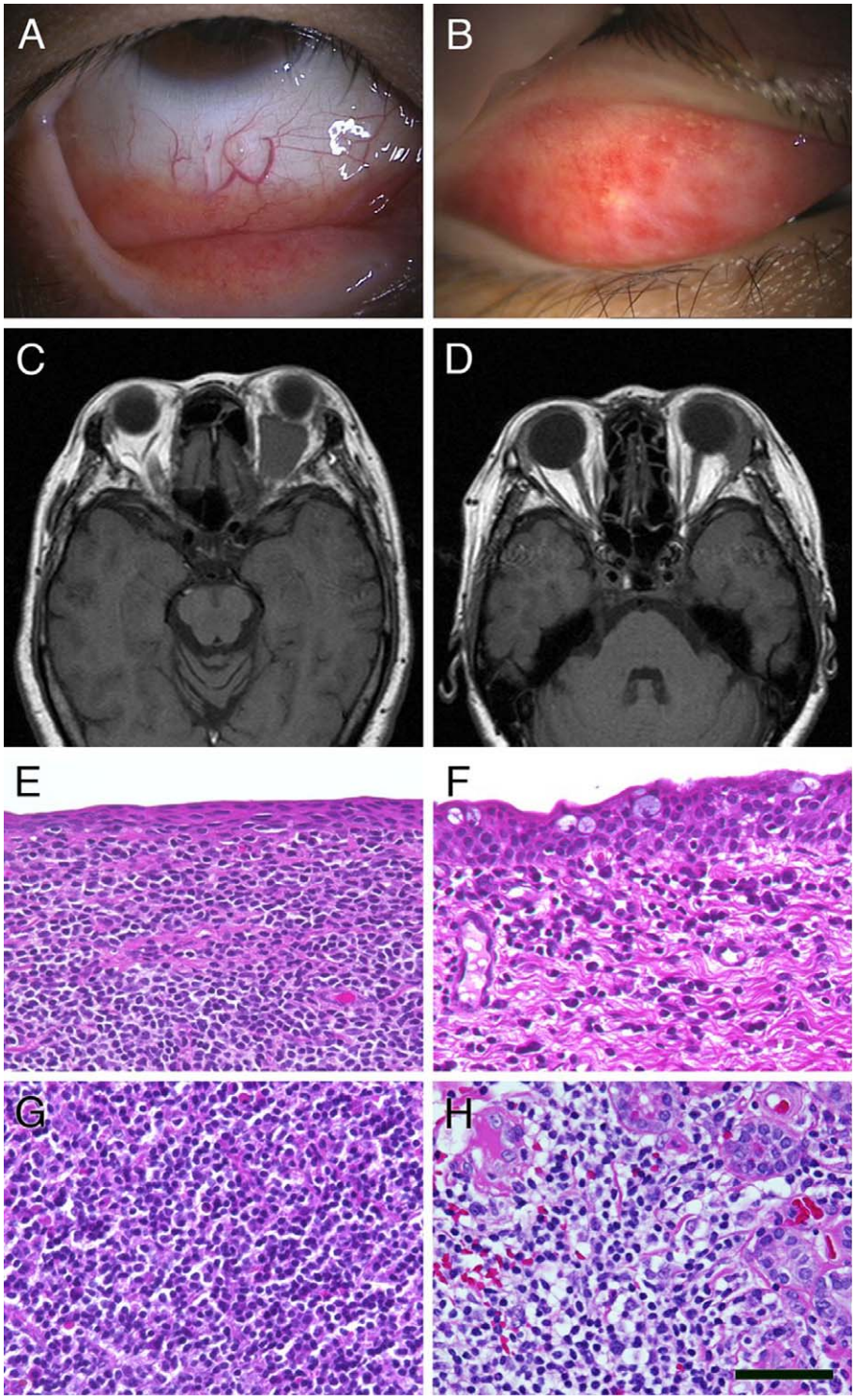

FIGURE 1. Slit-lamp examination in conjunctival EMZL (**A**) and RLH (**B**), MRI scan in orbital EMZL (**C**) and RLH (**D**), H&E staining in conjunctival EMZL (**E**) and RLH (**F**), and orbital EMZL (**G**), and RLH (**H**). Slit-lamp examination reveals a conjunctival tumor with a salmon-patch appearance (**A**, **B**). In an MRI scan of EMZL (**C**) and RLH (**D**), T1-weighted images show a mass lesion between rectus muscles and a lesion located in the lacrimal grand, respectively. Extranodal marginal zone B-cell lymphoma tissues show dense infiltration of lymphoid cells with mild nuclear membrane abnormalities and attenuation of the epithelium (**E**, **G**). Reactive lymphoid hyperplasia tissues show loose mononuclear cell infiltration without cellular atypia (**F**, **H**). *Scale bar*, 50 μm.

being 146.5 pg/mg total protein in a conjunctival EMZL. In contrast, the mean concentration was 19.6 pg/mg total protein in three orbital EMZLs. *Vascular endothelial growth factor* mRNA expression was also detected in conjunctival and orbital EMZL tissues, verified by RT-PCR (Fig. 2C). Vascular endothelial growth factor immunoreactivity colocalized with several CD20-positive cells (Figs. 3A–D; arrowheads) in EMZL tissues. Vascular endothelial growth factor

