Alternatively Spliced Form of Angiopoietin-2 as a New Vascular Rheostat

Miki Kamiyama[1] and Hellmut G. Augustin[1,2]

Angiopoietin (ANPGT)–TIE signaling serves as a critical regulator of vessel maturation controlling vascular quiescence, maintenance, and homeostasis (primarily through ANGPT1–TIE2 signaling), as well as enabling vascular plasticity and responsiveness to exogenous cytokines (primarily through antagonistically acting ANGPT2). An alternatively spliced form of ANGPT2 ($ANGPT2_{443}$) was first reported 20 years ago. Yet, little is known to this day about its biological functions. In this issue of *Cancer Research*, Kapiainen and colleagues report an elegant series of experiments adding to the complexity and contextuality of ANGPT–TIE signaling. The authors studied the function of $ANGPT2_{443}$ in cellular experiments as well as in a genetic model *in vivo*, revealing that it is proteolytically cleaved into a lower molecular weight isoform (termed $ANGPT2_{DAP}$) that lacks the superclustering domain necessary for multimer formation. When compared with full-length ANGPT2, $ANGPT2_{443}$ and $ANGPT2_{DAP}$ showed lower binding affinity to α5β1 integrin, but were more potent inhibitors of ANGPT1–TIE2 signaling. Functionally, $ANGPT2_{443}$ impaired vessel enlargement and vein morphogenesis during postnatal retinal angiogenesis. Tumor experiments in $Angpt2_{443}$-expressing mice showed enhanced destabilization of the lung vasculature, with varying effects on metastasis. Taken together, the study provides important insight into the significance of ANGPT2 alternative splicing and identifies $ANGPT2_{443}$ and $ANGPT2_{DAP}$ as a biological rheostat of ANGPT1–TIE2 signaling. Future work will need to characterize the relative ratios and functional contributions of the ANGPT2 variants in different pathophysiologic settings.

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VEGF acts as a hierarchically upstream master switch of the angiogenic cascade inducing tip cell formation and vascular sprouting from preexisting vessels. In contrast, angiopoietin (ANGPT)–TIE signaling regulates later steps of blood vessel formation related to vessel assembly, maturation, and acquisition of the quiescent endothelial cell (EC) phenotype. The default signaling axis of the ANGPT–TIE pathway results in activation of the vascular receptor tyrosine kinase TIE2 by the agonistically acting ligand ANGPT1 as well as the lesser characterized TIE2-ligand ANGPT4 (1). As alternative receptors, the angiopoietins are also capable of binding to and signaling through integrins, most notably in angiogenic EC with downregulated TIE2 expression (2). In addition, the coreceptor TIE1 and the TIE2 ligand ANGPT2 can act as modulators of constitutive ANGPT1–TIE2 signaling. TIE1 does not bind the ANGPT ligands, but interacts with TIE2 to contextually enhance or quench TIE2 signaling (3). In contrast to the still poorly understood TIE2-modulating activities of TIE1, the effects of ANGPT2 on ANGPT1–TIE2 signaling have been studied in much greater detail during the last two decades. In fact, based on its functional cooperativity with VEGF, ANGPT2 is probably the most intensely studied second-generation target for antiangiogenic therapies in the fields of oncology and ophthalmology. However, in contrast to promising preclinical results, combinations of anti-VEGF and anti-ANGPT2 have not proven superior to anti-VEGF alone in cancer-related clinical trials thus far. Studies to assess the efficacy of anti-ANGPT2 therapies to serve as facilitators of immune checkpoint inhibitors are ongoing. Likewise, advanced clinical trials with anti-VEGF/anti-ANGPT2 combination therapies are ongoing in the field of ophthalmology.

While ANGPT1 is a strong agonist for TIE2-mediated maintenance of vascular quiescence, ANGPT2 modulates TIE2 signaling agonistically or antagonistically in a context-dependent manner. Multimerization of the ANGPT ligands is considered as one of the critical determinants for their agonistic or antagonistic effects on TIE2 (1). In fact, ANGPT2 acts as a partial agonist on TIE2, that is, it quenches ANGPT1–TIE2 signaling in the presence of ANGPT1, whereas it acts as a weak agonist in the absence of ANGPT1.

