SCIENCE REPORT

VEGF, Dll4, and the Clinical Inhibition of Angiogensis

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Angiogenesis is the process by which new blood vessels are formed in normal development, wound healing, and pathological tumor growth. Because of its role in the growth and metastasis of tumors, a great number of research efforts have sought ways to treat cancer by inhibiting angiogenesis. This was accomplished with great success through the inhibition of vascular endothelial growth factor (VEGF), a protein that mediates the endothelial cell proliferation and sprouting that culminates in the formation of new blood vessels in both embryonic and pathological angiogenesis. The inhibition of VEGF has been displayed to be widely effective in limiting tumor growth through hindering angiogenesis, and due to this it has been approved for use as chemotherapy. While VEGF inhibition has been largely successful in this role, there have been definite problems in its clinical application, the most troubling of these being the ability of cancers to acquire drug resistance. Resistance to VEGF inhibition has prompted increased research into angiogenesis, and currently many of these efforts concern Delta-like ligand 4 (Dll4). Dll4 is a protein that inhibits endothelial cell proliferation while promoting effective angiogenesis, its inhibition resulting in larger and more complex but dysfunctional vascular networks. The inactivation of Dll4 in murine cancers has been observed to result in increased, yet 'nonproductive' angiogenesis in that the resulting vasculature does not support significant tumor growth, and because of this Dll4 inhibition is considered to be a serious candidate for future use as cancer treatment in humans.

INTRODUCTION

In its treatment of the various forms of cancer, chemotherapy is typically thought of as the targeted killing of malignant cells in order to combat tumor growth and prevent metastasis. While it is true that the majority of cancer therapies are directed at tumor cells, in recent years there have been significant efforts in both basic and clinical research aimed at treating tumors through attacking cells of the surrounding stroma, that is, normal cells that are hijacked by their cancerous neighbors so that the latter may survive and grow. Among the stromal cells that can function to facilitate tumor growth, a great deal of work has gone into the disruption of tumor-associated cells involved in the blood circulatory system (endothelial cells) and the process by which these cells proliferate and form new vessels, angiogenesis.

Angiogenesis is the branching of arterial and venous blood vessel components, resulting in the formation of a functional vascular network.[1] It is integral to embryonic development, during which it facilitates endothelial cell sprouting after the formation of primitive vascular structures by vasculogenesis. The necessity of angiogenesis to these early developmental processes is displayed by the fact that genetic abnormalities compromising it *in utero* have often been observed to result in embryonic lethality.[2-7] Angiogenesis is also important in later development, as well as for the repair of damaged blood vessels that occurs in wound healing.[8] It is thus clear that angiogenesis has an important physiological role in normal mammalian growth and survival; however, like many other bodily processes it can go badly awry. Pathological angiogenesis has been observed in a variety of diseases, but it is most commonly associated with cancer, given that it is often largely responsible for the rapid growth and invasiveness that is characteristic of aggressive tumors.

Cancer cells, like their normal counterparts, require access to actively circulating blood so that they may obtain the oxygen, nutrients, and removal of metabolic waste products that they need in order to survive, grow, and ultimately spread throughout the body in metastasis. Due to the fact that the diffusion limit of oxygen is only about 100 to 200 μm, cancer cells located at the periphery of rapidly growing tumors may quickly spread beyond the point of effective oxygenation by the nearest blood vessels.[9] Because of this, aggressive tumors are dependent on constant angiogenic vessel sprouting, given that in the absence of sufficient blood availability peripheral cells may become hypoxic and subsequently undergo necrotic cell death.[10] Disruptions in angiogenesis can thus hinder tumor growth and metastasis, and due to this many efforts aimed at inhibiting key angiogenic promoters have been undertaken. Recently, some of the most successful research efforts in this field have concerned the inhibition of the protein vascular endothelial growth factor (VEGF).

