Vascular endothelial growth factor-A is expressed both on lymphoma cells and endothelial cells in angioimmunoblastic T-cell lymphoma and related to lymphoma progression

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Vascular endothelial growth factor-A (VEGF-A), a main stimulator of endothelial cell proliferation, plays an important role on tumor angiogenesis. Angioimmunoblastic T-cell lymphoma (AITL) show the most prominent vascular component among lymphomas and their prognosis is difficult to predict. To assess the clinical significance of VEGF-A in AITL, *VEGF-A* gene expression was studied in the tumoral lymph nodes of 24 patients using laser microdissection and quantitative polymerase chain reaction. *VEGF-A* gene was overexpressed in both microdissected lymphoma and endothelial cells. Increased levels of *VEGF-A* gene expression in lymphoma cells, as in endothelial cells, were related to extranodal involvement and to short survival time. Accordingly, VEGF-A protein expression was also found in both types of cells in lymph nodes and bone marrows with lymphomatous involvement. Triple immunofluorescent labeling on lymph node sections showed that VEGF-A protein and its receptor VEGF-R1 were coexpressed on endothelial cells of microvessels in the areas of lymphoma invasion. In these areas, ultrastructural study showed dystrophic microvessels. Taken together, the value of *VEGF-A* gene expression as an adverse prognostic marker in AITL should thus be considered. In addition to lymphoma cells themselves, the vascular component, a critical pathologic characteristic in AITL, also contributes to lymphoma progression.
Laboratory Investigation (2004) **84,** 1512–1519, advance online publication, 16 August 2004; doi:10.1038/labinvest.3700145

Keywords: vascular endothelial growth factor-A; angioimmunoblastic T-cell lymphoma

Vascular endothelial growth factor-A (VEGF-A), an important angiogenic molecule, has direct effect on vascular endothelial cells, stimulates endothelial cell proliferation, migration, and increases vascular permeability.[1,2] The *VEGF-A* gene is alternatively spliced to yield major isoforms of 121, 165, and 189 amino acids (VEGF121, VEGF165, and VEGF189), which are essential for normal vascular development.[3] VEGF-A exerts its biological function through tyrosine kinase receptors, mainly as VEGF-R1 (Flt-1) and VEGF-R2 (KDR/flk-1).[4,5]

VEGF-A plays an essential role in embryogenesis, physiologic angiogenesis, and pathologic angiogenesis, particularly, the neovascularization of solid tumors and hematological malignancies.[2] In solid tumors, VEGF-A overexpression is associated with increased angiogenesis, tumor growth, and metastasis.[6] The tumor vessels are structurally heterogeneous and process morphological abnormalities.[1,7] In lymphoma, VEGF-A mRNA and protein

expression are also detected.[8,9] Elevated serum VEGF-A concentration is reported to indicate poor disease outcome in non-Hodgkin's lymphoma patients.[10] Experimentally, *VEGF-A* gene overexpression by lymphoma cells resulted in increased angiogenesis and correlated with lymphoma cell engraftment efficiency in severe combined immunodeficiency (SCID) mice.[11] However, the direct link between cellular VEGF-A and lymphoma progression has not yet been determined in lymphoma patients.

Angioimmunoblastic T-cell lymphoma (AITL) shows the most prominent vascular component among lymphoma.[12] The clinical outcome of the patients differs considerably, with rapid progression and fatal course in some patients, and durable remission and good prognosis in others.[13] VEGF-A protein expression has been observed by immunohistochemistry in lymphoma cells of AITL.[9] AITL thus provides a good model to evaluate the clinical significance of VEGF-A and its relation to lymphoma progression.

Materials and methods

Patients

In all, 24 AITL patients, 10 male and 14 female, 33–84-year-old (median 60 years), were included in this study. Histological diagnoses were established according to the WHO classification.[14] Induction chemotherapy consisted of six cycles of CHOP and CHOP-like regimen followed by consolidation and maintenance chemotherapies as previously reported.[15] The clinical and biological features of these patients are shown in Table 2.