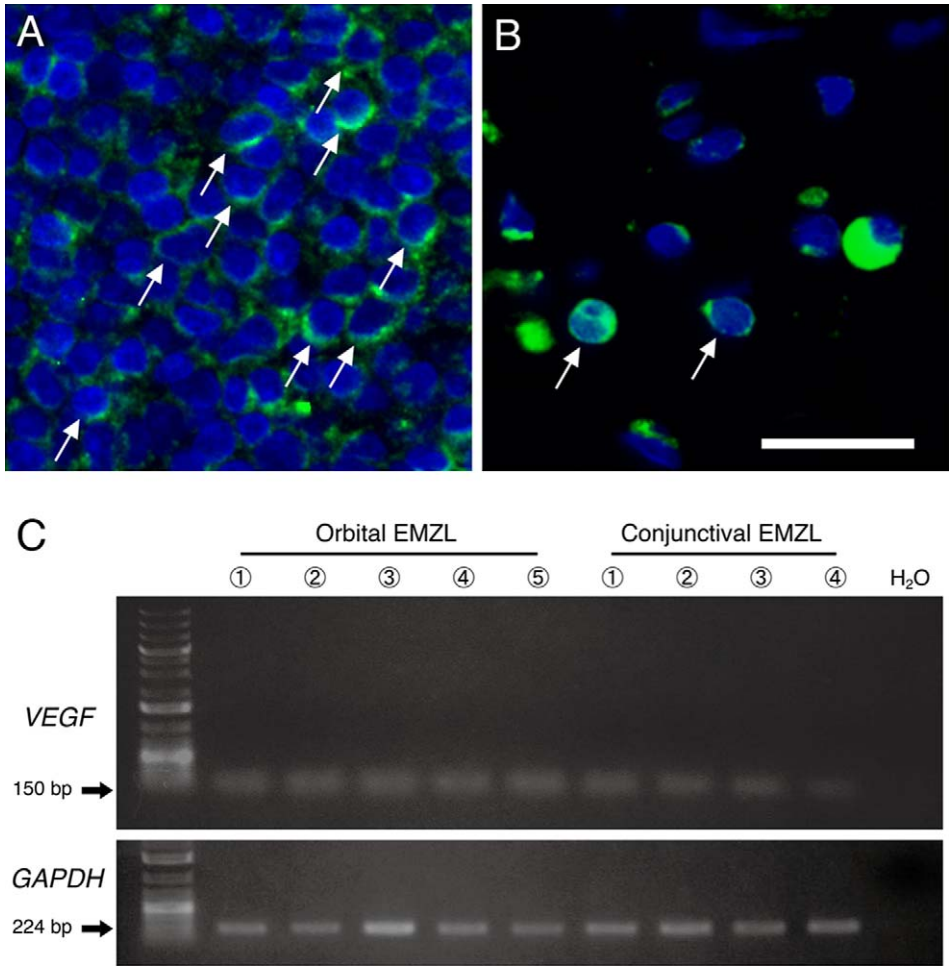

Figure 2. Immunoreactivity for VEGF (**A**, **B**; *green*) and DAPI nuclear staining (**A**, **B**; *blue*) in conjunctival EMZL (**A**), RLH (**B**), and VEGF gene expression by PCR (**C**). Immunofluorescence staining shows VEGF immunoreactivity in the cytoplasm of mononuclear cells in conjunctival EMZL (**A**, *arrows*) and RLH (**B**, *arrows*). *Scale bar*, 20 μm. (**C**) Gene expression analysis of *VEGF* in orbital and conjunctival EMZLs. Glyceraldehyde 3-phosphate dehydrogenase was used to evaluate RNA quality and to normalize for quantity.

immunoreactivity was also noted in the cytoplasm of lymphocytes in RLH tissues (Fig. 2B), where immunoreactivity for CD20 colocalized (data not shown). In the immunohistochemical results of VEGF in EMZL and RLH tissues, VEGF-immunopositive rate in B cells was significantly higher in EMZL than in RLH tissues in all ocular adnexal tumors (EMZLs, 45.4 ± 11.9 %, $n = 22$; RLHs, 26.8 ± 7.0 %, $n = 16$; $P < 0.00001$). Moreover, the higher expression was also noted in the orbit (EMZLs, 34.5 ± 5.6 %, $n = 10$; RLHs, 28.5 ± 6.3 %, $n = 12$; $P < 0.05$) and conjunctiva (EMZLs, 54.4 ± 7.3 %, $n = 12$; RLHs, 21.6 ± 6.2 %, $n = 4$; $P < 0.01$; Table. 