VEGF: A KEY PROMOTER OF ANGIOGENESIS

VEGF is a protein mitogen that promotes the endothelial cell proliferation and sprouting that is characteristic of angiogenesis.[11] Since its identification in 1983 and characterization as a mediator of angiogenesis in 1989, the gene encoding VEGF has been localized to the short arm of chromosome 6.[12-14] During the expression of the VEGF gene from this locus, its mRNA transcripts can be processed via alternative splicing to give rise to several isoforms of the protein, the most important of which are VEGF-A, which mediates angiogenesis through the tyrosine kinase receptors VEGFR 1 and 2 (vascular endothelial growth factor receptor), and VEGF-C and -D, which are involved in the production of lymph vessels in lymphogenesis.[10] Due to its central role in both normal and pathological angiogenesis, this report will focus on VEGF-A, which from here forward will be referred to simply as VEGF.

In normal development, VEGF is essential for the formation of the blood vascular system both *in utero* and during

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later growth. One of the best studied examples of fetal VEGF activity involves the development of retinal vasculature via angiogenesis. During the development of the retina, star-shaped glial cells of the nervous system called astrocytes gradually migrate away from neighboring blood vessels as the young sensory organ increases in size. As the retinal astrocytes move farther away from their blood supplies, they begin to suffer from hypoxia, a condition which causes them to produce the protein hypoxia-inducible factor 1 (HIF-1).[15] Once released, HIF-1 mediates a hypoxic response, and central to this is the expression of the VEGF gene, which contains a HIF-1 binding site in its promoter region.[16] VEGF proteins are then released by the hypoxic astrocytes towards existing blood vessels, where they initiate angiogenic vessel growth toward regions of low oxygen tension.[17] Finally, after the formation of new vessels, VEGF production by the now properly oxygenated astrocytes is downregulated; however, a certain concentration of the protein remains in the vicinity of the nascent vasculature in order to prevent endothelial cell apoptosis.[17] Similar mechanisms of vessel growth have been observed in the development of other organs, and the importance of VEGF to these processes is evidenced by the fact that loss of only one of the two VEGF alleles has been observed to cause embryonic lethality in mice.[2, 3] This indicates that sufficient amounts of the protein are needed for survival beyond the very early stages of development (i.e. the amount of VEGF produced from one allele is not enough for viability).

In stark contrast to its vital role in embryonic survival, VEGF can also be exploited by aggressive forms of cancer, which often upregulate its expression in order to facilitate sustained tumor expansion. In a manner that is remarkably similar to the vascularization of the retina, cancer cells on the periphery of growing tumors respond to hypoxia due to lack of a nearby blood supply by expressing HIF-1 and consequently VEGF. As in retinal development, VEGF is released from hypoxic cells in order to stimulate the growth of existing tumor vasculature towards oxygen-deprived regions.[10] In this way, VEGF-mediated angiogenesis provides tumors with the vasculature that they need in order to expand beyond their initial blood supplies. It is important to note that this not the only way that aggressive cancers can employ VEGF, given that, in addition to its role in stimulating angiogenesis and promoting endothelial cell survival, it has been observed to act as a powerful vasopermeability agent, that is, one that increases the permeability of blood vessels.[12] By upregulating VEGF expression tumors can increase the permeability of surrounding vessels and this serves to facilitate metastasis via the blood circulatory system. VEGF is therefore essential for both sustained tumor growth and expedited cancer metastasis, realities which made its inhibition an attractive avenue in the search for new chemotherapeutic methodologies.

Since its characterization as a mediator of tumor angiogenesis in 1989, a great deal of basic and clinical research has gone into identifying and testing potential inhibitors of physiological VEGF function. To date, VEGF activity has been suppressed in vitro and in murine models by methods including but not limited to the use of anti-VEGF monoclonal antibodies, RNA interference, and the expression of soluble VEGF receptors.[18-20] While not all of these strategies are at this point clinically viable, there have been major successes with anti-VEGF antibodies. Like any other antibody, these agents inhibit the activity of VEGF proteins by binding to and initiating an immune response against them. Currently there are two anti-VEGF monoclonal antibodies that have been approved for use as chemotherapy in humans, bevacizumab (Avastin) and ranibizumab (Lucentis), both of which have been shown to successfully inhibit tumor angiogenesis.[21, 22] In the abscense of VEGF activity and the angiogenesis that it mediates, tumors treated with either bevacizumab or ranibizumab have been observed not only to grow more slowly but in many cases to undergo significant regression.[23] Since its FDA approval, antibody-mediated VEGF inhibition has successfully treated a variety of humans cancers; however, its clinical application has not been without problems.