Reactive lymph node hyperplasia of eight age- and sex-matched patients were referred as controls. Approval was obtained from the Institut Universitaire d'Hématologie-Hôpital Saint-Louis institutional review board. All patients gave accordingly their informed consent.

Tissue Specimen

Lymph nodes, surgically removed for diagnostic purpose, were immediately cut into three parts: one part was fixed in formaldehyde and further processed for paraffin embedding, another part was snap frozen, and the third part was fixed in 2% glutaraldehyde and embedded in epoxy resin.

Bone marrow biopsies were performed in all 24 AITL patients and skin biopsies in 11 AITL patients with skin rash.

Laser Microdissection

Laser microdissection was performed on 7 μm lymph node-frozen sections of all 24 AITL and eight reactive hyperplasia. CD3$^+$ medium-sized lympho-

ma cells in AITL sections, CD3$^+$ small lymphocytes in interfollicular areas of reactive hyperplasia sections, and CD34$^+$ endothelial cells in both AITL and reactive hyperplasia sections, were laser microdissected (PALM, Bernried, Germany). A quantitative assessment was achieved by PALM Robo software. For each patient, approximately 1500 lymphoma and 1500 endothelial cells, corresponding to an average surface of 450 000 and 280 000 μm^2, respectively, were microdissected and catapulted into tubes for RNA extraction.

RNA Extraction and cDNA Synthesis

Total RNA was extracted using the acid–guanidinium thiocyanate–phenol–chloroform method.[16] First-strand cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen Corporation, CA, USA) and random hexamers primers (Amersham Biosciences, NJ, USA) according to the manufacturer's instructions.

Real-Time Quantitative PCR

The primers and probes of VEGF-A, its isoforms VEGF121, VEGF165, and VEGF189, and housekeeping gene β2-microglobulin (β2M) were synthesized as reported.[17,18]

The transcripts that encode for VEGF-A, its isoforms and β2M were amplified by PCR using corresponding primers. PCR products were separated on a 3% agarose gel, bands were purified by the QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany), and subsequently cloned by TOPO II TA Cloning reagent set pCRII (Invitrogen Corporation, San Diego, CA, USA). Plasmids were purified using the Qiagen Miniprep reagent set (Qiagen), sequenced (PE Applied Biosystems, Warrington, UK), and measured by spectrophotometry. Standard curves for VEGF-A, its isoforms and β2M were generated using serial dilutions from 10^{10} down to 10^1 copies/μl.

Quantitative PCR was realized on LightCycler instrument using LightCycler-FastStart DNA Hybridization Probes Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The amplification conditions for LightCycler consisted of an initial 8 min of incubation at 94°C for FastStart *Taq* DNA polymerase activation, followed by 45 cycles of denaturation at 94°C for 15 s and annealing/extension at 60°C for 20 s. All experiments were performed in duplicate.

Quantification of PCR products was determined by LightCycler software 3.1, according to the standard curve. The expression levels of VEGF-A and its isoforms were normalized by β2M and presented as copies of target gene per 10^3 copies of β2M.

Immunohistochemistry

Immunohistochemical analyses were carried out on 5-μm-paraffin sections with an indirect immunoperoxidase method, using antibodies directed against VEGF-A (mouse anti-human VEGF-A antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution, VEGF-R1 (goat anti-human VEGF-R1 antibody, kindly provided by Jean Plouet, Institut de Pharmacologie et Biologie Structurale, UMR CNRS 5089, Toulouse, France) at 1:100 dilution, and VEGF-R2 (goat anti-human VEGF-R2 antibody, R&D Systems Inc., MN, USA) at 1:10 dilution.

Triple Immunofluorescent Labeling

Triple immunofluorescent labelings were performed on 5-μm-frozen sections in lymphomatous areas of lymph nodes. The slides were incubated with the antibodies directed against CD34 (rabbit anti-human CD34 antibody, Santa Cruz Biotechnology), VEGF-A, and VEGF-R1 at 1:100 dilution, or CD3 (rabbit anti-human CD3 antibody, Zymed Laboratories, San Francisco, CA, USA), VEGF-A, and VEGF-R1 at 1:100 dilutions. Subsequently, the slides were incubated with FITC-conjugated swine anti-rabbit IgG (DAKOCytomation, Denmark), AMCA-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA), and rhodamin-conjugated donkey anti-goat IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) at 1:100 dilutions. The number of lymphoma cells, or of vascular sections with endothelial cells coexpressing VEGF-A and VEGF-R1 was assessed by systematic count of three different fields at magnification 400, for each case.