1). In EMZL tissues, although VEGF-immunopositive rate showed no significant difference between clinical stages (1E or 2E; orbital EMZLs, $P = 0.69$; conjunctival EMZLs, $P = 0.32$), the higher expression was found in conjunctival EMZLs (54.4 ± 7.3 %) compared with orbital EMZLs (34.5 ± 5.6 %, $P < 0.05$).

Intratumoral Microvessels in EMZL

To determine the microvessels in EMZL tissues, we performed immunohistochemical staining with anti-CD34 antibody and counted MVD (Table 2). There were vascular-rich tumor tissues containing abundant microvessels (Fig. 4A), and relatively hypovascular tumor tissues in cases with EMZL (Fig. 4B). The MVD of EMZL tissues showed a positive correlation with the VEGF-positive rate both in the orbit ($r^2 = 0.252$, Fig. 4C) and conjunctiva ($r^2 = 0.820$, Fig. 4C). However, the MVD showed no correlation with the number of CD20-positive cells (orbital EMZLs, $r^2 = 0.00031$; conjunctival EMZLs, $r^2 = 0.0552$).

Discussion

Extranodal marginal zone B-cell lymphoma is a common ocular adnexal malignant tumor; however, the mechanisms underly-

Table 1. The Rates of VEGF-Positive B Lymphocytes (%) in Ocular Adnexal Lymphoid Tumors

	Conjunctiva	Orbit	Total
EMZL	54.4 ± 7.3 ($n = 12$)	34.5 ± 5.6 ($n = 10$)	45.4 ± 11.9 ($n = 22$)
RLH	21.6 ± 6.2 ($n = 4$)	28.5 ± 6.3 ($n = 12$)	26.8 ± 7.0 ($n = 16$)

