Antiangiogenic VEGF-Ax: A New Participant in Tumor Angiogenesis

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Abstract

The transcript of the angiogenic factor vascular endothelial growth factor A (VEGF-A) is subject to a multitude of stimulus-dependent, posttranscriptional regulatory events, consistent with its unusually long 3′ untranslated region. We have recently reported translational readthrough of VEGFA mRNA whereby translating ribosomes traverse the canonical stop codon to a conserved, downstream stop codon, generating VEGF-Ax (‘x’ for extended), a novel, extended isoform with an additional 22 amino acids appended at the C-terminus. This event is the first vertebrate example of protein-regulated, programmed translational readthrough that generates a protein with a known function. Remarkably, VEGF-Ax exhibits potent antiangiogenic activity, both in vitro and in vivo, thus raising profound clinical implications, particularly with respect to cancer treatment. In this review, we discuss the potential of VEGF-Ax as a therapeutic agent and drug target, as well as its possible role in the failure of, or resistance to, conventional anti-VEGF therapies in many types of cancers. Cancer Res; 75(14); 2765–9. ©2015 AACR.

Introduction

More than 40 years ago, Judah Folkman proposed the contribution of blood vessel development, i.e., angiogenesis, to tumor growth and advocated a new modality of cancer treatment in which host angiogenesis is targeted rather than the tumor itself (1). To date, there have been some notable successes, but many attempts to treat human cancers by targeting angiogenesis have been met with disappointing outcomes (2). The process of angiogenesis and its regulation is certainly more complex than first believed, and new cellular processes, molecules, and pathways are continuously being discovered that contribute importantly to angiogenesis.

Vascular endothelial growth factor (VEGF)-A, discovered in the 1980s, is a critical factor that regulates angiogenesis both in physiologic and pathologic conditions, including tumorigenesis (3). VEGF-A has become a particularly important pharmacologic target in antitumor drug development, and in the current armamentarium for treatment for several tumor types. Many antiangiogenic drugs target either VEGF-A (e.g., bevacizumab, also known as Avastin) or its receptors (e.g., lenvatinib). The human VEGFA gene resides on chromosome 6, and multiple alternative splicing events generate several isoforms of VEGFA mRNA and protein. Overwhelming evidence from many investigators supports the potent proangiogenic activity of many VEGF-A splice variant isoforms in vitro and in vivo. Nonetheless, antiangiogenic isoforms in VEGFA mRNA were reported by Bates and colleagues (4). These isoforms, termed VEGF-Ab, are generated by an alternative splicing event with the 3′-most exon, resulting in a switch in the C-terminus of the encoded protein from canonical VEGF-A isoforms that end in CDKPRR to a C-terminus ending in SLTRKD. The different C-terminus is thought to be critical for the antiangiogenic activity of VEGF-Ab (5). Several laboratories have demonstrated antiangiogenic activity of VEGF-Ab in multiple systems, both in vitro and in vivo (6–8).

Discovery of VEGF-Ax and Its Generation by Translational Readthrough

Our laboratory has recently identified a novel antiangiogenic VEGF-A isoform, termed VEGF-Ax, in endothelial cells (EC; ref. 9). Our experiments to investigate the paracrine function of VEGF-A in cultured ECs revealed that ECs secrete an antiangiogenic isoform of VEGF-A. However, mRNA specific to VEGF-Ab, the alternatively spliced antiangiogenic isoform, was not detectable. This observation was consistent with the presence of a novel antiangiogenic VEGF-A isoform secreted by ECs and generated by an unknown mechanism.

