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World J Gastroenterol 2006 August 21; 12(31): 4937-4942
World Journal of Gastroenterology ISSN 1007-9327

EDITORIAL

Post-transcriptional regulation of vascular endothelial growth factor: Implications for tumor angiogenesis

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Received: 2005-12-01 Accepted: 2006-01-14

Abstract

Vascular endothelial growth factor (VEGF) is a potent secreted mitogen critical for physiologic and tumor angiogenesis. Regulation of VEGF occurs at several levels, including transcription, mRNA stabilization, translation, and differential cellular localization of various isoforms. Recent advances in our understanding of post-transcriptional regulation of VEGF include identification of the stabilizing mRNA binding protein, HuR, and the discovery of internal ribosomal entry sites in the 5'UTR of the VEGF mRNA. Monoclonal anti-VEGF antibody was recently approved for use in humans, but suffers from the need for high systemic doses. RNA interference (RNAi) technology is being used *in vitro* and in animal models with promising results. Here, we review the literature on post-transcriptional regulation of VEGF and describe recent progress in targeting these mechanisms for therapeutic benefit.

Key words: Vascular endothelial growth factor; Vascular endothelial growth hormone; Post-transcriptional regulation; mRNA stability; HuR; ELAV1; Internal ribosomal entry; IRES; siRNA; RNAi; Bevacizumab

Yoo PS, Mulkeen AL, Cha CH. Post-transcriptional regulation of vascular endothelial growth factor: Implications for tumor angiogenesis. *World J Gastroenterol* 2006; 12(31): 4937-4942

http://www.wjgnet.com/1007-9327/12/4937.asp

INTRODUCTION

The growth of any tissue *in vivo* relies on the adequacy of its vascular supply. Moreover, the notion of tumor angiogenesis, first advanced by Folkman in the early 1970s, posits that the growth of any tumor beyond 1-2 cubic centimeters depends on the growth of new blood supply[1]. In cases of insufficient vasculature, tissues become depleted of oxygen and nutrients, leading to the secretion of angiogenic factors[2]. These factors, which include hormones, interleukins, insulin, and growth factors, spur the ingrowth of new blood vessels. Vascular endothelial growth factor (VEGF) is one such key regulator of angiogenesis. It is a secreted mitogen and the most potent angiogenic factor, specific to endothelial cells and highly expressed in areas of active angiogenesis such as solid tumors.

The upregulation of VEGF expression in response to hypoxia plays a crucial role in tumor angiogenesis. Though it is not itself an oncogene, VEGF is upregulated in tumorigenesis and is important in blood vessel formation in solid tumors[3]. VEGF levels correlate with tumor progression and invasion, and a high VEGF level in colorectal carcinoma has been found to be an independent prognostic factor for long-term survival[4,5].

Hypoxic induction of VEGF appears to occur both by transcriptional activation and through stabilization of VEGF mRNA, which is otherwise labile in normoxic conditions. Transcriptional activation of VEGF relies largely on binding to the hypoxia inducible factor-1 (HIF-1), a heterodimeric basic helix-loop-helix protein that activates transcription of the human erythropoietin gene in hypoxic cells[6]. HIF-1 binds to a sequence in the 5'-flanking region of the VEGF gene called the hypoxic response element (HRE)[7-9]. Several other molecules have been implicated in the transcriptional control of VEGF. *Sp1* stimulates transcription by binding to G/C-rich boxes present on the VEGF promoter[10]. *AP-1* is a dimeric transcription factor of the leucine zipper family that is composed of jun/jun or jun/fos subunits. Hypoxia, oxidative stress, and cytokines may increase VEGF expression through the synthesis of jun and fos proteins and increased AP-1 binding activity. A number of other transcription factors also contribute to VEGF induction and regulation, including Stat-3[11].

Alternative splicing

In humans there are five alternatively spliced isoforms of VEGF, each is named for the number of amino acids along its length (VEGF 206, -189, -165, -145, 121). While varying amounts of each VEGF isoform mRNA can be generated to produce certain or all isoforms of VEGF[12],

each is predicted to have a characteristic extracellular localization based on biochemical differences. The larger isoforms bind neuropilin, matrix, and cell surface heparin proteoglycans, and are thought to act locally. The smaller isoforms do not display the heparin proteoglycan binding region and may diffuse to sites distant from the site of synthesis[13,14].

Post-transcriptional Regulation

A body of evidence is growing rapidly to demonstrate that post-transcriptional regulation of VEGF is critically important in the fine-tuned response required for both physiologic and malignant expression. The importance of post-transcriptional regulation of VEGF is based upon several key observations about the intrinsic nature of the VEGF mRNA. First, the mRNA is labile under conditions of normal oxygen tension[15]. Second, the 5' UTR of the VEGF mRNA possesses features that make efficient ribosome scanning and initiation of translation unwieldy.

