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INVESTIGATIVE REPORT

Microvessel Density and Expression of Vascular Endothelial Growth Factor and its Receptors in Different Subtypes of Primary Cutaneous B-cell Lymphoma

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A proangiogenic micromilieu is associated with a worse prognosis in systemic lymphoma. Hence, targeting the tumour microenvironment and its vasculature has evolved as a promising novel treatment strategy. The role of tumour neoangiogenesis in cutaneous B-cell lymphoma, however, has not yet been elucidated. Therefore, we examined the expression of vascular endothelial growth factor (VEGF) and its receptors VEGFR-1 and VEGFR-2, as well as microvessel density by immunohistochemistry in paraffin-embedded specimens of different subtypes of primary cutaneous B-cell lymphomas, systemic diffuse large B-cell lymphoma, and cutaneous B-cell pseudolymphoma. Primary cutaneous large B-cell lymphoma (PCLBCL) were characterized by significantly higher intratumoral expression levels of VEGF and its receptors in comparison with the indolent lymphoma subtypes. Moreover, PCLBCL exhibited significantly higher intratumoral microvessel counts. Our study provides evidence that the most aggressive subtype of cutaneous B-cell lymphoma, PCLBCL, is characterized by a proangiogenic micromilieu. ***Key words: angiogenesis; microvessel density; VEGF; cutaneous B-cell lymphoma; tumour microenvironment.***

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According to the World Health Organization/European Organisation for Research and Treatment of Cancer (WHO/EORTC) classification, primary cutaneous B-cell lymphomas (PCBCL) are subdivided into 3 major groups; namely, primary cutaneous follicle centre lymphoma (PCFCL), primary cutaneous marginal zone lymphoma (PCMZL), and primary cutaneous large B-cell lymphoma (PCLBCL) (1). While PCFCL and PCMZL show an indolent clinical course with an excellent prognosis, PCLBCL represents a more aggressive lymphoma. In contrast to diffuse PCFCL, in which small to large cleaved centrocytes dominate the tumour infiltrate, PCLBCL is histologically characterized by large sheets of centroblasts and immunoblasts. Age and tumour extent represent negative prognostic factors in PCLBCL. Moreover, impaired prognosis is observed when skin lesions are present at the lower extremities, and this has given the name to this lymphoma subgroup PCLBCL, leg-type (PCLBCL-LT)), a hitherto incompletely understood phenomenon. Similar to systemic diffuse large B-cell lymphoma (sDLBCL), gene expression profiling in PCLBCL has provided a molecular basis for the subdivision into this biologically distinct subgroup (2). Hierarchical clustering of PCLBCL, leg-type and PCFCL based on B-cell signatures demonstrated that PCLBCL-LT shows expression profiles resembling that of activated B cells. In sDLBCL, these expression patterns, indicative of different stages of B-cell differentiation, have strong prognostic significance (3, 4).

Besides molecular profiling of the tumour cells themselves, their interaction with the tumour microenvironment is currently the focus of interest in lymphoma research (5–7). In this context, Lenz et al. (8) have reported that the gene expression profile of the non-tumour cells significantly predicts biological behaviour, treatment response and survival in patients with sDLBCL. The so-called stromal-1 signature is related to extracellular matrix deposition and histiocytic infiltration. This genetic pattern portends a good prognosis. On the contrary, the denotated stromal-2 signature is associated with a poor prognosis. This signature comprises an overexpression of genes encoding proteins such as platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) or the receptor for vascular endothelial growth factor (VEGFR-2). Hence, it primarily reflects angiogenesis. Accordingly, high intratumoral microvessel density (MVD) (9) as well as overexpression of vascular endothelial growth factor (VEGF) and other proangiogenic cytokines are assigned a major role for the tumour biology of different types of systemic lymphoma (10–12). As a consequence, therapies targeting the microenvironment have gained increasing attention and tumour angiogenesis has evolved as an attractive molecular target for novel therapeutic strategies (6).

The angiogenic switch is essential for a continuous tumour growth beyond a certain threshold (13). The complex process of tumour neoangiogenesis is governed by a plethora of countervailing humoral and cellular factors, which either induce or repress angiogenesis (14). The multifunctional cytokine vascular endothelial growth factor VEGF-A (commonly referred to as VEGF) represents the leading proangiogenic factor. It is expressed by tumour as well as stromal cells belonging to the tumour microenvironment. Of note, beyond its paracrine effects on endothelial cells VEGF may serve as an autocrine growth factor for the tumour cell itself (10).