VEGF inhibition has been proven effective for and used extensively in the treatment of lung, breast, gastrointestinal tract, renal, and ovarian carcinomas.[21] As is expected with such a wide clinical application, a variety of side effects have been observed in patients treated in this manner, including but not limited to nausea, headache, fatigue, hypertension, proteinuria, menstrual cycle abnormalities, and bleeding at tumor sites.[21, 24] While some of these adverse effects can be cause for concern, at present the major problem facing the use of VEGF inhibition as chemotherapy deals with the ability of cancers to acquire drug resistance, a phenomenon that has been reported in both clinical and research settings.[25, 26] Tumor resistance to therapeutic VEGF inhibition has made alternative methods of angiogenic regulation an attractive avenue in the search for new cancer treatments, and a promising molecule by which this may eventually be accomplished is Delta-like ligand 4 (Dll4).

DLL4 AND THE CRITICAL INHIBITION OF ANGIOGENESIS

Delta-like ligand 4 or Dll4 is a membrane-bound ligand involved in the developmentally significant Notch signaling pathway.[27] Since its discovery by Shutter and others in 2000, the 4.3 kilodalton protein has been classified as a downstream target of VEGF that promotes ordered and productive angiogenesis by preventing an excessive response on the part of endothelial cells to angiogenic stimuli.[28-31] Dll4 is thus technically an inhibitor of angiogenic vessel growth; however, the inhibition that it mediates is often crucial to proper angiogenesis leading to the formation of a functional vascular network.[4-7] In the absence of Dll4-mediated angiogenesis inhibition, effective blood vessel growth is impossible in a number of physiological contexts, one of these being early vascular development.[4, 5, 7] It has been established that Dll4 protein activity is central to this early angiogenesis through the study of murine embryos lacking sufficient expression of the dll4 gene.

The essential role of Dll4 to early vascular development has been displayed through a series of experiments in which the development of mouse embryos lacking one or both of

the dll4 alleles (haploid or homozygous null for the gene, respectively) was observed.[4, 5, 7] Murine embryos lacking a functional copy of the dll4 gene have been found at normal Mendelian frequencies until approximately E9[5] (that is, 9.5 days after fertilization); however, by E10[5] all such homozygous null individuals suffer embryonic lethality.[5] It is thus clear that some level of Dll4 activity is needed for embryonic viability, and a number of studies have sought to access this level through the use of mice heterozygous for the dll4 gene.[4, 5, 7] Embryos haploid for dll4 develop normally until about E9.5, after which abnormalities including reduced recruitment of vessel-sheathing pericytes, reduction of blood vessel size, absence of yolk sac arterial organization, and vessel patterning defects due to hyperbranching have been observed.[4, 5, 7] These abnormalities typically result in embryonic lethality, and because of this dll4 has been properly classified as a haploinsufficient gene.[5] With few exceptions, murine dll4$^{+/-}$ embryos are not viable, and this indicates that the Dll4 protein is essential for proper angiogenic vascular development *in utero*.[7, 28]

In addition to early vascular development, Dll4 is also essential for effective angiogenesis in growing tumors. The role of Dll4 in pathological angiogenesis has been studied extensively in recent years through experiments in which its activity has been manipulated in tumor-bearing NOD-SCID (nude) mice.[29,30,32] Overexpression of the dll4 gene in nude mice has been observed to result in improved tumor vascular structure and more rapid tumor expansion, as compared to control mice expressing normal levels of the protein, indicating that the inhibitory effect of Dll4 paradoxically functions to facilitate tumor angiogenesis and growth.[32] Conversely, experiments in which Dll4 has been inhibited in murine models have shown that its regulatory effect is crucial for effective tumor angiogenesis.