Electron Microscopy

Lymph node samples fixed in 2% glutaraldehyde-buffered 0.1 M. cacodylate were embedded in epoxy resin. Semithin sections were stained with 2% toluidine blue, and ultrathin sections with uranyl acetate lead. The analysis focused on microvessels, with emphasis on endothelial cells, basal membranes, and pericytes. Presence or absence, dystrophy or damage of these structures was noted.

Statistical Analyses

Patient characteristics were compared using χ^2 and Fisher's exact tests for categorical variables, and Wilcoxon's test for continuous variables. Event-free survival (EFS) was calculated from the date of diagnosis to the date of progression, relapse, or death. Overall survival (OS) was measured from the date of diagnosis to either death from any cause or the stopping date of January 1, 2003. Survival rates were estimated using the Kaplan–Meier method and compared by log-rank test. Multivariate survival analysis was performed using a Cox regression model. Difference were considered significant when the two-sided P-value was <0.05. All statistical analyses were performed using SAS 8.2 software (SAS Institute Inc, Cary, NC, USA).

Results

Overexpression of *VEGF-A* Gene and Its Isoforms in Both Microdissected Lymphoma and Endothelial Cells in AITL

Compared to reactive hyperplasia, *VEGF-A* gene was overexpressed in microdissected lymphoma cells in AITL ($P = 0.0006$). Isoforms VEGF121 and VEGF165, but not VEGF189, were significantly increased ($P = 0.0009$ and 0.0048, respectively) (Table 1).

VEGF-A, VEGF121, and VEGF165 levels were also significantly higher in microdissected endothelial cells in AITL than in microdissected endothelial cells from reactive hyperplasia ($P = 0.0012$, 0.0040, and 0.0131, respectively) (Table 1).

VEGF-A Gene Overexpression Related to Poor Disease Outcome in AITL Patients

VEGF-A gene overexpression in both lymphoma and endothelial cells was significantly associated with advanced Ann Arbor stage, presence of bone marrow involvement, presence of skin involvement, and high-risk international prognostic index (Table 2).

Within a median follow-up of 51 months, 14 patients (58%) relapsed and 12 of them (50%) died. The 2-year EFS and OS rate were, respectively, 39.2 and 69.1%, with median EFS and OS at 17 and 38 months. Poor EFS and OS were correlated with high VEGF-A levels, in microdissected lymphoma cells ($P = 0.0078$ and 0.0091, respectively), and in microdissected endothelial cells ($P = 0.0152$ and 0.0342, respectively). Multivariate analyses revealed that VEGF-A levels in microdissected lymphoma cells were independent adverse prognostic factors for EFS and OS ($P = 0.0160$ and 0.0118, respectively).

VEGF-A and Its Receptor VEGF-R1 Protein were Expressed in Lymph Node and Bone Marrow Sections of AITL

To assess whether *VEGF-A* gene was biologically functional, immunohistochemical analyses were performed on lymph nodes and bone marrow specimens, using antibody directed against the VEGF-A protein and two receptors VEGF-R1 and VEGF-R2. VEGF-A protein was expressed in lymphoma cells, as in endothelial cells (Figure 1). Moreover, VEGF-A protein was expressed in the 24 AITL lymph nodes and in the 15 bone marrow biopsies with CD3$^+$ medium-sized lymphoma cells involvement, but not in the nine bone marrows without lymphoma involvement. The distribution of

