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A Hypothesis Concerning the Biphasic Dose-response of Tumors to Angiostatin and Endostatin

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Abstract

This manuscript proposes a hypothesis to explain the U-shaped dose-response observed for angiostatin and other high-molecular-weight drugs in various anti-cancer bio-assays. The dose-response curves for angiostatin and endostatin (measured as suppression of tumor growth) go through an optimum (i.e., minimum tumor growth) and then becomes less effective at higher doses. The literature suggests that at lower doses the primary action of these high-molecular-weight drugs is to counteract the angiogenic effects of vascular endothelial growth factor (VEGF). To do this, the drugs must pass out of the blood vessel and enter the extra-cellular matrix (ECM) where VEGF induces the growth and fusion of tip cells. Ironically, VEGF actually facilitates access of the drugs to the ECM by making the vascular endothelium leaky. At higher doses, the high-molecular-weight drugs seem to reverse VEGF-induced permeability of the endothelium. Thus, at high dose rates, it is hypothesized that the drugs are not able to enter the ECM and block the angiogenic effects of VEGF there. As a result, high doses of the drugs do not suppress vascularization of the tumor or tumor growth. Moreover, if the permeability of the vessels is suppressed, the VEGF released by the stroma is concentrated in the ECM where it amplifies the angiogenic activity around the tumor.

Keywords

angiostatin, endostatin, angiogenesis, macrophage, tip cell, biphasic, dose, response, cancer, tumor, VEGF, ECM

Angiogenesis and Cancer

In the early 1980s, it was recognized that a factor secreted by tumor cells caused leakage from blood vessels and the term ''vascular permeability factor'' (VPF) appeared in the literature (Senger et al. 1986; Clauss et al. 1990). It was also soon realized that VPF was associated with growth of blood vessels (i.e., angiogenesis) around tumors (Dvorak et al. 1991; Senger et al. 1993) and the term was changed first to ''vascular permeability factor/vascular endothelial growth factor'' (Senger et al. 1993) and finally to merely ''vascular endothelial growth factor'' (VEGF) by 1993 (Adamis et al. 1993).

Although some tumors attain nutrients by growing along existing blood vessels or otherwise coopting existing blood vessels (Dome et al. 2007; Donnem et al. 2013), by the late 1990s, it was widely accepted that conversion of hyperplasia into neoplasia is accompanied by (and facilitated by) formation of new blood vessels, i.e. an ''angiogenic switch'' (Hanahan and Folkman 1996; Bergers et al. 1999; Folkman 2002). Rapidly growing tumors tend to display an ''aerobic glycolysis'' phenotype (i.e., the Warburg effect) in which both aerobic and anerobic respiration provide energy (Sciacovelli et al. 2014) and proliferation is only limited by the availability of the blood supply. Sustained angiogenesis became recognized as one of the hallmarks of cancer (Hanahan and Weinberg 2000). Thus, great interest has developed in finding anti-angiogenic drugs (Folkman 1985; Folkman and Ingber 1992; Bergers et al. 1999).

The Tumor and its Stroma

Once a tumor has begun growing, inflammation and hypoxia cause the release of chemokines that attract immune cells that begin to form the stroma. VEGF/VPF isoforms are also cytokines that attract monocytes (Czepluch et al. 2011), macrophages (Li et al. 2011), pericytes (Grosskreutz et al. 1999; Yamagishi et al. 1999; Ribatti et al. 2011) and

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fibroblasts (Senger et al. 1993; Wynn 2008; Asumda and Chase 2011) to surround the tumor. The attraction of the pericytes to the tumor apparently pulls them from the endothelial walls of the local blood vessels and makes the blood vessels hyper-permeable (i.e., leaky) (Adams and Alitalo 2007; Zhao et al. 2007; Azzi et al. 2013).

It has been found that the concentration of VEGF/VPF in the microenvironment (i.e., immediately around specific clusters of cells) rather than the bulk concentration of VEGF/VPF is what is important in determining the course of angiogenesis (Dvorak et al. 2011; Fidler 2011). In an interesting series of experiments, Blau and coworkers (Springer et al. 2003; Ozawa et al. 2004; von Degenfeld et al. 2006) created four homogeneous clones of myoblast that secreted VEGF at different levels. They found that when they used mixtures of cell clones to produce average dose rates between 5 and 70 ng VEGF/10^6 cells/day, angiogenesis was observed with formation of normal blood vessels. At gross dose rates greater than about 100 ng VEGF/10^6 cells/day, abnormal development of blood vessels occurred leading to hemangiomas. More importantly, when homogeneous clones of myoblast that produced less than 70 ng VEGF/10^6 cells/day were used, no abnormal foci or abnormal angiogenesis were observed regardless of the number of myoblast injected; and the blood vessels that were formed were not leaky, had normal pericytes and were VEGF-independent. In contrast, when individual cells producing higher dose rates of VEGF were used, the blood vessels that formed were leaky, VEGF-dependent, and malformed. Clearly, new blood vessels tend to form only very near the source of VEGF/VPF (i.e., a very steep concentration gradient) (Springer et al. 2003). This observation may be related to the way that VEGF/VPF interacts with receptors (Kiba et al. 2003a; Kiba et al. 2003b). VEGF levels are normally very tightly controlled because too little or too much VEGF can be lethal to developing embryos (Carmeliet et al. 1996; Miquerol et al. 2000).

