standard DLBCL therapy.[5,6] However, results from the Nebraska Lymphoma Study Group as well as the Lymphoma/Leukemia Molecular Profiling Project have been consistent.[3,4,6]

Recently, significant progress has been made in the use of immunohistochemical markers to predict survival of DLBCL patients, as shown in the compelling and highly-significant results in the article by Perry et al.[1] Over the past decade, the authors of this paper have designed, conducted, and published many original papers on DLBCL that identify the most important diagnostic, prognostic and biologic markers (immunohistochemistry, gene expression profiling, microRNA profiling, and so on) for use in the daily diagnostic and clinical practice.[3,4,6-9]

The current study by Perry et al is carefully designed and conducted by a seasoned team of highly experienced lymphoma investigators from North America and Western Europe who have incorporated their most important published information on DLBCL.[4,7-9] Based on the results presented, the authors conclude that their new biologic prognostic model (BPM) delineates 2 clinically distinct groups of patients: one with a low biologic score (0-1) and good survival, and the other with a high score (2-3) and inferior survival.[1] This new BPM could be used in the future with the International Prognostic Index (IPI) to stratify patients for novel or risk-adapted therapies. The conclusions reached in this paper are based on the immunohistochemical stains for the cell-of-origin of the malignant cells (GCET1, MUM1, FOXP1, CD10, and BCL6), as well as admixed–stromal cells and benign histiocytes expressing secreted protein acidic and rich in cysteine (SPARC), and the microvessel density (MVD) determined by analyzing digital images of the CD31-stained slides. Using the BPM, 1 subset of DLBCL had a short overall and event-free survival in multivariate analysis after adjusting for the IPI, as evidenced by the presence of the non-GCB phenotype, low SPARC expression of $< 5\%$, and a high MVD score.[1] Importantly, their BPM based on immunohistochemistry is the first prognostic model in the rituximab era to combine the cell-of-origin of the malignant cells and stromal signatures of the benign component into one, integrated, numerical prognostic score that can be readily used in clinical practice.

Because the BPM model of Perry et al is newly introduced, it has not been tested by anyone else. Therefore, the authors emphasize that this BPM will need to be validated in future prospective clinical studies.[1] Because DLBCL is the most common lymphoma around the world and there is no similar model available, I also urge investigators to test this new BPM. Even though immunohistochemistry is widely available and easy to use, its reproducibility among different institutions has been variable due to differences in tissue processing, antibody clones used, and interobserver variability. Efforts should be made to standardize various aspects of immunohistochemistry and also use image analysis for quantification to promote better reproducibility so that the results from different studies can be compared in the future.

Furthermore, validation of this BPM model in formal clinical trials would be ideal. Toward this end, for new studies, participation of one of the coauthors of this study in the design and validation of this model could also be valuable for improving interobserver agreement, because reviewing of H&E-stained slides and extracting relevant histologic features and interpreting immunostains is subjective, and integrating all of this information with clinical and other information requires a high level of judgment that is surely dependent on the level of individual training, subspecialization, skills, and experience and, thus, making diagnosis is an art.[10,11] Beneath this art, there lies a solid foundation of scientific knowledge, but this new scientific information is highly complex, ever increasing, rapidly evolving, often uncertain, and continuously changing. This underlying complexity in each individual tumor accounts for at least, in part, the variability in the current and other predictive models. Future investigations may identify additional important markers that could be added to this model, not only to improve outcome prediction but also to guide target directed therapy.

Conflict-of-interest disclosure: The author declares no competing financial interests. ■

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● ● ● VASCULAR BIOLOGY

Comment on Hoeppner et al, page 2167

Zebrafish lead the way in control of vascular permeability

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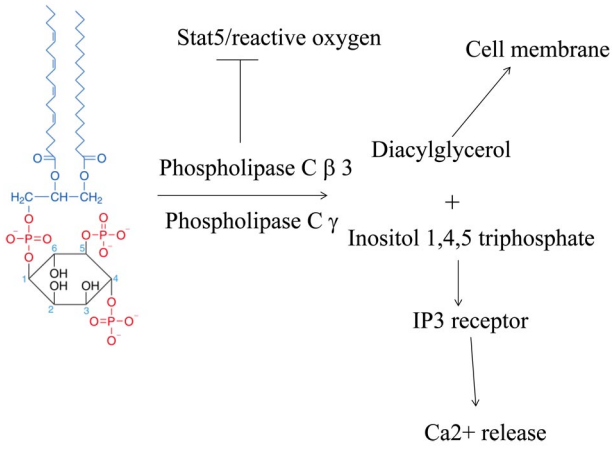
In this issue of *Blood*, Hoeppner et al create an extremely valuable model of vascular permeability that is amenable to high throughput chemical screening as well as genetic analysis.[1]