Lability

Whereas the average half-life of eukaryotic mRNAs is 10-12 h, the half-life of VEGF mRNA is less than one hour[16]. In 1995, Ikeda and colleagues first reported that although VEGF levels were elevated in cells cultured in hypoxic conditions, transcriptional activation alone could not account for the increase in VEGF mRNA levels. Nuclear run-on assays demonstrated upregulation of VEGF transcription under hypoxia that was apparent after three hours, and persisted after fifteen hours of incubation. VEGF mRNA levels were 8-10 times higher than baseline, and this persistent elevation of VEGF could only be explained by increased stability of the mRNA. They concluded that hypoxia could lengthen the half-life of VEGF mRNA by 2-3 fold[7].

The effect of stabilization is mediated by the RNA-binding protein HuR. Steitz and colleagues first established the association between mRNA degradation and HuR, a member of the Elav family of proteins found in *Drosophila*[17]. Beginning with the observation that some mRNAs are targeted for rapid degradation by the presence of AU-rich elements (AREs) in the 3' UTRs, it was determined that HuR exhibits affinity for AREs, and levels of HuR correlate with *in vitro* mRNA degradation[18].

In VEGF, it was observed that the increase in mRNA stability coincided with the binding of a protein to an ARE, forming an RNA-protein complex in a hypoxia-inducible fashion[16]. Using a tumor cell line lacking the wild type von Hippel-Lindau tumor suppressor gene and in which VEGF mRNA is constitutively stabilized, this RNA-protein complex was found to be constitutively elevated[19]. The protein was later identified as the HuR protein[15]. Inhibition of HuR expression by antisense sequences was found to inhibit the hypoxic stabilization of VEGF mRNA, demonstrating its critical role in post-transcriptional stabilization of VEGF expression. However, total cellular steady-state HuR was not altered by hypoxia, raising the possibility that HuR is a component of a hypoxia-inducible complex whose other components are regulated by oxygen tension[15].

HuR probably has a function as a nuclear-cytoplasmic shuttle as well. It is predominantly localized to the nucleus, where it likely binds newly-transcribed VEGF mRNA and transports it to the cytoplasm and protects it from the ARE-mediated degradation pathways[20].

Recent studies have suggested HuR and VEGF mRNA localize to the cytoplasm in response to cellular stress[21]. Upon hypoxic stress, HuR is found to be localized to the cytosol and bound to target mRNAs, preventing their decay. New experiments using double labeling immunofluorescence show that VEGF and HuR colocalize to the nucleus during hypoxia[22,23]. The nuclear role of VEGF mRNA, if any is unclear. Further studies to more clearly delineate the intracellular localization of VEGF mRNA and HuR are warranted.

The mechanism by which HuR protects VEGF from degradation has been further investigated. One hypothesis is that RNA binding proteins confer stability by binding directly to destabilizing sequences and making them unavailable to endonucleases. Goldberg and colleagues suggest that RNA-stabilizing factors may localize to a distinct binding site and thereby change the secondary or tertiary structure of the RNA, making a specific site unavailable to endonucleolysis. In support of this hypothesis, a 40 bp region adjacent to the HuR-binding site in the VEGF stability region has been identified that is susceptible to ribonucleases in the absence of HuR[24].

HuR's importance in carcinogenesis is underlined by a report Lopez de Silanes and colleagues[25]. Using a nude mouse model and the RKO colorectal cancer cell line, they reported that tumors modified to overexpress HuR grew significantly larger than controls, and conversely, tumors modified to repress HuR expression grew smaller. Whether this effect was brought about through stabilization of VEGF mRNA is yet unknown.

IRES

Internal ribosomal entry sites (IRESs) were first discovered in picornaviruses in which they initiate translation of naturally uncapped viral mRNA. Eukaryotic mRNA, however, possesses a 7-methylguanosine cap in the 5'UTR that is critical in the canonical model of translation. In this model, the eukaryotic initiation factors (eIFs) recognize and bind the 5' cap, and unwind the secondary structure of the 5'UTR, thereby making it sterically feasible for ribosomes to scan through the 5' UTR and translate the mRNA beginning at the AUG start codon[26].

However, perhaps 3%-5% of all cellular mRNAs are translated by a mechanism independent of the 5' cap structure[27]. Most of these mRNAs are likely to contain an internal ribosome-entry site (IRES) in the 5' untranslated region (UTR), as the ability to maintain efficient translation without utilizing the 5' cap-dependent mechanism relies on the presence of one or several IRESs. IRESs are typically found in mRNAs that possess unusually long 5'UTRs, greater than -300 nt, which consequently have significant secondary structure[28]. In eukaryotes, IRES-mediated translation bypasses the canonical initiation step by directly recruiting ribosomes to the start codon without the need for the 5' mRNA cap structure or the eukaryotic initiation

factor (eIF) complex[28,29].