In any event, the observations are consistent with the fact that VEGF/VPF acts locally; not systemically. The nutrient demand of rapidly growing tumors requires a blood supply that is initially provided by secretion of VEGF/VPF that dilates local blood vessels and makes them leaky. Leaky blood vessels appear to be caused by the migration of pericytes (Hasumi et al. 2007; Zhao et al. 2007; Cao and Cao 2010; Cao et al. 2010; Ribatti et al. 2011) that fill gaps between endothelial cells into the ECM.

The Modes of Action of VEGF/VPF

The mechanism of angiogenesis induced by VEGF/VPF is still poorly understood, but recent publications have provided some detail (Ferrara et al. 2003; Dvorak et al. 2011; Jeong et al. 2011; Ji 2011; Li et al. 2011; Ribatti et al. 2011; Tammela et al. 2011; Weis 2011; Indraccolo 2013). There seem to be two distinct activities of VEGF/VPF: (i) formation of leaks in existing vessels and (ii) formation of new vessels. In normal vessels the lumen accounts for about 30% of the vessel diameter, but under the influence of high concentrations of VEGF/VPF pericytes that maintain the integrity of normal vessels appear to be drawn away towards the tumor cells (Dvorak et al. 2011; Ribatti et al. 2011). Thus, the weakened vessels balloon outward and leak blood with macromolecules through the thin lining of endothelial cells (Dvorak et al. 1991). This effect of VEGF/VPF acts quickly (within 5 hr) but has a limited range of less than 0.5 mm from the tumor (Dvorak et al. 1991). The angiogenic effects of VEGF/VPF appear to act over greater distances (or at lower concentrations). For example, in the rabbit cornea model (Gimbrone et al. 1973) used to investigate angiogenesis, new vessels are induced to sprout many millimeters from the source of VEGF/PVF (Ryu and Albert 1979). The sprouting of new blood vessels may not begin for 1 to 10 days (Ryu and Albert 1979).

Anti-Angiogenic Agents

The clinical importance of biphasic angiostatic agents has recently been reviewed (Reynolds 2010).

Formation and Composition of Angiostatin and Endostatin

Concurrent with the establishment of angiogenesis and rapid growth of solid tumors, a variety of enzymes are used by the tumor to break down the ECM. The enzymes (e.g., matrix metalloproteinases (MMPs) and urokinase-type plasminogen activators (uPA)) cut polymers (e.g., glycosaminoglycans, proteoglycans and collagen) and proteins (e.g., plasminogen) into smaller soluble pieces (plasmin and angiostatin). Angiostatin (a 38 kDa protease fragment of plasminogen) (O'Reilly et al. 1994; Folkman 1995; Dong et al. 1997; Gately et al. 1997) was identified as a natural anti-angiogenesis agent in 1994 and was soon proven to have potent anti-tumor effects. Similarly, endostatin (a C-terminal, 20 kDa, zinc-binding protein cut from collagen XVIII) was discovered in 1997 (O'Reilly et al. 1997; Beecken et al. 2001).

Targets of Angiostatin

Angiostatin binds to cell surface glycoproteins, angiomotin (Troyanovsky et al. 2001), integrins (Tarui et al. 2001; Chavakis et al. 2005; Wahl et al. 2005), ATP synthase (Moser et al. 1999; Wahl et al. 2005; Chi and Pizzo 2006; Yamamoto et al. 2007). It also binds to mitochondrial ATP synthase (Lee et al. 2009).

Effects of Angiostatin

Angiostatin is known to promote apoptosis (O'Reilly et al. 1994; O'Reilly 1997; Lee et al. 2009). It inhibits recruitment of macrophages (Distler et al. 2002; Chavakis et al. 2005; Dineen et al. 2008; Lee et al. 2009; Chen et al. 2011; Li et al. 2011; Lin et al. 2011), which are normally attracted into

the stroma by signaling involving VEGFR-1 and VEGFR-2 (Li et al. 2011) and which are involved with completing vascular circuits with VEGFR-3 (Tammela et al. 2011). Angiostatin has recently been shown to inhibit migration and activation of neutrophils (Aulakh et al. 2014).