In PCBCL the impact of the tumour microenvironment, especially of the tumour-associated microvasculature, has not been systematically analysed to date. Therefore, we used immunohistochemical analyses to evaluate whether subtypes of PCBCL can be distinguished with respect to microvascular density and expression of VEGF with its auto- and paracrine functions. Indeed, all of these parameters were elevated in the more aggressive lymphoma subtype PCLBCL-LT and indicated a worse prognosis for this entity. Factors associated with angiogenesis could therefore serve as novel molecular biomarkers in cutaneous B-cell lymphoma and may represent attractive therapeutic targets in the future.

MATERIALS AND METHODS

Patients

Paraffin-embedded tissue of skin of 12 patients with PCLBCL-LT, 15 patients with PCFCL, 17 patients with PCMZL, 9 patients with cutaneous B-cell rich pseudolymphoma (PL) (including lymphadenosis cutis benigna) and secondary cutaneous infiltrates of 13 patients with sDLBCL were obtained from the archives of the Department of Dermatology and the Institute of Pathology, University of Wuerzburg. Tissues were collected during a time period from 1999 to 2011. Patients alive provided informed consent prior to entering the study according to the guidelines of the institutional ethics committe. Patients with cutaneous B-cell lymphoma were staged according to the WHO/EORTC classification, those with systemic B-cell lymphoma according to the Ann Arbor system. The clinical characteristics of the lymphoma patients are summarized in Table I.

Table I. *Clinical patient characteristics*

	PCLBCL-LT *n*	PCFCL *n*	PCMZL *n*	sDLBCL *n*
Patients	12	15	17	13
Mean age, years	70	55	48	65
Female	6	6	9	7
Male	6	9	8	6
Multi-locular lesions at time of diagnosis	6	7	7	12
Solitary lesion at time of diagnosis	6	8	10	1
Best response CR after first-line therapy	6	10	11	5
Overall response CR	5	11	11	6
Mean progression-free survival, months	12	25	26	26
Mean overall survival, months	27	60	60	43

CR: complete remission; PCLBCL-LT: primary cutaneous large B-cell lymphoma, leg-type; PCFCL: primary cutaneous follicle centre lymphoma; PCMZL: primary cutaneous marginal zone lymphoma; sDLBCL: systemic diffuse large B-cell lymphoma.

Immunohistochemical studies

Immunohistochemical analysis. Immunohistochemistry for CD31, VEGF, VEGFR-1 and VEGFR-2 was performed on 5-µm tissue sections, which were placed on glass slides. For immunohistochemistry, tissue sections were deparaffinized in xylol and rehydrated in graded alcohol. For antigen retrieval, slides were overlaid with antigen retrieval solution (Dako, Hamburg, Germany) and incubated in saturated steam for 20 min. For staining, slides were incubated with a murine polyclonal anti-CD31/PECAM-1 antibody (Dako, Hamburg, Germany), which highlights both blood and lymphatic endothelial cells, rabbit polyclonal anti-VEGF antibody (VEGF (A-20), Santa Cruz, Heidelberg, Germany), which recognizes the $VEGF_{165,121}$ and $_{189}$ splice variants, rabbit polyclonal anti-VEGFR-1 antibody (Flt1, Spring Bioscience, Pleasanton, CA, USA) and rabbit polyclonal anti-VEGFR-2 antibody (Flk-1, Lab Vision, Thermo Science, Cheshire, UK). For detection of VEGFR-1 and -2, Ultravision Quanto Detection System based on HRP-polymer (TL-125-QHL, Lab Vision, Thermos Science, Chesire, UK) was used, for visualization of VEGF and CD31 the Dako Real Detection System based on HRP (K5003, Dako, Hamburg, Germany). Control antibody staining using polyclonal rabbit antiserum was performed accordingly for VEGF, VEGFR-1 and VEGFR-2 using appropriate protocols.