Table 1 VEGF-A gene expression in AITL

	VEGF-A[a]		VEGF121[a]		VEGF165[a]		VEGF189[a]	
	Microdissected lymphocytes X (s.d.)	Microdissected endothelial cells X (s.d.)	Microdissected lymphocytes X (s.d.)	Microdissected endothelial cells X (s.d.)	Microdissected lymphocytes X (s.d.)	Microdissected endothelial cells X (s.d.)	Microdissected lymphocytes X (s.d.)	Microdissected endothelial cells X (s.d.)
AITL (n=24)	4.00 (1.67)	7.54 (2.74)	2.17 (0.99)	4.62 (2.01)	1.62 (0.86)	3.47 (1.61)	0.10 (0.05)	0.70 (0.28)
Reactive lymph node hyperplasia (n=8)	0.51 (0.06)	2.99 (0.79)	0.32 (0.05)	1.51 (0.38)	0.23 (0.03)	1.19 (0.34)	0.06 (0.02)	0.67 (0.17)
P	0.0006	0.0012	0.0009	0.0040	0.0048	0.0131	0.1765	0.8393

[a]All data presented as copy numbers of target gene per 1000 copy numbers of $\beta 2M$.

VEGF-A protein expression corresponded to nodal and extranodal invasion by lymphoma in the 24 patients.

VEGF-R1 was also expressed on lymphoma cells as on endothelial cells in lymph node and in bone marrows with $CD3^+$ medium-sized lymphoma cells involvement. The staining for VEGF-R1 was particularly strong in the areas of adipose tissue invasion in lymph node sections (Figure 1). However, we failed to detect VEGF-R2 expression in the same areas.

VEGF-A and Its Receptor VEGF-R1 Protein were Coexpressed on Endothelial Cells and on Lymphoma Cells in the Areas of Lymphoma Invasion

In order to determine if lymphoma cells and/or endothelial cells coexpressed VEGF-A and VEGF-R1, we performed a triple immunofluorescent labeling on lymph node sections, combining antibodies against CD3, VEGF-A and VEGF-R1, or CD34, VEGF-A and VEGF-R1. In the areas of adipose tissue invasion by lymphoma, endothelial cells of the microvessels coexpressed VEGF-A and VEGF-R1 (Figure 2). Quantitative assessment showed that a mean of 46% (range 38–51%) of vascular sections coexpressed the two markers, when a mean of 33% (range 25–38%) of lymphoma cells coexpressed them.

Presence of Dystrophic Vessels without Pericytes in the Areas of Lymphoma Invasion

On lymph node sections, electron microscopy study in the areas of the adipose tissue invasion showed the coexistence of normal microvessels with both endothelial cells and pericytes, and of dystrophic vessels with absence of pericytes. In these dystrophic vessels, endothelial cells were either normal, even turgescent, or thinned and compacted along their basal membrane (Figure 2).

Discussion

VEGF-A is an important angiogenic cytokine with critical roles in tumor angiogenesis.[6] In lymphoma, to our knowledge, only experimental data are available for *VEGF-A* gene expression: VEGF-A and isoforms VEGF121 and VEGF165 were expressed in cell lines of cutaneous T-cell lymphoma and Burkitt's lymphoma.[8] Using *in situ* hybridization, Foss *et al*[19] detected VEGF mRNA expression in peripheral T-cell lymphoma (including five AITL); however, double immunolabeling showed that VEGF was mainly observed in reactive nonlymphoid CD68-negative cells. Using laser microdissection and quantitative PCR, we demonstrated that lymphoma cells in AITL overexpressed VEGF-A mRNA, and the same two

Table 2 VEGF-A gene expression in microdissected lymphoma and endothelial cells according to clinical characteristics in angioimmunoblastic T-cell lymphoma patients ($n = 24$)