An important insight came from inspection of the proximal 3′ untranslated region (UTR) of VEGF mRNA in multiple mammalian species. Interestingly, the VEGFA 3′ UTR has an evolutionarily conserved stop codon in-frame with the canonical stop codon. Even more surprising, the two stop codons and their in-frame nature are conserved despite mutation, deletion, and insertion events during evolution. This analysis enticingly suggested that VEGFA mRNA translation might extend beyond the canonical stop codon to terminate at the downstream stop codon in what is considered to be 3′U TR. Progression of translating ribosomes beyond the stop codon is known as translational readthrough or stop codon readthrough, and is most often observed and best understood in certain viruses (10). The putative translational readthrough event in VEGFA mRNA would generate a protein with a 22-amino acid extension (21 amino acids encoded by the 63-nt extension plus a stop codon replacement) terminating with SLTRKD. This is the same C-terminus in VEGF-Ab thought to
Confer the antiangiogenic property. We termed the putative extended isoform VEGF-Ax ("x" for extended).

The generation of VEGF-Ax by translational readthrough was validated by multiple experimental approaches. An antibody was raised against a 15-amino acid segment in the C-terminal extension, and validated to detect VEGF-A, but not any other VEGF-A isoform (including VEGF-Ab). The antibody detected endogenous VEGF-Ax in lysates of primary ECs from multiple mammalian species, as well as in serum samples from healthy human subjects. Mass spectrometric analysis not only detected the readthrough sequence of VEGF-Ax, it also identified Ser as the amino acid inserted in place of the canonical UGA stop codon. Translational readthrough was also demonstrated using a construct containing luciferase cDNA downstream of the VEGF-A cDNA after the canonical stop codon. Robust luciferase expression was observed when this construct was transfected in ECs, and also the canonical stop codon. Robust luciferase expression was comparable with authentic readthrough observed in some viruses (10, 11).

Readthrough events can be programmed by downstream cis-acting RNA elements, termed programmed translational readthrough (PTR; ref. 12). Readthrough of VEGFA mRNA is executed by the 63-nt RNA sequence (termed Ax element) between the canonical and the evolutionarily conserved downstream stop codon, thereby utilizing a PTR mechanism. The Ax element can program readthrough even in a heterologous context. Thus, the Ax element performs a dual function; it not only encodes the peptide extension, but it also acts as an RNA element that programs readthrough. We showed that heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1, a known RNA-binding protein, binds this element and promotes readthrough and VEGF-Ax generation.

VEGF-Ax Function
Anti–VEGF-Ax antibody stimulated migration and proliferation of cultured EC consistent with a paracrine, antiangiogenic activity of endogenous VEGF-Ax. Likewise, recombinant VEGF-Ax<sup>ΔN</sup> (an isoform in which the upstream stop codon is replaced by an Ala codon to facilitate efficient expression) reduced EC migration, proliferation, and tube formation in Matrigel in vitro. The in vivo activity of VEGF-Ax was tested using a human xenograft system in nude mice. Subcutaneous administration of recombinant VEGF-Ax markedly reduced the progression of HCT116 (human colon carcinoma cell)-derived tumors and associated angiogenesis, demonstrating antiangiogenic property of VEGF-Ax. The finding that the dominant activity of VEGF-A released by EC is antiangiogenic was unexpected, but consistent with a previous report that aortic rings from mice heterozygous for EC-specific VEGFA gene deletion exhibit increased sprouting (13).

VEGF-Ax binds VEGFR2 with an affinity comparable with VEGF-A, but does not bind the coreceptor, neuropilin 1. Binding of VEGF-A to neuropilin 1 is essential for proangiogenic activity (14). Moreover, neuropilin 1 expression is required for development of angiogenesis in mice (15). Thus, the inability of VEGF-Ax to bind neuropilin 1 is likely to contribute to its antiangiogenic function. Also, VEGF-Ax does not activate the canonical VEGFR2 signaling pathways as determined by phosphorylation of key receptor Tyr residues (9). VEGF-Ax is likely to act as a decoy ligand of VEGFR2, competing with VEGF-A. Moreover, it is possible that VEGF-Ax induces an alternative, inhibitory signaling pathway leading to arrest of EC migration and proliferation, and consequent antiangiogenic function. Currently, we are far from a mechanistic understanding of the receptor-mediated, cellular function of VEGF-Ax. The physiologic function of VEGF-Ax likewise remains unclear. Prevention of unnecessary angiogenesis is almost certainly essential for vascular homeostasis and organismal well-being. Interestingly, antiangiogenic VEGF-Ax is predominant in the conditioned medium derived from EC compared with proangiogenic isoforms, possibly due to preferential secretion. We propose that the antiangiogenic activity of VEGF-Ax secreted by ECs contributes to the maintenance of vascular homeostasis. In this context, a robust source of proangiogenic agents, for example, VEGF-A secreted by tumors or during wound healing, can locally overwhelm VEGF-Ax and induce angiogenesis (Fig. 1).
Regulation of Translational Readthrough and VEGF-Ax Expression