The eIF-4F complex is one of the key mediators of ribosome recruitment in cap-dependent translation and the eIF-4E subunit of this complex can be rate-limiting[2]. Under normal cellular conditions, eIF-4E is bound to its binding protein 4E-BP1 and translation is limited by the availability of the unbound fraction of eIF-4E[30]. When 4E-BP1 is phosphorylated, eIF-4E is released and becomes available to bind mRNA and participate in the eIF-4F complex. This phosphorylation is instigated by the downstream effects of various mitogenic pathways. Hypoxia, however, has the opposite effect, increasing the binding of eIF-4E with 4E-BP1, which may mediate the global decrease in protein synthesis seen in stressed cells[31].

Several observations about the 5' UTR of VEGF mRNA led to the hypothesis that it may contain IRES elements. First, cap-dependent translation of VEGF is cumbersome because the 5' UTR (1038 bp in humans) is much longer than typical eukaryotic 5' UTRs (-300 nt) and does not allow efficient ribosomal scanning. Second, it has a high G+C content, predisposing it to form stable secondary structures. Third, the 5'UTR contains a short open reading frame with in-frame initiation and termination codons[32]. Furthermore, in order to maximize its effect, translation of VEGF must be upregulated during periods of stress such as cellular hypoxia, when cap-dependent protein synthesis is globally inhibited.

In 1998, VEGF mRNA was shown to possess two IRES sites (IRES A and B) in the 5' UTR[32-34]. IRES A is contained in a 293nt segment just upstream from the AUG codon and is believed to control translation initiated at the AUG codon[35]. IRES B is contained in the early portion of the 5' UTR[33]. These two sites appear to bind different proteins, and the cellular milieus in which each functions optimally are not well understood.

IRES-mediated translation *in vivo* and *in vitro* yields a distinct isoform of VEGF, called "Large VEGF" or "L-VEGF." Whereas initiation mediated by the 5' cap and IRES A is at the AUG start codon it shares with cap-dependent translation, the initiation of the L-VEGF isoform occurs at an upstream CUG start codon (nt 499) within the 5'UTR. The L-VEGF isoform contains an N-terminal extension of 180 a.a., which is cleaved to yield a C-terminal peptide that is nearly identical to VEGF 189. This N-terminal extension is highly conserved through human, bovine, and murine cells, but whether it has any function is unknown[36].

Inquiry into the control of IRES A has uncovered a complex model of translational regulation. The five classic isoforms (excluding L-VEGF) are the translation products of five distinct mature mRNAs; each of these mature mRNAs is the product of alternative splicing of the transcription product or pre-mRNA. Bornes and colleagues have reported that differences in exon content of the differentially spliced mRNA can lead to preferential activity of one IRES or the other[35].

Though it is becoming clear that the IRES mechanism in VEGF translation is a plausible pathway in normal physiology, it remains debatable whether this pathway plays a significant role in tumor angiogenesis. The controversy relies on the observation that there is a correlation between eIF-4E and protein synthesis in malignancy. This correlation has been demonstrated in breast carcinomas, head and neck squamous cell carcinomas, soft tissue sarcomas, and colon carcinoma[37-40], and is bolstered by the observation that cells that over-express eIF-4E increase secretion of VEGF by greater than 100-fold[41]. Therefore, it is conceivable that VEGF production in transformed cells relies on the cap-dependent pathway as well, though this has not been definitively demonstrated.

Whether hypoxia is a sufficient stimulus to induce IRES activity, or if other mitogenic stimuli can trigger internal ribosomal entry remains unknown. In a study comparing the IRES elements of VEGF, hypoglycemia was seen to significantly activate IRES activity[42]. In both transformed and benign cells, the balance between IRES and cap-dependent translation in VEGF requires further investigation.

Deranged expression of the *c-myc* oncogene is associated with many human malignancies, as its over-expression promotes cell growth and angiogenesis[43]. This effect is in part due to the fact that *myc* over-expression increases secretion of VEGF[44]. The mechanism behind this increase was recently described by Mezquita and colleagues[45]. They demonstrated that *myc* interacts with VEGF mRNA to upregulate initiation of translation capable of bringing about a 10-fold increase in VEGF production. They observed no increase in eIF activity and attribute the translational upregulation to VEGF-specific increase of translation initiation, either through *c-myc* or VEGF directly.