Characteristics		No. (%)	VEGF-A[a] Microdissected lymphoma cells		VEGF-A[a] Microdissected endothelial cells	
			X (s.d.)	P[b]	X (s.d.)	P[b]
Clinical features						
Age (years)	<60	12 (50)	3.66 (1.39)	0.4456	6.44 (2.19)	0.1277
	>60	12 (50)	4.35 (1.88)		8.63 (3.09)	
Gender	Male	10 (42)	4.63 (1.65)	0.8371	7.63 (2.01)	0.8895
	Female	14 (58)	4.43 (1.84)		7.20 (2.75)	
Hepatosplenomegaly	Yes	18 (75)	4.39 (1.79)	0.1320	7.95 (2.56)	0.3252
	No	6 (25)	2.84 (1.17)		6.30 (2.70)	
ECOG	0-1	10 (42)	3.59 (1.43)	0.4455	6.57 (2.09)	0.2583
	2-4	14 (58)	4.30 (1.73)		8.23 (3.05)	
Ann Arbor Stage	II	2 (8)	2.04 (0.71)	0.0146	3.56 (0.65)	0.0104
	III	6 (25)	2.40 (0.78)		4.99 (0.78)	
	IV	16 (67)	4.85 (1.78)		8.99 (2.62)	
B-symptoms	A	4 (17)	2.40 (0.96)	0.1076	4.99 (1.17)	0.1109
	B	20 (83)	4.32 (1.67)		8.05 (2.76)	
Bone marrow involvement	Yes	15 (63)	5.16 (0.42)	<0.0001	9.49 (2.08)	<0.0001
	No	9 (37)	2.08 (0.95)		4.29 (0.96)	
Skin involvement	Yes	8 (33)	5.80 (1.89)	0.0020	9.83 (2.51)	0.0200
	No	16 (67)	3.10 (1.22)		6.40 (2.42)	
International prognostic index	Low risk–intermediate low risk	8 (33)	2.73 (0.88)	0.0389	5.26 (1.58)	0.0202
	Intermediate high risk–high risk	16 (67)	4.64 (1.76)		8.68 (2.56)	
Biological features						
Lactate dehydrogenase	<Normal	5 (21)	2.87 (1.30)	0.1983	5.72 (0.99)	0.1979
	>Normal	19 (79)	4.30 (1.77)		8.02 (2.94)	
Coomb's test	Positive	10 (42)	3.65 (1.44)	0.3573	8.17 (3.25)	0.4690
	Negative	14 (58)	2.10 (0.36)		7.09 (2.28)	
Hypereosinophilia	Yes	9 (38)	2.54 (1.12)	0.0558	5.76 (2.21)	0.0779
	No	15 (62)	4.74 (1.64)		8.43 (2.55)	
Hypergammaglobulinemia	Yes	10 (42)	3.32 (1.97)	0.8188	6.74 (2.69)	0.8594
	No	14 (58)	3.06 (1.07)		6.68 (2.22)	

[a]All data presented as copy numbers of target gene per 1000 copy numbers of β2M.

[b]P-value obtained when comparing the subgroups of each characteristic.

isoforms VEGF121 and VEGF165. Moreover, in this series of AITL, the expression of VEGF-A, VEGF121 and VEGF165 was also significantly increased in microdissected endothelial cells. This confirms that although the malignant cells themselves are primarily responsible for VEGF-A expression in tumors, vascular endothelium may also express VEGF-A.[6,20]

The levels of these three markers, in microdissected lymphoma cells as in microdissected endothelial cells, correlated with adverse clinical prognostic factors and decreased survival time. Particularly, VEGF-A overexpression in lymphoma cells was an independent prognostic factor, suggesting that lymphoma cells play an important role on disease progression. As far as we know, no previous study addressed the prognostic value of *VEGF-A* gene expression in lymphoma. In acute myeloid[21,22] and lymphoblastic leukemia,[23] a direct link between high cellular VEGF-A level and short survival has been reported. In solid tumors, *VEGF-A* gene overexpression was associated with advanced stage, metastasis, and poor prognosis in lung[24] and colon cancer.[25]

The correlation we found between high levels of *VEGF-A* gene and its two isoforms with poor disease outcome could be linked to tissue invasion by lymphoma. The pattern of VEGF-A protein expression, on lymphoma and endothelial cells, is in accordance with *VEGF-A* gene overexpression in the two cell populations selected from AITL sections by laser microdissection. This suggests the involvement of both the lymphoma and the vascular component of AITL in VEGF-A synthesis and expression. A striking feature in our study was that the distribution of VEGF-A protein expression corresponded to nodal and extranodal involvement by lymphoma in the 24 patients: VEGF-A protein was strongly expressed on lymphoma cells invading perinodal adipose tissue, or bone marrows, while it was not found in bone marrows without lymphoma involvement.