Evaluation of immunohistochemical staining. For evaluation of MVD, slides were scanned in the light microscope at × 40 magnification and so-called hot spots were identified. MVD was determined by counting the number of CD31+ microvessels per measurement field at × 200 magnification (× 20 lens, × 10 ocular, measurement field of 0.3136 mm^2) using an Olympus BX45 microscope, as described (15). A microvessel was defined as any distinct CD31+ cell cluster with or without a vessel lumen. The cell surface molecule CD31, which is expressed both by blood as well as by lymphatic endothelium, was chosen for the immunohistochemical analyses because *CD31/PECAM-1* is one of the genes included in the formerly described stromal-2 signature (8). Counting was performed at least in 2 different representative fields of the tumour. The microvessel counts in the analysed areas were averaged and denoted MVD. To evaluate differences in microvasculature dependent on body location among patients with PCLBCL-LT, MVD inside the tumour and the non-involved surrounding tissue was assessed separately for location at the leg and non-leg location.

A visual semiquantitative scoring system was used for evaluating the staining results of VEGF and VEGFR-1 and -2. In case of VEGF, less than 5% cellular staining inside the tumour was scored negative (i.e. 0), 5–30% staining was scored weakly positive (i.e. 1), 30–60% staining intermediately positive (i.e. 2) and more than 60% staining strongly positive (i.e. 3). Due to lower frequencies of VEGFR-1- and VEGFR-2- positive cells, 5–20% of stained cells was graded as weakly (i.e. 1), 20–40% as intermediately (i.e. 2) and > 40% staining as strongly positive (i.e. 3). Physiologically VEGF/VEGFR-positive structures, such as keratinocytes, endothelial cells or sweat glands, served as internal controls (16). Images of the immunohistological staining were acquired using a Zeiss Axiophot microscope and Axiocam digital camera.

Statistical analysis

Statistical analysis was performed with Prism Graph Version 3.0. Survival curves were estimated using log-rank test and

presented in Kaplan–Meier curves. Multivariate analysis was performed according to the Cox proportional hazards regression model. The χ^2 test for independence was used to test for correlations between categorical variables. Test results with p-values ≤ 0.05 were considered significant.

RESULTS

PCLBCL-LT exhibits a proangiogenic micromilieu

The patient characteristics are presented in Table I. Microvascular density was assessed to determine if tumour-associated vasculature correlates with the prognostic subtype of cutaneous B-cell lymphoma. All analysed tumours showed a significantly higher microvascular density than the surrounding normal tissue ($p \leq 0.001$). However, the tumour vascularity varied within and between the different subgroups (Fig. 1a). PCLBCL-LT (Fig. 1b) and sDLBCL displayed a significantly higher vascular density ($p \leq 0.001$) compared with PCFCL (Fig. 1c), PCMZL and PL. The score of intratumoral microvessel density was comparable among the subgroups PL, PCFCL and PCMZL. In accordance with high MVD PCLBCL-LT and sDLBCL showed significantly higher frequencies ($p \leq 0.0001$) of VEGF-positive tumour cells within the tumour than the other lymphoma subtypes and pseudolymphoma (Fig. 2 a, b). MVD both inside the tumour and in the surrounding tissue did not significantly differ for location at the leg and other locations in PCLBCL-LT (Fig. 3a). However, VEGF scores were higher in tumours and surrounding non-involved tissue when located in the lower extremities (Fig. 3 b, c).

Increased expression of VEGFR-1 and VEGFR-2 in tumour cells from PCLBCL-LT

In order to elucidate whether VEGF may potentially act on lymphoma cells in an autocrine manner, we analysed the expression pattern of VEGF receptors VEGFR-1 and VEGFR-2. Immunohistochemical scores for VEGFR-1 and VEGFR-2 expression were significantly higher in PCLBCL-LT and sDLBCL in comparison with PCFCL, PCMZL and PL ($p \leq 0.001$) (Fig. 2 c–f).

Microvessel density and prognosis in PCLBCL-LT

Overall survival (OS) and progression-free survival (PFS) of 12 patients with PCLBCL-LT being treated with R-CHOP ± radiotherapy were correlated with MVD (Fig. 3 d, e). There was no statistically significant difference between the 2 groups, and confirmation in a larger cohort is required.