Through the expression of a genetic construct encoding an inactive, soluble version of Dll4, the development of tumor vasculature in the absence of its inhibition of angiogenesis has been observed *in vivo* in mice.[30] Expression of this inactive form of Dll4 in tumor-bearing mice resulted in tumor vasculature that was significantly more branched and interconnected than in control tumors as a result of excessive endothelial cell proliferation and sprouting.[30] The inhibition of Dll4 activity therefore increased angiogenesis and gave rise to larger, more complex vascular networks; however, it is clear that these networks were not fully functional, given that they did not support significant tumor growth, with histological assessment displaying that peripheral tumor cells exhibited extensive hypoxia.[30] These results, combined with those obtained through inhibition via Dll4-targeted antibodies, indicate that tumor vasculature formed under Dll4 inhibition is nonfunctional and the angiogenesis that engenders it "nonproductive."[29-30] Dll4 is thus a negative regulator of angiogenesis that, when inhibited, results in decreased tumor growth despite increased vessel proliferation. Because of this, Dll4 inhibition may represent an avenue toward new cancer treatments.

It has been established by the studies cited above that Dll4 inhibition results in angiogenesis that is nonproductive and characterized by excessive endothelial cell sprouting leading to the formation of nonfunctional and poorly perfused blood vessels.[28-30] When its expression is induced by VEGF, Dll4 acts as a critical negative regulator of endothelial cell proliferation in actively growing vasculature, and this fact makes its inhibition especially attractive as a future chemotherapy, given that normal, fully developed tissues would not be adversely affected.[28, 29] Additionally, Dll4 inhibition has been proven effective in treating human cancers resistant to anti-VEGF therapy *in vivo* in mice, suggesting that it could eventually serve to hinder angiogenesis in tumors in which a VEGF-targeted approach is not possible.[29] There certainly appears to be many advantages to targeting Dll4 in the treatment of cancers; however, it is apparent more research has to be done before this is possible.

The inhibition of Dll4 has been shown to hinder tumor growth, but it has not yet been established that the malformed vasculature resulting from such inhibition would allow for the perfusion of chemotherapeutic drugs required to fully eliminate the tumor in question. If it is found that Dll4 inhibition does in fact lower the efficacy of other chemotherapies, it may become much less attractive as a mode of cancer treatment, and thus studies seeking to answer this question would be of great interest. In addition to this, more research must be conducted to access the extent to which, if any, Dll4 is involved in the angiogenesis that occurs during wound healing. Such studies would be helpful in predicting what adverse side effects would be expected, should Dll4 inhibition undergo clinical trials as a means of chemotherapy. These and other research efforts concerning this protein are warranted, given that Dll4 may eventually succeed VEGF as the principal target in anti-angiogenesis chemotherapy.

CONCLUSION

The formation of new blood vessels is required for the sustained growth of aggressive tumors, and because of this the ways to inhibit angiogenesis have been researched extensively in the search for new cancer treatments. In recent years, great successes have been had with the antibody-mediated inhibition of VEGF, a key promoter of the endothelial cell proliferation and sprouting that is characteristic of angiogenesis. While VEGF inhibition has been shown to be efficacious in treating a of variety of cancers, problems with its clinical application including but not limited to treated tumors acquiring drug resistance have prompted renewed efforts in angiogenesis inhibition research. At present, the inactivation of Dll4, a molecule that inhibits vessel proliferation and sprouting while at the same time promoting effective angiogenesis, appears promising. Tumors in which Dll4 has been knocked down have displayed increased angiogenesis; however, this angiogenesis has been called "nonproductive," given that it does not give rise to a vascular network that can support significant tumor growth. Dll4 inhibition thus has the potential for successful therapeutic application, but before this is possible more research is needed into the potential advantages and pitfalls of such treatment.

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