Both phospholipase Cβ3 and phospholipase Cγ have similar enzymatic activities in cleavage of PIP2 into diacylglycerol and Ins (1,2,4) triphosphate. Diacylglycerol activates canonical protein kinase C, while Ins (1,2,4) triphosphate binds to its receptor IP3R, resulting in calcium release. Loss of phospholipase Cβ3 results in activation of stat5 and possibly reactive oxygen in the face of unopposed phospholipase Cγ, resulting in proliferation and increased vascular permeability. Structure of PIP2 from Wikipedia.[10]

Vascular permeability is a major cause of morbidity and mortality in human disease. The edema of glioblastoma multiforme, which increases intracranial pressure, is due to tumor vasculature leakage. Cerebral malaria, which also causes death, is characterized by heightened vascular permeability, and mortality is associated with high levels of Angiopoietin-2 (Ang-2), a major mediator of vascular permeability.[2] Tumors with high levels of Ang-2 and vascular permeability are associated with a poor prognosis, possibly because vascular leak causes increased tumor hypoxia due to ineffective perfusion, and hypoxia promotes increased secretion of vascular endothelial growth factor, epithelial mesenchymal transformation, and increased stem cell characteristics. Finally, bacterial sepsis is a major cause of death in humans, and is poorly responsive to pressors that increase blood pressure, because increasing blood pressure merely pushes fluid out of the blood vessels, leading to the phenomenon called third spacing. Given that recent trials for sepsis-reducing agents have met little success, a greater understanding of vascular permeability is required.[3]

Currently, the gold standard of vascular permeability studies is the Miles Blue assay, in which a dye, Miles Blue, is infused into mice with various pathologic conditions. The quantity of dye that leaks into the resulting tissue is assessed spectrophotometrically. This type of assay is laborious and not amenable to high throughput analysis. In addition, the mouse system makes genetic analysis of vascular permeability difficult, and requires not only the time-consuming process of knocking out a gene, but also that the mice survive the knockout. Here, Hoeppner et al have devised an innovative zebrafish model to study vascular permeability.[1] In this transgenic model, heat shock is used to activate the induction of VEGF in a highly reproducible fashion. Using this model, vascular permeability can be divided into basal vascular permeability (likely reflecting vascular tone), acute vascular permeability, and chronic vascular permeability. Chronic vascular permeability likely represents the phenomena seen in human cancer and chronic infections.

VEGF, which was initially described as vascular permeability factor (VPF), activates multiple signaling pathways, mostly downstream of activation of VEGFR2. The ability to separate the promigratory pathways that stimulate endothelial chemotaxis, versus the vascular permeability function, is clinically relevant. Notably, one of the major side effects of the anti-VEGF factor bevacizumab (Avastin), is hypertension, and often limits use of bevacizumab. In the article by Hoeppner et al, the authors describe a positive role of phospholipase Cγ in promoting vascular permeability, which is opposed by phospholipase Cβ3. The role of phospholipase Cβ3 in opposing vascular permeability is confirmed in knockout mice for phospholipase Cβ3. The opposing roles of these phospholipases appear to be at the level of intracellular calcium. An additional upstream factor may be increased reactive oxygen. Of interest, mice that have an endothelial deficiency of NADPH oxidase 2 (Nox2) or the classic transient receptor potential channel 6 are protected from lung ischemia-reperfusion injury, and reactive oxygen was shown to induce phospholipase Cγ, placing reactive oxygen species upstream of phospholipase Cγ.[4]

The availability of these zebrafish has the potential to revolutionize the study of vascular permeability. First, these models will allow high throughput screening of compounds to assess their impact on vascular permeability. Second, it can be used to assess whether all forms of vascular permeability use the same pathways. For instance, does Ang-2, one of the major instigators of vascular permeability, induce phospholipase Cγ or repress phospholipase Cβ3?

Third, it allows the assessment of antiangiogenic agents on vascular permeability. Do hemangiomas of infancy, the classic manifestation of vascular permeability, have high levels of phospholipase Cγ and/or low levels of phospholipase Cβ3? Do agents that inhibit hemangioma growth, such as NADPH oxidase inhibitors (fulvenes, gentian violet) and β blockers, reverse the imbalance between phospholipase Cγ and phospholipase Cβ3 (angiogenic switch)[5-8]? Finally, both phospholipase Cγ and phospholipase Cβ3 have similar enzymatic activity, but phospholipase Cβ3 has been shown to be a tumor suppressor, because of nonenzymatic functions of phospholipase Cβ3, namely activation of the stem cell factor stat5 (see figure).[9] It is the unopposed action of phospholipase Cγ that likely accounts for the angiogenic switch to increased vascular permeability.