VEGF-Ax expression exhibits tissue specificity. Healthy human colon, cerebrum, and spleen express substantial amounts of VEGF-Ax, whereas the protein is not detected in skeletal muscle and heart. Furthermore, whereas normal colon exhibits robust expression of both VEGF-Ax and VEGF-A, colon cancer tissues show expression of VEGF-A only, consistent with the vigorous angiogenesis observed in colon carcinoma (9). Together, these observations provide compelling evidence for physiologic and pathologic regulation of translational readthrough of VEGFA mRNA. It will be of great interest with respect to a mechanistic understanding, as well as potential therapeutic application, to determine if these readthrough changes are caused by alterations in expression or activity of hnRNP A2/B1, or possibly as-yet-unidentified trans-acting factors.

Our mechanistic understanding of regulation of translational readthrough is currently very limited in any system. In yeast, hypoxia-induced hydroxylation of Pro46 in ribosomal protein S23p in the decoding center of ribosomes affects both translational accuracy and translational readthrough efficiency in a context-dependent manner (16). Intriguingly, transcriptome-wide analysis of mRNA methylation revealed several mRNAs that undergo methylation at the N6 position of adenosine near stop codons (17). Possibly, site-dependent methylation influences the efficiency of translation termination and therefore translational readthrough, in a regulatable manner analogous to transcriptional regulation. Pseudouridine, the most prevalent mRNA modification, is generated from uridine residues by a catalytically regulated, posttranscriptional event (18). Interestingly, pseudouridylation of uridine residues in stop codons can elicit translational readthrough (19).

Translational Readthrough as a Mechanism of Proteome Expansion in Vertebrates

The contribution of translational readthrough to proteome expansion in less complex organisms, such as viruses and yeast, is well established; however, the significance of its role in vertebrate proteome expansion is only beginning to be recognized. For nearly 30 years, rabbit β-globin was the only reported example of readthrough in vertebrate transcripts (20). In 2012, Yamaguchi and colleagues demonstrated readthrough in the myelin P0 transcript (21). More recently, readthrough has been shown in several mammalian transcripts, including lactate dehydrogenase B (LDHB), mitogen-activated protein kinase 10 (MAPK10), aquaporin 4 (AQP4), opioid receptor kapa 1 (OPRK1), and opiate receptor-like 1 (OPRL1; refs. 22, 23). Our genome-wide bioinformatic analysis, followed by experimental confirmation, revealed two additional transcripts that exhibit readthrough: argonaute 1 (AGO1) and mitochondrial carrier 2 (MTCH2; ref. 9). The function of the extended readthrough product has not been determined in any of these cases, nor trans-acting regulators identified. A ribosome profiling study in human fibroblasts revealed ribosomal footprints on the 3′UTR of 42 mRNAs, suggesting that they are the targets of translational readthrough (24). However, a more recent report has shown that ribosomes can gain access to the 3′UTR of mRNAs without translating it (25). Clearly, experimental demonstration of the generation of readthrough product is essential to rigorously establish an mRNA as an authentic translational readthrough target. Nonetheless, ribosome profiling has substantial potential for identifying new readthrough candidate mRNAs in vertebrates and other organisms (24).