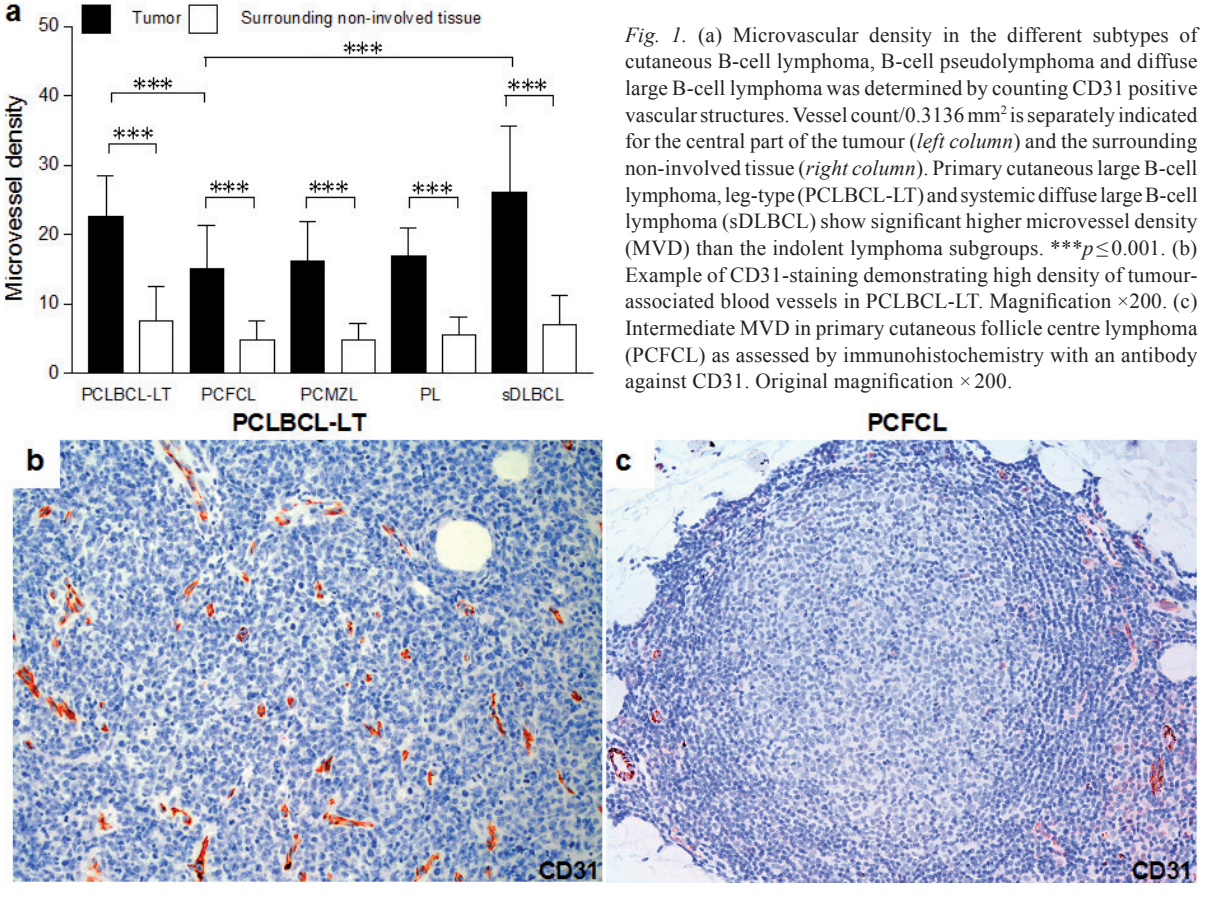

Fig. 1. (a) Microvascular density in the different subtypes of cutaneous B-cell lymphoma, B-cell pseudolymphoma and diffuse large B-cell lymphoma was determined by counting CD31 positive vascular structures. Vessel count/0.3136 mm^2 is separately indicated for the central part of the tumour (*left column*) and the surrounding non-involved tissue (*right column*). Primary cutaneous large B-cell lymphoma, leg-type (PCLBCL-LT) and systemic diffuse large B-cell lymphoma (sDLBCL) show significant higher microvessel density (MVD) than the indolent lymphoma subgroups. ***$p \leq 0.001$. (b) Example of CD31-staining demonstrating high density of tumour-associated blood vessels in PCLBCL-LT. Magnification ×200. (c) Intermediate MVD in primary cutaneous follicle centre lymphoma (PCFCL) as assessed by immunohistochemistry with an antibody against CD31. Original magnification × 200.

