CD5-positive diffuse large B cell lymphoma (DLBCL) is the least frequent immunohistochemical subgroup of DLBCL. The relatively little available data suggests a worse outcome in this population, resulting from a resistance to chemotherapy.

The aim was the comparative assessment of angiogenesis in both CD5-positive and CD5-negative DLBCL, as well as in lymphatic tissues without lymphoproliferative diseases.

The analysis included 36 cases of CD5-positive DLBCL (19 females and 17 males) aged 29–87 years (mean age 69), diagnosed and treated in the Maria Sklodowska-Curie Institute and Oncology Center and Medical University of Warsaw in 2002–2013. The control group comprised 28 cases of CD5-negative DLBCL (14 females and 14 males) aged 24–82 years (mean age 58.5). The secondary control group (13 cases) consisted of normal lymphatic tissue obtained from patients without lymphoproliferative diseases. The level of angiogenesis was assessed on the basis of immunohistochemical CD34, vWF and HIF1α expression measured using morphometric methods.

CD5-positive DLBCL, in comparison to CD5-negative DLBCL, was characterized by: (1) higher mean of total blood vessel area, (2) higher mean total ratio of blood vessel area and staining intensity, (3) higher mean of total blood vessel area in regions defined as hot spots, (4) higher mean of total ratio of blood vessel area and staining intensity in hot spots. The measurements in lymph nodes without lymphoproliferative diseases lay between the values obtained in both DLBCL subgroups.

We observed a significant exacerbation of angiogenesis in CD5-positive DLBCL in comparison to the CD5-negative subgroup, possibly explaining its more aggressive clinical course. Our data does not substantiate the hypothesis that angiogenesis is more pronounced in frequent CD5-negative DLBCL subgroup in comparison to benign lymphatic tissue (Adv Clin Exp Med 2016, 25, 6, 1149–1155).

Key words: CD5-positive DLBCL, angiogenesis, CD34, vWF, HIF1α.
tion, resulting mainly from resistance to chemotherapy [2]. It is still unclear whether CD5-positive DLBCL constitutes an independent clinical entity or merely an immunophenotypic variant of DLBCL.

Angiogenesis is a complex process of the formation of new blood vessels on the basis of the already existing network. An initial step is the proteolytic degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs). Subsequent steps are the migration and proliferation of endothelial cells, as well as the formation and maturation of blood vessels [3]. Judah Folkman in 1971 was the first to point out the potential role of angiogenesis in cancer development [4].

There are multiple factors either promoting or inhibiting angiogenesis. The supremacy of one group of factors results in either induction or inhibition of the angiogenesis, respectively. The formation of new vessels is stimulated by various cytokines like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (bFGF), transforming growth factor β (TGFβ), etc. On the contrary, antithrombin, thrombospondin, angiostatin, platelet factor 4 (PF4) and metalloproteinase inhibitors possess antiangiogenic properties [5].

Rapid tumor growth is frequently accompanied by hypoxia, which stimulates the expression of genes encoding proangiogenic factors and thus promotes formation of new vessels, enabling both the survival and proliferation of malignant cells. A hypoxic environment is associated with activation of a transcription factor – hypoxia inducible factor 1α (HIF1α), which strongly enhances signalization via VEGF protein [6].

The aim of the present study was the comparative assessment of angiogenesis in both CD5-positive and CD5-negative DLBCL, as well as in lymphatic tissues without lymphoproliferative diseases.

**Material and Methods**

The analysis included 36 specimens of CD5-positive DLBCL (27 lymph nodes, 5 stomachs, 2 brains, 1 intestine, 1 testis) obtained from 14 women (50%) and 14 men (50%), aged 24–82 years (mean age 58.5). The secondary control group included normal lymphatic organs (11 lymph nodes, 2 tonsils) obtained from persons without lymphoproliferative diseases.

**Histological Procedures**

Tissue samples for histological analysis were fixed in a 4% formalin buffer, embedded in paraffin blocks according to standard procedure and then cut into slices 3–4 μm thick. The slices were subsequently stained with hematoxylin and eosin (H&E).