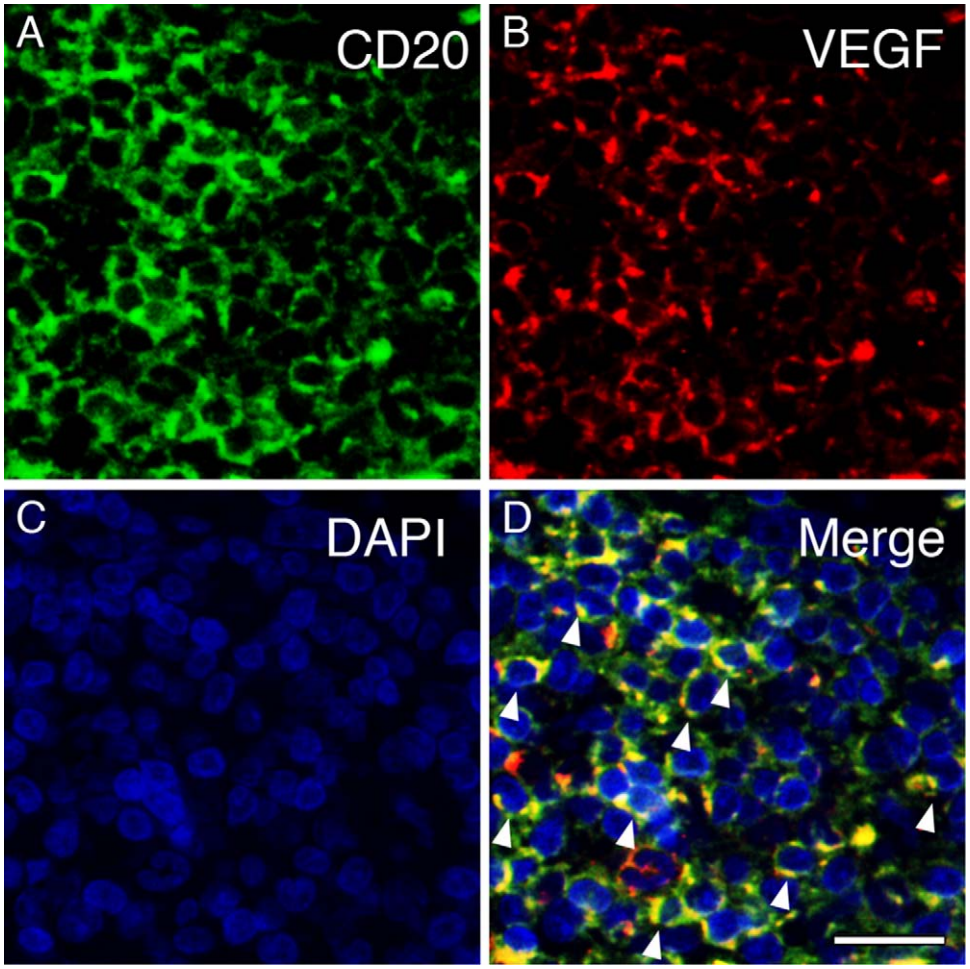

Figure 3. Double-staining immunofluorescence image in conjunctival EMZL with CD20 (**A**, **D**; *green*), VEGF (**B**, **D**; *red*), and DAPI nuclear staining (**C**, **D**; *blue*). *Arrowheads* indicate colocalization. *Scale bar*, 20 μm.

Table 2. Clinicopathologic Profiles of Patients With Ocular Adnexal EMZL Examined in This Study

N	Age, y	Sex	Side	Clinical Stage	Location	VEGF-Positive Rate, %	MVD, vessels/mm²
1	55	F	R	2E	Conjunctiva	55.7	30.4
2	63	F	R	1E	Conjunctiva	47.6	35.5
3	76	M	R	2E	Conjunctiva	62.1	45.6
4	45	M	R	1E	Conjunctiva	48.3	15.2
5	63	F	R	1E	Conjunctiva	47.8	10.1
6	67	F	R	1E	Conjunctiva	61.2	81.1
7	55	F	L	1E	Conjunctiva	52.2	35.5
8	53	F	L	2E	Conjunctiva	56.6	55.7
9	53	F	R	2E	Conjunctiva	44.8	10.1
10	45	F	R	2E	Conjunctiva	55.0	50.7
11	45	F	L	2E	Conjunctiva	71.8	106.4
12	35	F	L	1E	Conjunctiva	50.8	15.2
13	66	F	R	2E	Orbit	29.2	64.2
14	80	F	R	1E	Orbit	35.1	67.6
15	69	M	R	2E	Orbit	26.3	50.7
16	56	M	R	1E	Orbit	31.6	37.2
17	52	F	L	2E	Orbit	28.3	74.3
18	55	F	R	1E	Orbit	33.1	114.8
19	62	F	R	2E	Orbit	43.9	98.0
20	71	M	L	1E	Orbit	36.2	87.8
21	68	F	L	1E	Orbit	41.8	81.1
22	72	M	R	1E	Orbit	40.4	77.7