In addition to full-length *ANGPT2*, an alternatively spliced form of *ANGPT2*, termed $ANGPT2_{443}$, was isolated from human EC 20 years ago (4). $ANGPT2_{443}$ is generated by excision of exon 2, which codes for the coiled-coil domain and is necessary for multimerization. Although $ANGPT2_{443}$ was subsequently reported to be expressed by patient-derived leukemic cells (5) in addition to EC and macrophages (4), no functional studies of this isoform have been performed to date. In this issue of *Cancer Research*, Kapiainen and colleagues report a series of elegant cellular and genetic *in vivo* experiments that provide intriguing insight into the function of this ANGPT2 variant (6). When performing Western blot analyses of human ECs overexpressing full-length ANGPT2 or $ANGPT2_{443}$, the authors unexpectedly detected two bands in ECs overexpressing $ANGPT2_{443}$. The band with lower molecular weight than expected from calculation was identified as a new truncated isoform of $ANGPT2_{443}$. Mass spectrometric analysis revealed that the $ANGPT2_{443}$-derived low molecular weight isoform (termed $ANGPT2_{DAP}$) lacks the superclustering domain necessary for multimer formation (6). The authors thereupon demonstrated that $ANGPT2_{443}$ inhibits ANGPT1 [confirming the earlier report (4)] as well as ANGPT4 activity more potent than full-length ANGPT2, resulting in less activation of TIE2 signaling. Notably, $ANGPT2_{DAP}$

[1]Division of Vascular Oncology and Metastasis, German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), Heidelberg, Germany. [2]European Center for Angioscience (ECAS), Medical Faculty Mannheim, Heidelberg University, Heidelberg, Germany.

Corresponding Author: Hellmut G. Augustin, European Center for Angioscience (ECAS), Medical Faculty Mannheim, Heidelberg University, and German Cancer Research Center Heidelberg (DKFZ-ZMBH Alliance), Im Neuenheimer Feld 280, Heidelberg D-69120, Germany. Phone: 4962-2142-1500; Fax: 4962-2142-1515; E-mail: augustin@angioscience.de

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was found to even be more potent in inhibiting ANGPT1- and ANGPT4-mediated TIE2 activation (6).

An analysis of The Cancer Genome Atlas data of splice variants in patients with breast cancer revealed that the transcript ratio of *ANGPT2*$_{443}$ to full-length *ANGPT2* was shifted toward *ANGPT2*$_{443}$ in human breast tumor tissues. On the basis of this observation, the authors generated *Angpt2*$_{443}$ mice, in which exon 2 was deleted. Studying these mice in comparison with wild-type mice (expressing full-length ANGPT2) as well as heterozygous mice (expressing one full-length and one exon 2–deleted allele), the authors performed a series of developmental and cancer-related phenotyping experiments to define the biology of *Angpt2*$_{443}$. Disruption of full-length *Angpt2* with forced expression of *Angpt2*$_{443}$ in mice impaired vessel enlargement and vein morphogenesis in the retina in a dose-dependent manner (6). The authors further investigated the effect of ANGPT2$_{443}$ in cancer progression, employing three different lung metastasis models: mouse mammary tumor virus-*polyoma middle tumor-antigen* (MMTV-*PyMT*), melanoma B16F10 tail vein injection, and breast cancer E0771 orthotopic transplantation. Compared with wild-type mice, heterozygous *Angpt2*$^{+/443}$ mice had less primary tumor growth and reduced lung metastasis, with increased vascular leakage in the MMTV-*PyMT* model. In contrast, heterozygous *Angpt2*$^{+/443}$ and homozygous *Angpt2*$^{443/443}$ mice had increased lung metastasis with destabilized lung vasculature compared with wild-type mice upon B16F10 melanoma tail vein injection. In the E0771 orthotopic transplantation model, primary tumor growth was not different. However, overexpression of ANGPT2$_{443}$—but not ANGPT2$_{DAP}$ or full-length ANGPT2—enhanced E0771 lung metastasis with destabilization of the lung vasculature, with no effect on primary tumor growth (6).

