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Tumorigenesis and Neoplastic Progression

Microvessel Density and Expression of Vascular Endothelial Growth Factor and Its Receptors in Diffuse Large B-Cell Lymphoma Subtypes

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Angiogenesis is known to play a major role in neoplasia, including hematolymphoid neoplasia. We assessed the relationships among angiogenesis and expression of vascular endothelial growth factor and its receptors in the context of clinically and biologically relevant subtypes of diffuse large B-cell lymphoma using immunohistochemical evaluation of tissue microarrays. We found that diffuse large B-cell lymphoma specimens showing higher local vascular endothelial growth factor expression showed correspondingly higher microvessel density, implying that lymphoma cells induce local tumor angiogenesis. In addition, local vascular endothelial growth factor expression was higher in those specimens showing higher expression of the receptors of the growth factor, suggesting an autocrine growth-promoting feedback loop. The germinal center-like and nongerminal center-like subtypes of diffuse large B-cell lymphoma were biologically and prognostically distinct. Interestingly, only in the more clinically aggressive nongerminal center-like subtype were microvessel densities significantly higher in specimens showing higher vascular endothelial growth factor expression; the same was true for the finding of higher vascular endothelial growth factor receptor-1 expression in conjunction with higher vascular endothelial growth factor expression. These differences may have important implications for the responsiveness of the two diffuse large B-cell lymphoma subtypes to anti-vascular endothelial growth factor and anti-angiogenic therapies. ***(Am J Pathol 2007, 170:1362–1369; DOI: 10.2353/ajpath.2007.060901)***

Angiogenesis and the proangiogenic growth factor vascular endothelial growth factor (VEGF; also known as vascular permeability factor) have a known role in solid neoplasia, and there is increasing evidence that they also play a role in hematolymphoid neoplasia. Increased microvessel density has been noted in a range of hematolymphoid disorders, including multiple myeloma, non-Hodgkin lymphoma, acute and chronic leukemias of myeloid and lymphoid lineages, and myelodysplastic disorders.[1] VEGF promotes angiogenesis and vascular permeability via its receptor VEGF receptor (VEGFR)-2 (also known as Flk-1).[2] In addition, VEGF also has a direct role in hematolymphoid cell development; an autocrine loop involving VEGF and its receptor VEGFR-1 (also known as Flt-1) modulates *in vivo* hematopoietic stem cell survival and proliferation.[3] Cell lines derived from a variety of hematolymphoid malignancies have been shown to express both VEGF and VEGFR-1,[4] suggesting a role for a similar autocrine loop in neoplasia.

We assessed the interaction among microvessel density and local expression of VEGF and VEGFR-1 and -2 in diffuse large B-cell lymphoma (DLBCL). We further examined these findings in the context of clinically and biologically relevant subtypes of DLBCL. DLBCL is both common and aggressive, with a high mortality within the 1st year after diagnosis and 5-year overall survival of less than 50%.[5] Recently, gene expression profiling has uncovered at least two biologically and prognostically distinct subgroups of DLBCL.[6] These two subgroups are defined by the expression of sets of genes that they have in common with germinal center B cells (GC-like subtype) and activated peripheral blood B cells (ABC or non-GC-like subtype).

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Although gene expression profiling is crucial in the research setting for identifying biologically relevant gene products, immunohistochemical analysis has the advantages of being readily available in the clinical setting and of enabling morphological evaluation of expression patterns and such higher order features as tumor vascularity. We have used immunohistochemistry to assess tissue microarrays of DLBCL for microvessel density (highlighted with the vascular marker CD34) and expression of VEGF and its receptors VEGFR-1 and VEGFR-2. We report the relationships among these variables both within the group of DLBCL cases as a whole and within the GC-like and non-GC-like subgroups individually.

Materials and Methods

Tissue Microarray Construction

A tissue microarray containing 94 DLBCL cases from the Institute of Pathology at Aarhus University Hospital (Aarhus, Denmark) was used for this study. Only *de novo* DLBCL cases were included. The cases were not collected consecutively. No cases of primary mediastinal DLBCL, intravascular DLBCL, T-cell or histiocyte-rich B-cell lymphoma, or other World Health Organization-recognized special variants of DLBCL were included. Tissue microarrays were previously constructed as described previously[7] using a tissue arrayer (Beecher Instruments, Silver Spring, MD).[8] Institutional review board approval was obtained for these studies.