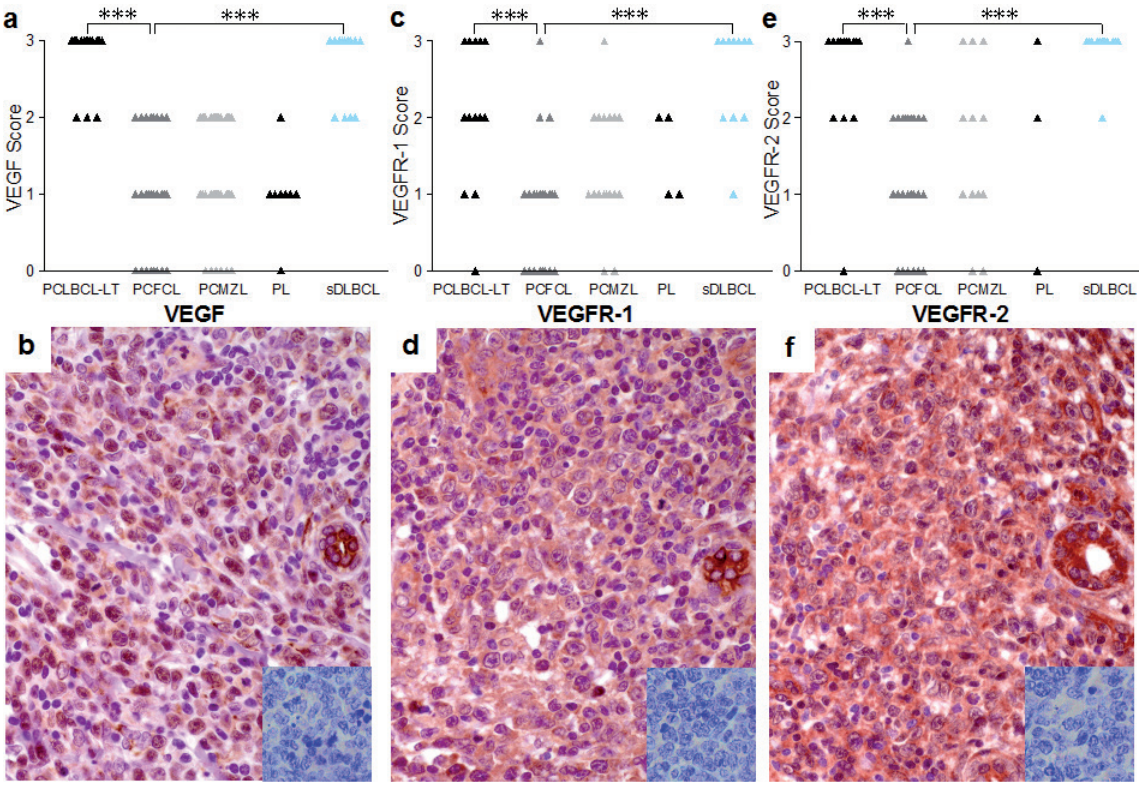

Fig. 2. Primary cutaneous large B-cell lymphoma, leg-type (PCLBCL-LT) and systemic diffuse large B-cell lymphoma (sDLBCL) exhibit significantly higher scores of (a) vascular endothelial growth factor (VEGF) and its receptors (c) VEGFR-1 and (d) VEGFR-2 than primary cutaneous follicle centre lymphoma (PCFCL), primary cutaneous marginal zone lymphoma (PCMZL) and pseudolymphoma (PL). ***$p \leq 0.001$. Representative photomicrographs showing immunohistochemical stains for (b) VEGF, (c) VEGFR-1 and (e) VEGFR-2, in a representative specimen of the same patient with PCLBCL-LT. Original magnification ×400. Control antibody staining (inset). Magnification × 400.