Since VEGF-A exerts its activity through the binding of specific receptors, we studied the expression and distribution pattern of two receptors, VEGF-R1 and VEGF-R2, by immunohistochemistry. VEGF-R1, but not VEGF-R2, was expressed in lymphoma cells and in endothelial cells. As
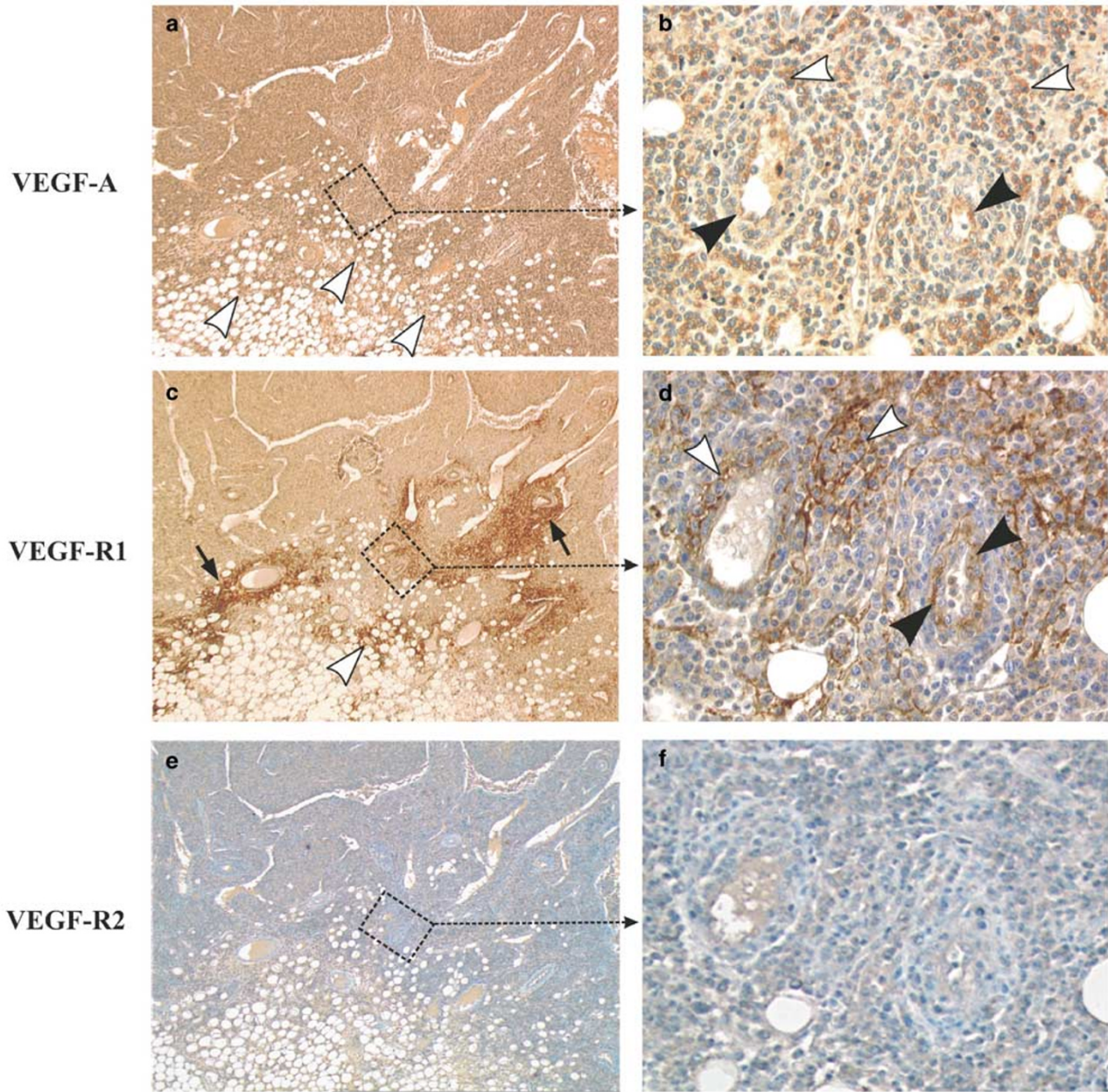
Figure 1 Lymph node of AITL: Immunohistochemical study of the expression of VEGF-A and its receptors (VEGF-R1 and VEGF-R2) on following sections (indirect immunoperoxidase method (**a, c, e**, ×40). (**b, d, f**) corresponded to higher magnification (×400) of the lymphomatous areas framed by dotted lines in (**a, c, e**). (**a**) VEGF-A was expressed on lymphoma cells invading lymph node capsule and adipose tissue (empty arrowheads), ×40. (**b**) Higher magnification (×400) of the area framed with dotted line in (**a**): VEGF-A was expressed on lymphoma cells (empty arrowheads) and on endothelial cells of medium-sized vessels (full arrowheads). (**c**) VEGF-R1 was expressed around vessels (arrows) and on lymphoma cells invading lymph node capsule and adipose tissue (empty arrowheads), ×40. (**d**) Higher magnification (×400) of the area framed with dotted line in (**c**): VEGF-R1 was expressed on lymphoma cells (empty arrowheads) and on endothelial cells of medium-sized vessels (full arrowheads). (**e**): VEGF-R2 was not expressed on lymphoma cells or around vessels on the same areas of the following sections, ×40. (**f**) Higher magnification (×400) of the area framed with dotted line in (**e**): VEGF-R2 was not expressed on endothelial cells of the same vessels as (**d**).