**Immunohistochemical Procedures**

Immunohistochemical staining (IHC) was performed in an Autostainer Link 48 automatic staining system manufactured by Dako or manually, at room temperature (20–25°C). The staining protocols were based on standard methodology and the manufacturers’ recommendations in the case of the following antibodies used to confirm DLBCL diagnosis: CD20, CD3, CD5, CD10, ki67, MUM1, Bcl2, Bcl6, CyclinD1, CD138 and CD30. The assessment of angiogenesis was performed by means of 3 immunostains: CD34 (QBEnd10, IR632, Dako), vWF (IR527, Dako) and HIF1α (H1alpha67, MS-215, ThermoScientific). In the case of HIF1α, an individual staining procedure was developed, including the selection of a buffer for antigen retrieval, concentration and duration of incubation (pH 6.0, dilution 1/50, incubation time 20 min).

All specimens were evaluated by two experienced hematopathologists (Bogna Ziarkiewicz-Wróblewska and Beata Gierej).

**Morphometric Analysis**

Analysis of CD34, vWF and HIF1α expression was performed using morphometric methods, namely the automatic image analyzer and Image ProPlus software (Media Cybernetics, USA). The evaluation included both the total area of visualized blood vessels and the staining intensity. Ten representative visual fields were assessed in each specimen; the final result was the mean of all values. The study parameters included the total blood vessel area in μm², as well as total ratio of blood vessel area and staining intensity (expressed in arbitrary units, AU). Additionally, three regions with highest vessel density were analyzed separately (hot spots).
Statistical Methods

Statistical analysis was performed using IBM SPSS Statistics 19 software. The Fisher exact test with 2x2 contingency tables was used to assess the significance of the observed distribution of study parameters between CD5-positive and CD5-negative DLBCL. The comparison of morphometric indexes between study groups (CD5-positive DLBCL, CD5-negative DLBCL and the additional control group without hematologic disorders) was performed in two steps: firstly, analysis of variance with multiple-comparison Tukey’s test was implemented; secondly, linear regression analysis was performed. The results were regarded as statistically significant if the p-values were below 0.05 for each of the aforementioned tests.

Results

The intensity of staining differed greatly for each investigated antibody. Reaction with CD34 was more pronounced than with vWF, whereas the reaction with HIF1α was distinctly weaker in comparison to both remaining stains (Fig. 1).

Mean total blood vessel area (µm²) and mean total ratio of blood vessel area and staining intensity (AU), measured by means of CD34 and vWF expression, was highest in the CD5-positive DLBCL group, whereas the lowest values were noted in the CD5-negative DLBCL group. The respective values observed in lymphatic organs acquired from patients without lymphoproliferative diseases lay between both these extremes. In the case of HIF1α, both tested parameters were distinctly lower in comparison to the values obtained using CD34 and vWF. Similarly, the highest HIF1α expression was noted in the CD5-positive DLBCL subgroup. In patients with CD5-negative DLBCL, the study parameters were borderline higher than in patients without lymphoproliferative diseases (Table 1).

Likewise, the analysis of areas with the highest blood vessel density (hot spots) showed the highest values in CD5-positive DLBCL, intermediate values in patients without lymphoproliferative disease (Table 1).

![Fig. 1. Blood vessels visualized by immunohistochemical reactions with (A) CD34, (B) vWF, (C) HIF1α in specimens of CD5-positive DLBCL (magnification ×150)](image)

Table 1. Angiogenesis parameters in three study groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>CD34</th>
<th>vWF</th>
<th>HIF1α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean total blood vessel area (µm²)</td>
<td>mean total ratio of blood vessel area and staining intensity (AU, arbitrary unit)</td>
<td></td>
</tr>
<tr>
<td>DLBCL CD5(+)</td>
<td>6579.79 (100%)</td>
<td>5210.02 (100%)</td>
<td>174.02 (100%)</td>
</tr>
<tr>
<td>DLBCL CD5(−)</td>
<td>354.47 (5.39%)</td>
<td>227.39 (4.36%)</td>
<td>61.18 (35.16%)</td>
</tr>
<tr>
<td>Normal lymphatic organs</td>
<td>3137.13 (47.68%)</td>
<td>2968.87 (56.98%)</td>
<td>48.13 (27.66%)</td>
</tr>
<tr>
<td>DLBCL CD5(+)</td>
<td>692327.27 (100%)</td>
<td>514550.9 (100%)</td>
<td>19970.03 (100%)</td>
</tr>
<tr>
<td>DLBCL CD5(−)</td>
<td>35695.94 (5.15%)</td>
<td>19970.96 (3.88%)</td>
<td>6878.55 (34.44%)</td>
</tr>
<tr>
<td>Normal lymphatic organs</td>
<td>353895 (51.11%)</td>
<td>270088.3 (52.49%)</td>
<td>5479.68 (27.43%)</td>
</tr>
</tbody>
</table>
cases, and lowest in patients with CD5-negative DLBCL. This general tendency was also observed in the HIF1α analysis, although the values noted in CD5-negative DLBCL and in the group without lymphoproliferative diseases were very similar (Table 2).