Presenting novel factors that contribute to translational readthrough regulation (26). Eukaryotic initiation factor 3 (eIF3), along with its associated factor, high-capacity suppressor tRNA (HCR1), regulates translational readthrough by interacting with releasing factors in yeast. They show that inactivating mutations in eIF3 and HCR1 decrease translation readthrough efficiency. A deep mechanistic understanding of readthrough will likely require detailed information on the interaction, and possibly the structure, of terminating ribosomes in conjunction with the cis-acting RNA signal element and its bound proteins.

Clinical Implications of VEGF-Ax

Disappointing results from current anti-VEGF therapy for cancers

Because of its critical role in angiogenesis, agents that selectively target VEGF-A, or its receptor or downstream signaling pathway, have received enormous attention as a pharmacologic target: bevacizumab (Avastin) and ranibizumab (Lucentis) are antibodies that target VEGF-A, and sintilimab (Sutent) and sorafenib (Nexavar) target VEGF-A receptors. These agents have shown remarkable effectiveness in many mouse models of cancer. Bevacizumab is currently used as first-line treatment for metastatic renal cell carcinoma and colorectal cancer, and has been approved for combination treatment, with chemotherapy, of platinum-resistant recurrent ovarian cancer (27). However, the improvement obtained with bevacizumab, and other VEGF-related therapeutics, has for the most part been significant but marginal; tumor shrinkage is often transient and followed by tumor re-growth (2, 28). In fact, none of the antiangiogenesis agents and treatments have met the high early expectations of the field initiated by Folkman (1).

As one example among many, bevacizumab is approved by the federal FDA as an adjuvant for the treatment of metastatic colon carcinoma in combination with standard FOLFOX (5-fluorouracil, leucovorin, and oxaliplatin) chemotherapy. However, the addition of bevacizumab increases the overall survival by only about 5 months, and it is not effective in nonmetastatic (i.e., stage IV) colon carcinoma (29). Furthermore, even this successful application has been debated due to the high cost and rather serious adverse effects (30). Similar findings have been observed in other cancers and other anti-VEGF drugs.

Possible mechanisms underlying the ineffectiveness of anti-VEGF therapies: role of VEGF-Ax

VEGF-A is arguably the most important proangiogenic factor for tumor growth; however, other potent proangiogenic cytokines...
have been investigated, including members of ephrin, fibroblast growth factor, and angiopoietin families. Expression of these factors, and activation of their angiogenic pathways, can circumvent anti-VEGF therapies (28). Tumor hypoxia can attract bone marrow–derived cells, including vascular progenitor cells, resulting in neovascularization, which can reduce the effectiveness of anti-VEGF treatments. Conceivably, local overexpression of VEGF-A, for example by hypoxia, can overwhelm the drugs (2). Anti-VEGF therapies can induce vascular normalization, i.e., reduce the vessel tortuosity and hyperpermeability observed in many tumors, and increase the pericyte coverage around blood vessels (31). Tumor cells can take advantage of the increased perfusion due to vessel normalization to invade adjacent healthy tissue (28). In this manner, anti-VEGF agents can increase tumor invasiveness, contrary to the goals and expectations of the treatment.

Current anti-VEGF drugs indiscriminately target both canonical proangiogenic and antiangiogenic VEGF-Ax (and VEGF-Ab) isoforms. Clearly, inhibition of an antiangiogenic agent such as VEGF-Ax can present negative consequences in cancer treatment. Therefore, the presence of VEGF-Ax can contribute to the ineffectiveness of anti-VEGF therapies. We propose that the level of antiangiogenic VEGF-Ax in the tumor or circulation can be a strong predictor of the effectiveness of anti-VEGF therapy. As an example, VEGF-A treatment might not be effective in grade 1 colon carcinoma where there is robust VEGF-Ax expression (9). This hypothesis can be tested by clinical studies evaluating VEGF-Ax levels in responders versus nonresponders to anti-VEGF therapies. Alternatively, agents that specifically target proangiogenic VEGF-A isoforms, for example, a C-terminal–specific antibody that does not recognize VEGF-Ax, might be more effective than current anti-VEGF drugs, and possibly with fewer adverse side effects. Finally, the potential of VEGF-Ax as a cancer biomarker needs to be reconsidered as these studies are generally done with antibodies that detect all VEGF-A isoforms.