previously reported, only VEGF-R1 was found in a series of lymphoma cell lines.[8] In endothelial cells, this could be due to hypoxia, by which VEGF-R1 is selectively induced with VEGF-R2 downregulated.[20] Interestingly, VEGF-R1 was also detected in the areas of lymphoma invasion in lymph nodes (extension in perinodal adipose tissue) and in bone marrows with lymphoma involvement, where VEGF-A protein was expressed. Using triple immunofluorescent labeling, we proved that VEGF-A and VEGF-R1 were coexpressed on lymphoma cells. *In vitro* models of T-leukemia cells[26] and myeloma cells[27] coexpressing VEGF-A and VEGF-R1 showed that VEGF-A participates in cell migration through
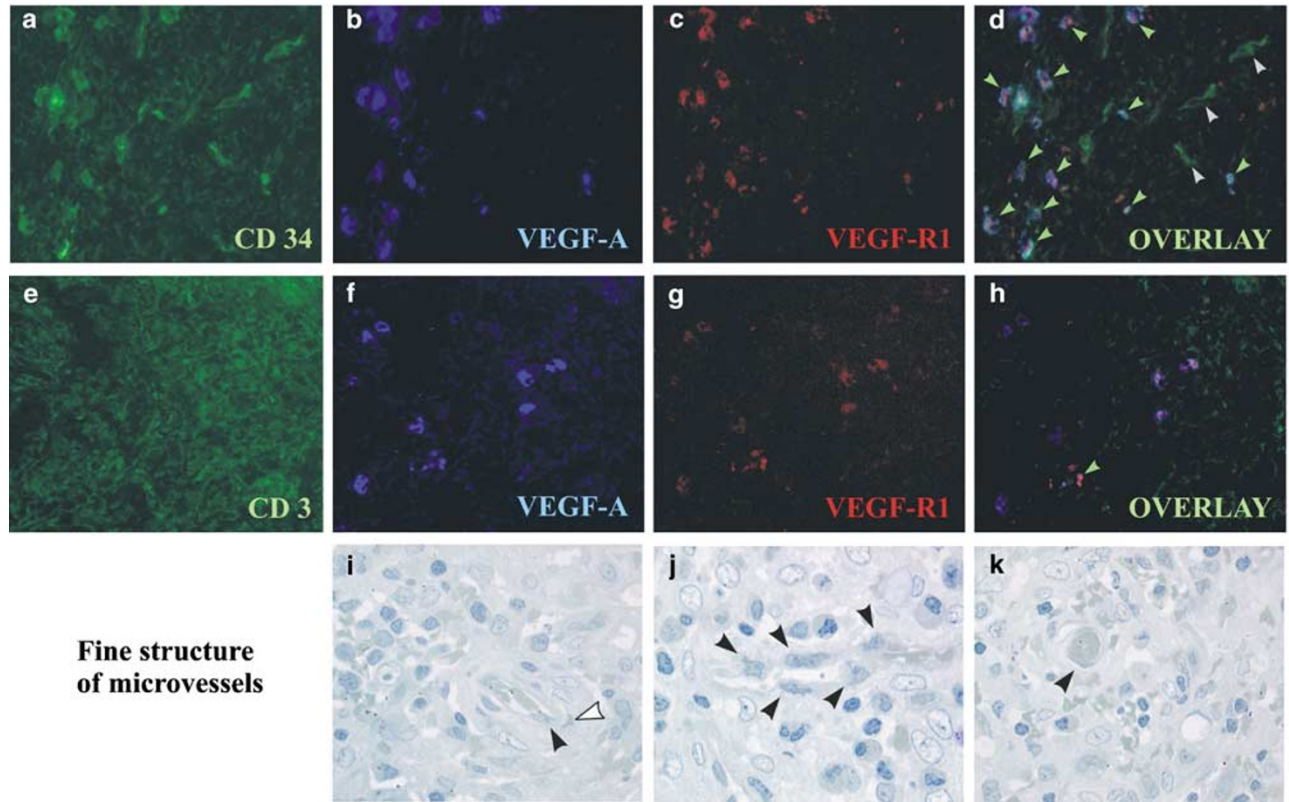
Figure 2 Lymph node of AITL: (**a–h**) Expression of VEGF-A and VEGF-R1 in the areas of lymphoma invasion, × 100. (**i–k**) Fine structure of microvessels in semithin sections, × 800. The combined triple immunofluorescent labeling of CD34 (**a**), VEGF-A (**b**) and VEGF-R1 (**c**) showed that most endothelial cells coexpressed VEGF-A and VEGF-R1 (**d**) (green arrowheads), when only few endothelial cells identified by fluorescent CD34 staining did not express VEGF-A or VEGF-R1 (white arrowheads). The combined triple immunofluorescent labeling of CD3 (**e**), VEGF-A (**f**), and VEGF-R1 (**g**) showed that many lymphoma cells expressed VEGF-A (**f**), but only few of them expressed VEGF-R1 (**g**) and coexpressed VEGF-A and VEGF-R1 (**h**) (green arrowheads). (**i**) Microvessel with normal structure: endothelial cells (full