The study has importantly added new dimensions to the current understanding of ANGPT–TIE signaling. Yet, as with essentially any good study, it provokes many more new questions. What can mechanistically be learnt from the varying phenotypes in the different tumor models, whereas enhanced destabilization of the lung vasculature appears to be a more common phenotype in different tumor models? The authors discuss some possibilities. Compared with the MMTV-*PyMT* model, E0771 orthotopic tumors had less TIE2-positive ECs in primary tumors and, thus, ANGPT2 effects on tumor progression might be less dependent on TIE2 and more dependent on integrin signaling. In fact, enhanced inhibition of TIE2 with reduced integrin binding and activation of ANGPT2$_{443}$ would likely be compatible with this phenotype. Conceptually, ANGPT2$_{443}$ would thereby serve as a modulating rheostat of full-length ANGPT2 differential effects on TIE2 and integrin signaling.

Previous studies have shown that genetic deletion or antibody blockade of ANGPT2 delays primary tumor growth and metastasis, but detailed temporal analysis of resulting tumor phenotypes revealed that ANPGT2-mediated vascular destabilization primarily affects early stages of tumor growth. ANGPT2 was found to be dispensable for later stages of tumor progression (7), an observation that may explain the limited efficacy of ANGPT2 targeting in oncology clinical trials. The failure of clinical trials has in recent years stimulated work aimed at (i) exploring drug combinations involving anti-ANGPT2, (ii) identifying specific tumor entities, in which ANGPT2 may be a bottleneck, and (iii) validating different therapeutic windows for ANGPT2-targeted therapies. All three avenues hold substantial potential. The recent breakthrough results of the IMbrave trial in hepatocellular carcinoma [combination of immune checkpoint blockade (PD-L1) and antiangiogenesis (VEGF; ref. 8)] have created an urgent need to elucidate the mechanisms of antiangiogenic priming of immune checkpoint therapy. In this context, research should not just concentrate on VEGF, but similarly focus on ANGPT2 as well as VEGF/ANGPT2 combinations. Concerning stratification for specific tumor entities, not all tumors are alike—mechanistic studies and biomarker validation may pave the way into personalized antiangiogenic therapy. For example, recent work has shown that in a subtype of patients with melanoma, ANGPT2 is produced not just by EC, but also by tumor cells to drive tumor progression and metastasis, providing a strong scientific rationale for individualized, biomarker stratification-driven antiangiogenic therapy (9). Finally, exploring different therapeutic windows, ANGPT2 was recently discovered as key regulator of lymphatic metastasis. Neoadjuvant ANGPT2 blockade prior to primary tumor surgery led in preclinical experiments to a substantial reduction of metastasis—even when surgery was performed at late stage in fairly advanced primary tumors (10).

Integrating the findings of the Kapiainen and colleagues study into the pathophysiologic assessment of ANGPT2-mediated tumor progression and metastasis, as well as in determining its promise as a therapeutic target, will require both additional mechanistic preclinical experimentation and validation work in relevant clinical specimens. Forced genetic expression or deletion of a candidate molecule in a manipulatory preclinical model grants insight into its molecular function. Yet, it does not provide information on the expression and regulation of a molecule in the natural setting, particularly as it relates to differential splicing mechanisms. As such, determining the relative ratio of full-length ANGPT2 versus ANGPT2$_{443}$ expression in different pathophysiologic settings will be important to put the findings of the Kapiainen and colleagues study into perspective. Intriguingly, this ratio may well reflect different patterns of angiogenesis (i.e., balancing ANGPT2 effects on integrins affecting tip cells vs. ANGPT2 effects on TIE2 affecting stalk cells) and it may be worthwhile to explore whether this ratio can be exploited as an informative biomarker of angiogenic activity.

Authors' Disclosures

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