Conflict-of-interest disclosure: J.L.A. is an inventor on triphenylmethane and fulvene technology mentioned in the commentary. ■

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VASCULAR BIOLOGY

Comment on Jiang et al, page 2330

CD146: a new partner for VEGFR2

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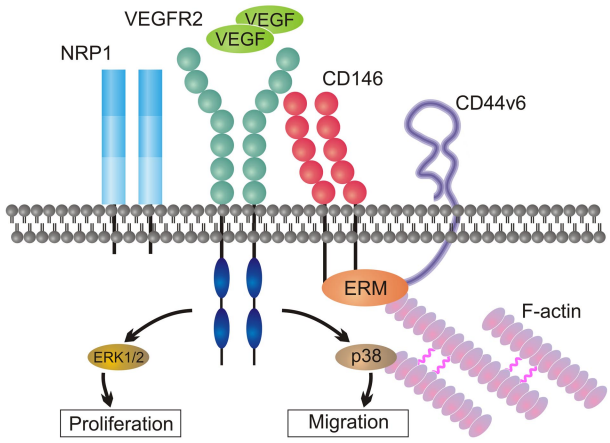
In this issue of *Blood*, Jiang et al identify the cell adhesion molecule CD146 as novel co-receptor for vascular endothelial growth factor receptor 2 (VEGFR2).[1]



Vascular endothelial growth factor receptor 2 (VEGFR2) signalosome. Within the cell membrane, VEGFR2 is associated with several co-receptors such as neuropilin-1 (NRP1), CD146, and CD44v6, together representing the so-called VEGFR2 signalosome. Co-receptors enable efficient ligand binding to VEGFR2 by their extracellular domains. Furthermore, their cytoplasmic tail binds ezrin-radixin-moesin (ERM) proteins that recruit cytoskeletal elements that function as scaffold for VEGFR2 downstream mediators. Depending on the combination of VEGFR2 with its co-receptors, VEGFR2 may cause activation of diverging downstream signaling cascades resulting in discriminative biologic processes.

The cell adhesion molecule CD146 was first described in 1987 because of its expression on malignant melanocytes and was correlated with a negative prognosis of melanoma patients. Based on sequence homology analysis, CD146 could be identified as a member of the cell adhesion molecules (CAMs) of the immunoglobulin superfamily. Therefore, it is also known as M-CAM or Mel-CAM (melanoma cell adhesion molecule). Not long afterward, CD146 was identified as a cell-surface antigen of endothelial cells. CD146 became popular as marker of circulating endothelial cells (CECs), which are increased in pathologic conditions such as cardiovascular diseases, inflammation, or cancer. Through the use of activating and inhibiting antibodies, involvement of several intracellular pathways in CD146 signaling including focal adhesion kinase or p38 kinase was identified. In this regard, CD146 mediates cell-cell interactions and migration of endothelial cells.[2]

Jiang et al demonstrate the direct binding between CD146 and VEGFR2 in coimmunoprecipitation experiments. Furthermore, they found that the interaction takes place in the extracellular protein domain as the antibody AA98, which recognizes an extracellular CD146 epitope could block the interaction between VEGFR2 and CD146. Introduction of a mutation in this protein domain confirmed this observation. In addition, evidence was provided that the interplay of CD146 with VEGFR2 is mandatory for functional VEGFR2 signaling. Using an anti-CD146 antibody or CD146 siRNA, VEGF-induced phosphorylation of VEGFR2 was suppressed in human umbilical vein endothelial cells. Furthermore, inhibition of CD146 resulted in abrogation of the downstream cascade of p38 and Akt signaling, whereas ERK signaling was not affected by anti-CD146 antibody or CD146 siRNA.

VEGFR2 mediates the full range of VEGF responses in endothelial cells including proliferation, regulation of survival, migration, and vascular tube formation. VEGFR2 has multiple tyrosine phosphorylation sites, which may explain its manifold biologic functions.[3] For instance, phosphorylation of Tyr^{1175} results in activation of protein kinase C and downstream induction of the ERK pathway leading to cell proliferation.[4] On the other hand, upon phosphorylation of tyrosine residue Tyr^{1214}, tyrosine kinase Fyn is activated, which results in subsequent activation of Cdc42 and MAP kinase p38 inducing reorganization of the actin cytoskeleton and thus enhanced cell migration.[5] Although the exact molecular mechanisms of how stimulation of VEGFR2 induces diverging downstream signals have not yet been elucidated in detail, ligand diversity and availability as well as interaction with co-receptors might explain most of these effects. VEGF-A has at least 9 different splicing forms that induce distinct cellular functions due to different binding affinities to their receptors or extracellular matrix components.[3] Chen et al previously reported that VEGFR2 signaling induced from soluble versus matrix-bound VEGF resulted in distinct molecular activation patterns. Upon exposure to matrix-bound VEGF, clustering and internalization of VEGFR2 were potentiated compared with soluble VEGF, which resulted in prolonged VEGFR2 phosphorylation of tyrosine residue Tyr^{1214} and thus extended p38 signaling.[6] In addition, interaction with co-receptors is essential for functional signaling of many tyrosine kinases. Co-receptors identified for VEGFR2 include neuropilin-1, the hyaluronic acid receptor CD44, vascular endothelial cadherin, and β integrins.[7]