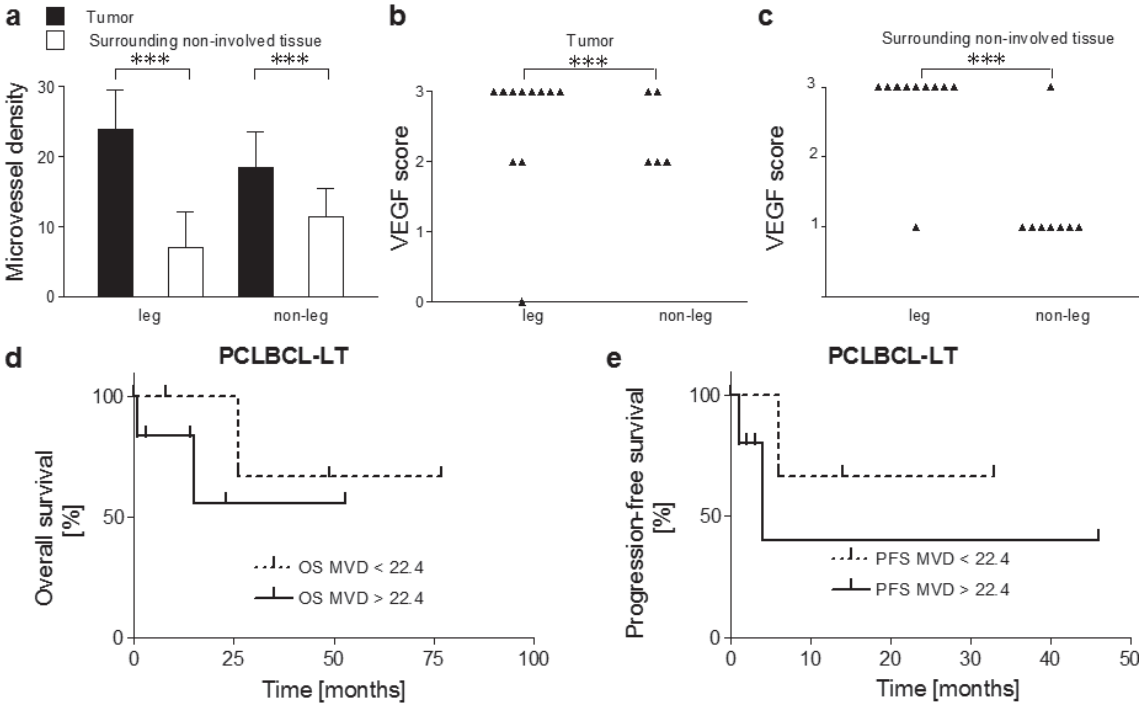

Fig. 3. Microvessel density (MVD) and expression of vascular endothelial growth factor (VEGF) expression was assessed dependent on body site. (a) MVD in primary cutaneous follicle centre lymphoma-leg-type (PCLBCL-LT) inside the tumour (*black columns*) and in the non-involved surrounding tissue (*white columns*) did not differ for location at the leg and non-leg location. VEGF score inside (b) the lymphoma infiltrate and (c) the surrounding tissue of PCLBCL-LT showed significantly higher values for location at the legs. No statistical difference is detected for low or high MVD on (d) overall and (e) progression-free survival in PCLBCL-LT.

DISCUSSION

In various systemic B-cell neoplasms increased tumour neoangiogenesis was found to be associated with a more aggressive behaviour and a poor prognosis (17). Recently, the underlying molecular basis has been delineated in sDLBCL (8). To date, no study has systematically addressed this topic in PCBCL. Therefore, we aimed to evaluate the role of tumour neoangiogenesis for the prognostically different subtypes PCFCL, PCMZL and PCLBCL-LT.

In the current study, all analysed specimens, both malignant lymphomas and benign pseudolymphomas, showed a signifcantly higher mean MVD compared with the surrounding normal tissue. Physiological angiogenesis is known from benign conditions, such as wound healing, hair growth cycle, acute and chronic inflammation (18). Likewise, it is herein observed in reactive inflammatory pseudolymphoma. Inflammatory processes induce vessel dilatation and enhanced permeability. Angiogenic response to inflammation promotes a reversible enlargement of pre-existing vessels (19) rather than sprouting of new vessels.

On the other hand, new vessel formation is induced via aberrant and constant overexpression of proangiogenic cytokines due to oncogene-driven gene expression. In our study, the microvessel count did not discriminate between benign inflammatory processes, such as PL and the indolent cutaneous lymphoma subtypes PCFCL and PCMZL. Only one prior study has to date assessed MVD in different types of indolent primary cutaneous B-cell lymphoproliferative disorders with comparable results (20). However, concerning the more aggressive lymphoma subtypes characterized by a large cell morphology (PCLBCL-LT, sDLBCL), significantly higher levels of mean MVD were detected. This is in line with previous studies in systemic B-NHL, where more unfavourable lymphoma subtypes showed higher MVD (9, 21). In systemic B-cell lymphomas the extent of MVD distinguishes between reactive benign lymphadenopathy and lymph nodes enlarged due to infiltration by non-Hodgkin's lymphoma (22). Hence, the assessment of MVD can serve as an important adjunct in the diagnostic algorithm for the diagnosis of large B-cell lymphoma of the skin (23).