M, male; F, female.

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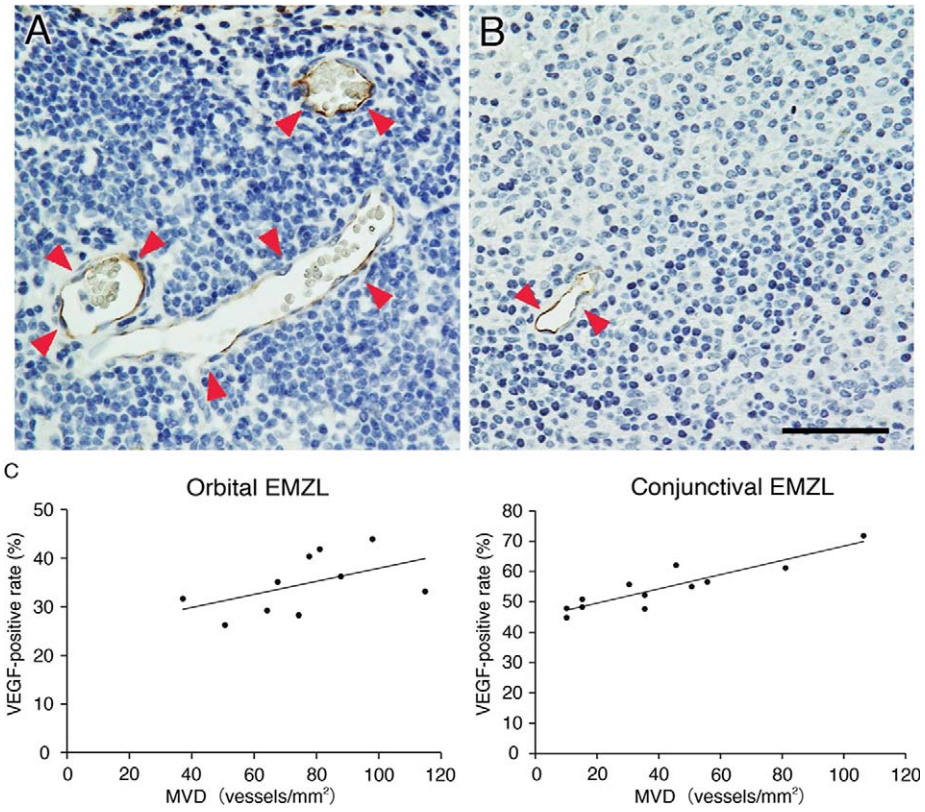

FIGURE 4. CD34-positive intratumoral microvessels (**A**, **B**), and the correlation with the VEGF-positive rate and MVD in orbital and conjunctival EMZLs (**C**). Variable staining with CD34 in conjunctival EMZL showing abundant microvessels (**A**, *arrowheads*) and few microvessels (**B**, *arrowheads*) in different lesions. *Scale bar*, 50 μm. Microvessel density shows a positive correlation with the VEGF-positive rate (**C**).

ing the pathogenesis and tumor development have yet to be elucidated. It has been demonstrated that VEGF plays an important role in intratumoral neovascularization, tumor cell infiltration, and metastasis.[14] This study demonstrated protein expression of VEGF in orbital and conjunctival EMZL tissues, where cytoplasmic immunoreactivity for VEGF was detected in CD20-positive B cells. Moreover, we confirmed VEGF protein and gene expressions by ELISA and RT-PCR, respectively. These results may support the immunoreactivity of VEGF in this study. Importantly, the VEGF-positive rate correlated well with MVD in ocular adnexal EMZL, particularly in conjunctival lesions (Fig. 4). This is the first report showing increased VEGF expression in human ocular adnexal EMZL, indicating a potential role for VEGF in the pathobiology of adnexal lymphoma.