The observed differences in the expression of markers of angiogenesis were statistically significant in all three study groups (Table 3).

Discussion

The results of the present study show that CD5-positive DLBCL is characterized by a more abundant blood vessel network in comparison to the DLBCL subtype without CD5 expression, as well as normal lymphatic organs (Table 1, 2). To our knowledge, in the available literature there are no reports on angiogenesis intensity in CD5-positive DLBCL.

CD5-positive DLBCL is considered a DLBCL subtype with aggressive clinical course. It occurs in the elderly with female sex predominance, more frequently in extranodal localization. In comparison to the CD5-negative DLBCL subtype, patients with CD5-positive DLBCL have more disseminated disease at diagnosis, worse performance status, higher serum lactate dehydrogenase (LDH) activity, and a propensity towards central nervous system involvement, as well as worse response to treatment [7].

The high blood vessel density promotes progression of the neoplastic process and may be an indirect indicator of high malignant potential. Cardesa-Salzmann et al. assessed the vasculature of DLBCL using antibodies specific to CD31 and computerized picture analysis and concluded that high angiogenesis intensity was correlated with malignant clinical behavior and shorter overall survival (OS). The authors showed significantly lower values of microblood vessel density (MVD): \(11.8 \times 10^3; 78.67\%\) in the GCB subtype vs. ABC subtype \(15 \times 10^3; 100\%\), regarded as a more aggressive variant of DLBCL [8]. Similarly, Vacci et al. documented richer tumor vasculature in

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**Table 2. Hot spot analysis in three study groups**

<table>
<thead>
<tr>
<th>Study group</th>
<th>CD34</th>
<th>vWF</th>
<th>HIF1α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean total blood vessel area (µm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DLBCL CD5(+)</td>
<td>11850.39</td>
<td>9240.26</td>
<td>297.44</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(100%)</td>
<td>(100%)</td>
</tr>
<tr>
<td>DLBCL CD5(−)</td>
<td>497.71</td>
<td>326.97</td>
<td>92.16</td>
</tr>
<tr>
<td></td>
<td>(4.20%)</td>
<td>(3.54%)</td>
<td>(30.98%)</td>
</tr>
<tr>
<td>Normal lymphatic organs</td>
<td>5577.35</td>
<td>5832.06</td>
<td>97.32</td>
</tr>
<tr>
<td></td>
<td>(47.06%)</td>
<td>(63.11%)</td>
<td>(32.72%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>mean total ratio of blood vessel area and staining intensity (arbitrary unit, AU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLBCL CD5(+)</td>
<td>1264203.83 (100%)</td>
</tr>
<tr>
<td></td>
<td>924424.41 (100%)</td>
</tr>
<tr>
<td></td>
<td>33788.49 (100%)</td>
</tr>
<tr>
<td>DLBCL CD5(−)</td>
<td>50112.23 (3.96%)</td>
</tr>
<tr>
<td></td>
<td>29194.49 (3.16%)</td>
</tr>
<tr>
<td></td>
<td>10300.92 (30.49%)</td>
</tr>
<tr>
<td>Normal lymphatic organs</td>
<td>643884.9 (50.93%)</td>
</tr>
<tr>
<td></td>
<td>537933.7 (58.19%)</td>
</tr>
<tr>
<td></td>
<td>11025.78 (32.63%)</td>
</tr>
</tbody>
</table>

**Table 3. The analysis of variance for differences of means in CD5-positive DLBCL, CD5-negative DLBCL and normal lymph nodes subgroups**

<table>
<thead>
<tr>
<th>Parameter tested by the analysis of variance for differences of means in CD5-positive DLBCL, CD5-negative DLBCL and normal lymph nodes subgroups</th>
<th>CD34</th>
<th>vWF</th>
<th>HIF1α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean total blood vessel area</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.026</td>
</tr>
<tr>
<td>Mean total ratio of blood vessel area and staining intensity</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.024</td>
</tr>
<tr>
<td>Hot spots – mean total blood vessel area</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.018</td>
</tr>
<tr>
<td>Hot spots – mean total ratio of blood vessel area and staining intensity</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.015</td>
</tr>
</tbody>
</table>
Angiogenesis in CD5-positive DLBCL

Aggressive lymphomas in comparison to subtypes with an indolent clinical course [9]. The number of blood vessels per 0.78 mm² ± SD in lymphomas with low, intermediate and high malignant potential was 7 ± 2, 12 ± 3 and 14 ± 5, respectively. This observation was also confirmed by other investigators: Arias et al., Crivelatto et al. and Ribatti et al. [10−12]. The increased angiogenesis in CD5-positive DLBCL observed in the present study may thus be associated with worse clinical course of this subtype of lymphoma.