To elucidate the pathophysiological background of neoangiogenesis in PCBCL, we analysed the protein expression of VEGF. As VEGF-A represents the leading proangiogenic mediator for regulating the complex process of neoangiogenesis (24), we restricted our analysis to this major cytokine. No difference in VEGF expression was observed in PL and indolent PCFCL and PCMZL corresponding to equal MVD in these subtypes. These findings may indicate that a proangiogenic milieu probably does not gain relevance until advanced stages of malignant transformation. In PCLBCL-LT and sDLBCL, VEGF was significantly more strongly expressed, which corresponded to a higher vascularity. Similar studies in systemic haematological disorders have demonstrated higher levels of VEGF expression in tissue and circulation in more aggressive lymphoma subtypes (21, 25). To date, only 2 case reports have demonstrated elevated levels of VEGF in tissue and blood of a single patient with PCLBCL-LT (26, 27).

The exact nature of VEGF overexpression in PCLBCL-LT is so far unknown. One may speculate that higher VEGF expression in tumour cells of this lymphoma subtype may result from aberrant oncogenic signalling (28). PCLBCL-LT exhibits, among others, an upregulation of bcl-2 and c-myc (2, 29). Beside antiapoptotic and prosurvival properties (30), these factors are known to promote tumour angiogenesis (31–35). Hence, overexpression of bcl-2, which was detected in >90% of our PCLBCL-LT specimens (data not shown) and upregulation of c-myc may thus, at least partially, explain the observed VEGF induction in tumour cells of PCLBCL-LT. Of note, in our study VEGF expression was not only restricted to lymphoma cells. In addition, various stromal cells stained positive. This underlines the importance of the tumour microenvironment for the angiogenic response (36).

Induction of VEGF gene expression in response to hypoxia (37) strongly depends on the proliferative activity of the tumour (38). In this context, high expression of hypoxia inducible factors (HIF-1α, HIF-1β) and consecutive VEGF upregulation (39, 40) was demonstrated in aggressive lymphomas (41, 42). Intratumoral hypoxic conditions are supposed to be especially relevant for PCLBCL-LT. Its rapidly growing, ulcerating and necrotic tumours predominantly arise at body sites with restricted blood and oxygen supply, such as the lower legs (43). In chronic venous insufficiency, lipodermatosclerosis and peripheral arterial ischaemia of the lower extremities upregulation of VEGF was observed in response to hypoxia and inflammation (44–46). Likewise, in our study, higher amount of VEGF expression was observed in different specimen derived from the lower extremities. Hence, overexpression of VEGF in soft tissue of the lower extremities may establish a protumorigenic micromilieu and provides a rationale explanation for the prognostically unfavourable location at the legs in PCLBCL-LT.

To elucidate whether VEGF may exert beyond its paracrine, i.e. proangiogenic, also autocrine, i.e. protumorigenic, properties in PCBCL (10), we analysed the expression of VEGFR-1 and -2. In skin, VEGFR-1 and -2 are physiologically expressed on endothelial cells, adnexal structures and keratinocytes (16). Both receptors were also aberrantly upregulated on the tumour cells. Of interest, expression of both tyrosine kinase receptors was significantly higher in PCLBCL-LT and sDLBCL than in the indolent lymphoma subtypes and PL. A

functional autocrine loop with constitutive mitogenic self-stimulation in these more aggressive lymphomas may be the consequence. That could contribute to the high proliferation index, as observed in PCLBLT; accordingly, all analysed PCLBCL-LT showed a high proliferation index, as determined by Mib1 staining (data not shown).

PCLBCL-LT with higher mean baseline MVD (> 22.4 $CD31^+$ microvessels/0.3136 mm^2) exhibited a slightly worse prognosis; however, without statistically significant difference. Traditional prognostic parameters for PCLBCL-LT include age, extent and location of skin lesions and expression of bcl-2, MUM-1 (29, 47). Further studies will clarify whether the easily-applicable assessment of VEGF- and VEGFR-expression as well as MVD by immunohistochemical staining with CD31 may provide a useful tool to more accurately predict the prognosis of PCBCL and to deduce the adequate therapeutic regimen. In systemic large B-cell lymphoma and other lymphoma subtypes antiangiogenic therapeutic modalities are already applied in clinical routine, and clinical studies are currently evaluating antiangiogenic therapies, including bevacizumab, endostar and thalidomide (www.clinicaltrials.gov).

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