Immunohistochemistry

Immunohistochemistry for CD34 (1:10 dilution; Becton Dickinson Biosciences, San Jose, CA), VEGFR-1 (1:50; Lab Vision Corp., Fremont, CA), VEGFR-2 (1:400 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and VEGF (1:200 dilution; Santa Cruz Biotechnology) was performed on 4-μm sections, which were placed on glass slides, baked for 1 hour at 60°C, deparaffinized in xylene, and hydrated in a graded series of alcohol. Antigen retrieval was as follows: mild heat retrieval for CD34, ethylenediamine tetraacetic acid retrieval for VEGF, Tris retrieval for VEGFR-1, and Tris retrieval for VEGFR-2. Endogenous peroxidase was blocked, and the chromogen used was diaminobenzidine. Immunohistochemistry for HGAL, BCL2, BCL6, CD10, and MUM1 (also known as IRF4) was previously described.[7] Images of the immunostained tetramethylammonium slides for CD34, VEGF, VEGFR-1, and VEGFR-2 were scanned, digitized, and stored at *http://tma.stanford.edu/tma_portal/mvd* (Stanford University School of Medicine, accessible as of 8/1/2006).

Scoring of Immunohistochemical Stains

Immunohistochemical stains for HGAL, BCL2, BCL6, CD10, MUM1,VEGFR-1, VEGFR-2, and VEGF were scored as follows based on the percentage of lymphoma cells stained: more than 30% staining was scored strong positive; 5 to 30% staining was scored weak positive; and less than 5% staining was scored negative. Staining was performed on two separate cores from each case of DLBCL; for each of the stains listed, at least 86 were evaluated on two duplicate cores, and at least 88 were evaluated on a single core. Where two scores were available for cores taken from the same specimen, the higher value was used in the analysis. For microvessel density quantitation, where two scores were available for cores taken from the same specimen, the average was used in

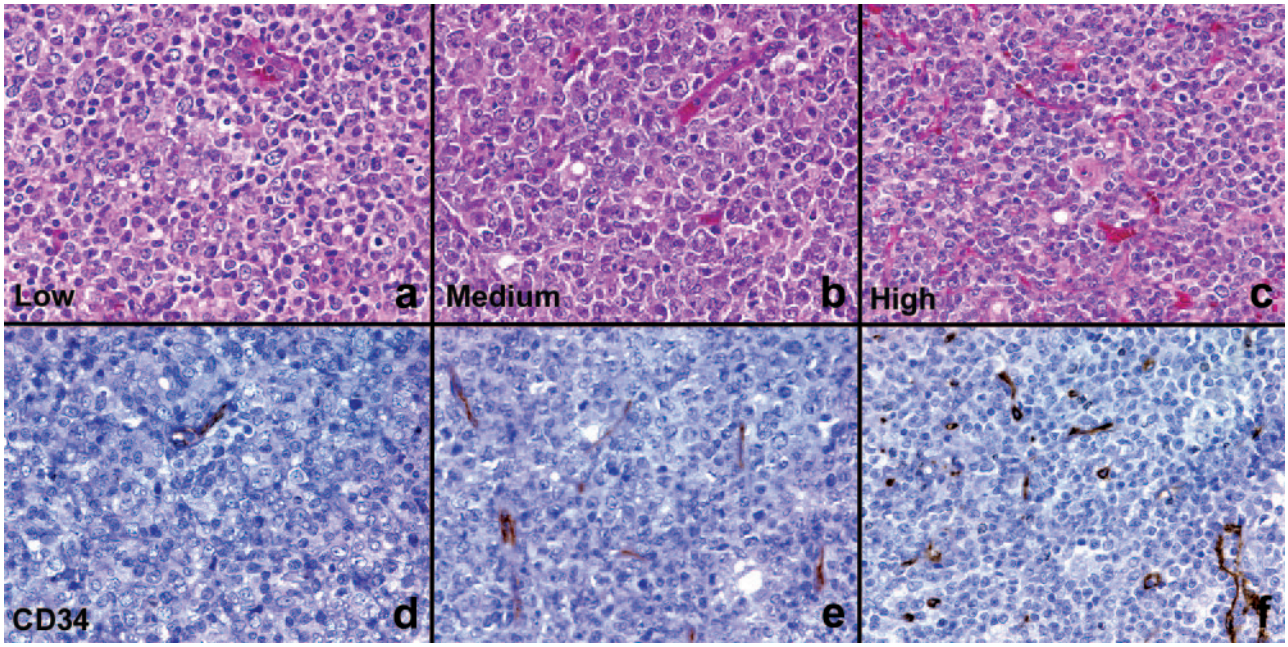

Figure 1. Vascularity in DLBCL. Examples of DLBCL illustrating low (**a** and **d**), medium (**b** and **e**), and high (**c** and **f**) vascularity (H&E, anti-CD34; magnification, ×400).