Implications for Tumor Angiogenesis

In 1993, Kim and colleagues reported that anti-VEGF antibodies had a strong inhibitory effect on the growth of several tumor types in nude mice. Since then, anti-VEGF therapies to target angiogenesis have gained further attention. Investigators have reported inhibited tumor growth with other anti-VEGF applications including anti-sense nucleotides, anti-receptor antibodies, soluble VEGF receptors, and a retrovirus-delivered Flk-1 mutant.[46-49] A recent phase three FDA trial demonstrated improved survival when patients with metastatic colorectal cancer were treated with a humanized monoclonal anti-VEGF antibody (bevacizumab) in combination with standard chemotherapy (irinotecan, 5-FU, and leucovorin), resulting in FDA approval. The FDA has also approved its use in non-small cell lung cancer. Bevacizumab is being tested in several other tumor models with and without adjunctive traditional chemotherapy agents.

Anti-angiogenic therapies have targeted the normal endothelial cell's ability to respond to angiogenic factors, either by primarily inhibiting endothelial cell proliferation or by using antibodies to prevent angiogenic factors from activating endothelial cells[50]. However, some investigators have observed resistance to this therapy due to the ability of tumor cells to upregulate the expression of VEGF and thereby negate the anti-angiogenic effect[51]. There is further evidence suggesting that high tumor levels of VEGF may promote tumor survival[52].

One potential anti-angiogenic strategy is to silence gene expression of angiogenic factors at the mRNA level.

While antisense oligonucleotides have been used for this strategy, the high concentration required and non-specific effects have limited this approach. First described in *C. elegans*, RNA interference (RNAi) is a process in which double stranded RNA (dsRNA) is processed by the enzyme Dicer, resulting in short interfering RNAs (siRNA) 21-25 nucleotides in length[53,54]. These siRNA molecules are incorporated into an RNA-induced silencing complex (RISC) that binds complementary target mRNAs, leading to their degradation[55,56].

There is great potential for using RNAi technology in therapeutic applications that target critical signaling pathways involved in cancer, due to its high specificity for targeted genes and its potency at low concentrations. However, relatively few studies have examined the use of RNAi technology to suppress VEGF production.

In 2003, three reports proved the concept. Filleur and colleagues used a rat fibrosarcoma model and systemically delivered siRNA to achieve a 66% decrease in tumor size when compared to controls[57]. Reich and colleagues used direct injection of anti-VEGF siRNA to decrease choroidal neovascularization in laser-induced retinal injury in the rat[58]. A plasmid-based siRNA delivery system driven by a Pol III promoter was used by Zhang and colleagues to show isoform-specific knockdown of VEGF[59].

These three experiments provided the conceptual basis for two further experiments to demonstrate the efficacy of RNAi inhibition of VEGF. In 2005, Yoon and colleagues injected HT1080 fibrosarcoma cells stably transfected with anti-vegf siRNA into the subcutaneous tissue of rats. They reported substantial decrease in tumor volume and vessel density in resected specimens[60]. In the same year, Kwon and colleagues described a bi-level approach to silencing VEGF. In their experiment, they combined zinc finger-mediated repression of transcription with RNAi technology to achieve knockdown of VEGF of over 90%[61].

The critical problem with RNAi-based therapy is one of delivery. Whereas *in vitro* transfection of cells is easily accomplished using lipid-based solution, *in vivo* targeting of the siRNA molecules to cancer cells is far more challenging. Systemic delivery of naked siRNA is problematic as the drugs localize poorly and the degree of dilution is often unacceptable[62]. Though synthetic vectors offer several advantages such as their relative safety and the ease of incorporating target-specific ligands, there are significant barriers to their effective use that have not yet been overcome[63]. Viral vectors may offer specific advantages as well[64].

CONCLUSION

Although much has been learned about VEGF since its discovery in 1989, much more remains to be understood. First, the relevance of hypoxic stabilization of VEGF mRNA must be established in the settings of both neoplasia and normal physiologic conditions. Does our current understanding of hypoxic stabilization provide a sufficient explanation with regard to tumor angiogenesis? Are there other mitogenic factors that can overwhelm the cell's innate response to hypoxia? Second, the balance between cap-dependent and IRES-mediated translation must be elucidated. What are the factors that mitigate the switch from cap-dependent to IRES-mediated translation? Finally, it is clear that post-transcriptional regulation of VEGF has profound clinical implications with regard to targeting tumor angiogenesis. Will RNAi technology prove to be an efficacious therapeutic modality? If so, how will we deliver the drug to achieve a safe, durable, and clinically significant outcome? It will be a challenge for coming years to determine how best to exploit these molecular mechanisms for our patients' benefit.

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