arrowheads) and pericytes (empty arrowheads). (**j**) Dystrophic microvessel with turgescent normal endothelial cells (full arrowheads) but no pericyte. (**k**) Dystrophic microvessel with thinned endothelial cell (full arrowheads) but no pericyte.

activation of VEGF-R1 and facilitate tissue invasion. Importantly, VEGF-A and VEGF-R1 were coexpressed on endothelial cells in areas of lymphoma invasion. Recent experiments have demonstrated the importance of anti-Flt-1 therapy in the inhibition of tumor angiogenesis.[28] In murine models of lung carcinoma and colorectal carcinoma, VEGF-R1 expression in endothelial cells was significantly related to tumor growth and metastasis.[29] VEGF-A, both expressed on lymphoma cells and endothelial cells can bind to VEGF-R1 and facilitate lymphoma invasion.

Ultrastructural study showed the microvessels deprived of pericytes in the areas of perinodal adipose tissue invasion in our series of AITL. In solid tumors expressing VEGF-A, as ovarian cancer,[30] or glioma,[31] dystrophic microvessels with pericyte loss were also found within the tumors. Therefore, in this type of lymphoma, the vascular component, and not only the lymphoma cells, could contribute to tumor growth and metastasis.

In conclusion, VEGF-A was both expressed in lymphoma and endothelial cells, and involved in lymphoma progression in these 24 cases of AITL.

Further biological studies are needed to assess if angiogenesis inhibitors could be efficient agents in AITL treatment.

Acknowledgements

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