Human and murine VEGF-A are very similar in amino acid sequence (90% identity) and proangiogenic activity. However, the peptide extensions in VEGF-Ax generated by translational sequence (90% identity) and proangiogenic activity. However, antibodies that detect all VEGF-A isoforms.

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VEGF-Ax as a diagnostic or therapeutic agent

VEGF-A, determined in tumors, serum, or plasma, has been used as a diagnostic for tumor severity, as prognostic indicator, and to predict effectiveness of treatment in a multitude of studies on nearly all tumor types (32). In most cases, correlations between VEGF-A levels and tumor growth are observed; however, determination of VEGF-A by itself generally is not an accurate diagnostic due to low sensitivity (33). The presence of VEGF-Ax in tumors and in serum almost certainly adds a confounding variable (9). Conceivably, low levels of VEGF-Ax in tumors (or serum) might predict robust angiogenesis and rapid tumor growth. In contrast, high levels of VEGF-Ax might predict ineffective anti-VEGF-A therapy due to inactivation of both pro- and antiangiogenic activity. Clearly, there is a need for detailed preclinical and clinical studies of the utility of VEGF-Ax (possibly as a ratio with VEGF-A) as a diagnostic tool in many cancer types.

The potent antiangiogenic activity of VEGF-Ax in cell culture and in mice suggests that it is a promising candidate for the treatment of solid tumors, retinopathies, and other pathologic conditions that depend on angiogenesis. Current anti-VEGF drugs function “passively” by inhibiting VEGF-A function by binding and inactivating the growth factor or its receptor; they do not exhibit “active” antiangiogenic function. Furthermore, proangiogenic VEGF-A is required for the maintenance of the normal adult vasculature. Complete or near-complete inhibition of this essential function underlies complications of anti-VEGF agents, including hypertension. In this context, the dual antiangiogenic properties of VEGF-Ax, namely active as well as passive inhibitory function, might provide more effective antiangiogenic activity than currently available anti-VEGF therapeutic agents.

The VEGF-Ax element as a cis-acting RNA signal, hnRNP A2/B1 as a trans-acting protein regulator, and specific anti-VEGF-Ax antibody provide unique opportunities for pharmacologic interventions that target the generation or activity of VEGF-Ax. Potentially, they can be applied to promote (e.g., in ischemic ulcers or myocardial infarction) or inhibit (e.g., in cancer or pulmonary hypertension) angiogenesis. Antisense oligonucleotides, such as morpholinos, can be used to target the Ax element to disturb its secondary structure or inhibit its binding to hnRNP A2/B1, both resulting in decreased VEGF-Ax expression. Small molecular compounds that bind and stabilize the secondary structure of Ax element, and increase binding to trans-acting factors, can be sought to increase VEGF-Ax expression.

Concluding Remarks

The cells, processes, and molecules that contribute to tumor angiogenesis are far more complex than originally believed. Multiple cytokines and their receptors, redundant pathways, and tumor adaptability have combined to make targeting angiogenesis an exceptionally challenging endeavor. The exciting discovery of VEGF-Ax and other antiangiogenic VEGF-A isoforms should encourage the field to reconsider brute-force strategies targeting VEGF, in the light of the opposing activities of these new-found molecules. Instead of ignoring their presence, the time is right to embrace the potential of antiangiogenic VEGF-A isoforms in guiding and enhancing therapeutic strategies targeting cancer.

Disclosure of Potential Conflicts of Interest

P.L. Fox and S.M. Eswarappa have ownership in a patent (application PCT/ US2014/013109).

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References

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