Surprisingly, the intensity of angiogenesis was higher in normal lymphatic organs obtained from patients without lymphoproliferative diseases than in specimens of CD5-negative DLBCL (Table 1, 2). The results of other studies undertaking this question are inconsistent. Some authors found lower blood vessel density in lymphomas than in reactive lymph nodes, as in the present study [13, 14]. Kodowaki et al. performed an MVD analysis in normal lymph nodes and in various types of lymphomas and obtained the following results: 7818 ± 3533 and 6243 ± 4027, respectively. On the basis of CD34 immunostain, the researchers found that angiogenesis in DLBCL in general (without division into subtypes) is less prominent than in normal lymph nodes: 3243 ± 1453 and 7818 ± 3533, respectively [14]. Korkolopoulou et al. [15] and Mazur et al. [16] did not observe any relationship between blood vessel density and the morphological variant of lymphoma.

According to other reports, new vessel formation in nodes involved with lymphoma is more pronounced in comparison with non-neoplastic lymph nodes [17−19]. Vacca et al. analyzed specimens from 30 patients with benign lymphadenopathy and 71 patients with various lymphomas and assessed the number of blood vessels per area of 0.78 mm² ± SD, obtaining the following values: 3 ± 1 and 11 ± 5, respectively [17].

It should be noted that proliferation of small blood vessels remains a classic feature of T zone reaction in lymphatic organs, frequently observed as an unspecific response to various stimuli. It is probably important to precisely select the control group with exclusion of all lymph nodes with even minor histological abnormalities.

In line with the results presented by others, CD34 was the most sensitive marker for the detection of angiogenesis in the present study [20], whereas the staining reaction was weakest in the case of HIF1α (Fig. 1). HIF1α induces the biosynthesis of over forty protein regulators preparing cells for hypoxic conditions [21], which also increase the propensity of the tumor to develop distant metastases [22−24]. Owing to the uneven distribution of hypoxia, the neoplastic tissue is characterized by heterogeneous HIF1α expression, which is in turn correlated with regional blood vessel density [25, 26]. The morphometric analysis of the study parameters using antibodies specific to both CD34 and vWF gave comparable results. The analysis using HIF1α did not show any differences in angiogenesis intensity between lymphatic organs obtained from patients without lymphoproliferative diseases and CD5-negative DLBCL. This observation may be explained by the low intensity of the staining with this antibody, below the threshold of sensitivity of the implemented method.

Identification of a subgroup of patients with high risk lymphomas on the basis of increased angiogenesis forms a mainstay for the introduction of antiangiogenic therapies. Currently, multiple clinical trials assessing antiangiogenic molecules are ongoing. These are inhibitors of integrins and metalloproteinases, as well as soluble receptors of proangiogenic cytokines and others. These agents have already become standard treatment of certain malignancies, like sorafenib in unresectable hepatocellular carcinoma [27]. Another effective antiangiogenic treatment indicated in metastatic colon cancer is bevacizumab, a recombinant monoclonal antibody directed against vascular endothelial growth factor (VEGF) [28].

Therefore, inhibitors of angiogenesis may be a very promising therapeutic option in the treatment of certain lymphoma subtypes, which may substantially complement the standard chemotherapy and improve its effects [8, 29]. However, further clinical trials are mandatory to confirm the preliminary results presented in this report.

The study showed a higher intensity of angiogenesis in CD5-positive DLBCL in comparison to the CD5-negative variant, which may contribute to its more aggressive clinical behavior. The results do not substantiate the hypothesis that the more frequent CD5-negative DLBCL subtype was better vascularized than non-neoplastic lymphatic organs.

In view of the rapid progress of antiangiogenic therapy, the characterization of a subgroup of lymphomas as a potential target of this treatment modality has great clinical importance.
References

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