Immunohistochemically, the VEGF-immunopositive rate in EMZL tissues was significantly higher than that in RLH tissues. The VEGF-positive rate in EMZL was similar to those in other systemic malignancies (77.3% in nonsmall cell lung cancer,[15] 63.3% in gallbladder cancer,[16] 55% in lobular breast cancer, and 90% in ductal breast cancer[17]). Additionally, the VEGF-positive rate correlated with a higher clinical stage in gallbladder cancer[16] and with the rate of lymph node metastasis in squamous cell carcinoma of the esophagus.[18] In diffuse large B-cell lymphoma, the VEGF-positive rate is a predictor of overall survival.[9] These findings suggest that VEGF contributes to the proliferation and development of malignant lymphoproliferative lesions. Along with the results of VEGF immunoreactivity in this study, we also previously reported that conjunctival EMZL can arise from RLH.[7,8] These results allow us to speculate that VEGF expression is upregulated during lymphomagenesis of conjunctival RLH lesions. Wong et al.[19] demonstrated that VEGF expression in the adenocarcinoma was higher than the adenoma, a premalignant tumor of the colon. Taken together, VEGF expression may be a potential biomarker for malignant versus reactive lymphoproliferative lesions. Additionally, VEGF expression in conjunctival EMZLs was higher than that of orbital EMZLs in our study. These data suggest that proliferation and development of conjunctival EMZL may be more VEGF-dependent than those of orbital EMZL.

Given that VEGF expression is associated with MVD in several malignant tumors,[17,18] and that overexpression of VEGF is correlated with increased MVD in a premalignant lesion,[20] VEGF might lead a benign tumor to develop into a malignant lesion through angiogenic switch. In the current study, the VEGF-positive rate correlated with MVD in conjunctival and orbital EMZLs, suggesting that VEGF also plays a significant role in the intratumoral angiogenesis of ocular adnexal lymphoma. In addition, stronger association between VEGF expression and MVD was noted in the conjunctival EMZLs ($r^2 = 0.820$) than the orbital EMZLs ($r^2 = 0.252$). These results indicate that the relatively vascular-rich microenvironment in conjunctival EMZL may be the reason for its salmon-patch appearance on slit-lamp examination.

To date, the treatments in ocular adnexal EMZL consist of mainly surgical excision and irradiation, because EMZL is a low-grade and radiosensitive malignancy.[21] Recent studies showed that intralesional injection of interferon-α,[5] or rituximab, anti-CD20 monoclonal antibody,[22,23] could be one of the therapeutic approaches for EMZL. However, there still exist cases that do not achieve complete remission, even though such treatment, including conventional radiotherapy,[21] is successfully conducted. Therefore, development of further therapeu-

tic options will be required. This study summarized VEGF expression and the possible association with MVD in lymphoproliferative disorders, indicating that blockade of VEGF function may prevent the onset and tumor growth of ocular adnexal lymphoma. In order to prove that anti-VEGF therapy may be one of the effective therapeutic options in patients with conjunctival and orbital EMZL, randomized clinical trials will be required. Based on the previous trials in other malignancies,[24,25] there are two possible routes of administration of anti-VEGF agents to treat ocular adnexal EMZL: intralesional or systemic administration. Considering the potential severe side effects of systemic administration like thromboembolic and bleeding events,[25] intralesional injection may be initially recommended for orbital or conjunctival EMZL. As a first step, it is mandatory to confirm whether local injection of anti-VEGF agents leads to tumor regression in future clinical trials.

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