Hypothesis for Action

One hypothesis for its mechanism of action is that angiostatin blocks the action of hepatocyte growth factor (HGF) (Wajih and Sane 2003), which activates the c-met receptor. The c-met receptor of endothelial and smooth muscle cells initiates migration and facilitates proliferation essential to establishment of new blood vessels (Chang et al. 2013). There is very active research on this pathway in a number of cancer types. The mechanism appears to be dependent on the kringle 5 (K5) domain of angiostatin which is shared with HGF (Ansell et al. 2010). The mechanism of endostatin is less studied but appears to facilitate apoptosis and suppress autophagy by modifying the effect Bcl-2 via its complex with Beclin 1 (Ramakrishnan et al. 2007; Wu et al. 2011; Ibrahim et al. 2014).

Disappointing Clinical Results and the U-shaped Dose-Response Curve

Following the 1996 report from the Folkman group on the preclinical effects of angiostatin (O'Reilly et al. 1996) there was much optimism that at last a "unifying concept" had been discovered for cancer treatment (Saphir 1997). Numerous candidate compounds went into clinical trials (Folkman 2003) and the ownership of intellectual property was aggressively contested (Brower 2000). But, as time passed, the dream of a universal chemotherapy for cancers began to wane as clinical trials produced less than overwhelming results (Gupta and Zhang 2005). Reynolds has provided a comprehensive review of the findings and show that a u-shaped dose-response curve may be the problem in many cases (Reynolds 2010; Javaherian et al. 2011). Here, I want to dissect the U-shaped curve into its component parts.

A Hypothesis for U-Shaped Dose Response for Angiostatin

Mathematically, a U-shaped curve can be derived from two S-shaped dose-response curves as indicated in Figure 1. Many biochemical mechanisms could be invoked to account for the two S-shaped curves. The key factor here seems to be that VEGF/VPF released by the tumor is known to have two distinct modes of action: It is angiogenic and it causes permeability of the nearby blood vessels. Based on our understanding of the roll of VEGF/VPF in angiogenesis (discussed above), a conceptual model can be proposed (Figure 1). The first drug target (with a rather low effective dose 50%, ED_{50}) blocks angiogenesis and inhibits tumor growth as expected. At higher doses, a second target (with higher ED_{50}) blocks the access of the drug to the first target. This model has the

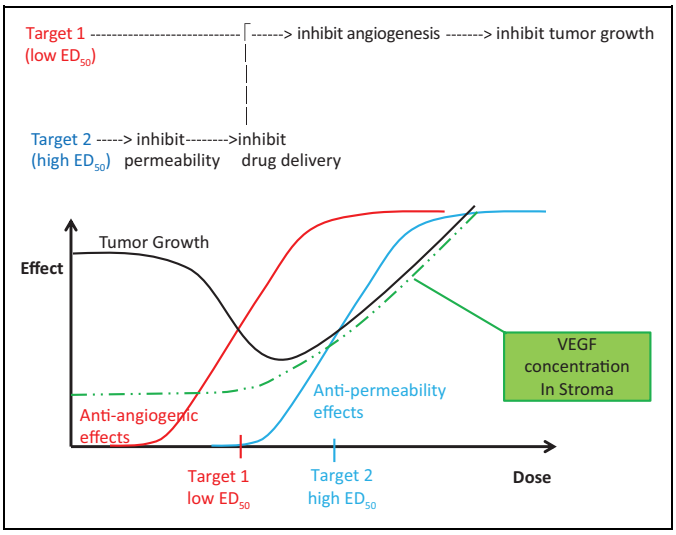

Figure 1. Two S-shaped Curves Produce a U-shaped Curve. Based on inspection of typical S-shaped curves, we estimate that the ED_{50} for the anti-angiogenic effect is about half-way down the slope and the ED_{50} for the anti-permeability effect is about at the bottom of the U.

virtue of requiring a minimum number of assumptions. The second target is merely nullifying the first target. Although it is easy to postulate such a model with some confidence, it is harder to demonstrate the actual biochemical processes that are being impacted by the drug.

Discussion

As discussed above, VEGF/VPF has very localized effects on angiogenesis (Springer et al. 2003), whereas the angiostatin and endostatin effects are systemic (i.e., they suppress angiogenesis at distant metastases) (Fisher et al. 1989a; Fisher et al. 1989b; Fisher et al. 1990). The U-shaped dose-response of angiostatin on angiogenesis is probably traceable to its different affinity and/or efficacy towards two (or more receptors).

Low Dose Rates of Angiostatin

The effects of angiostatin, especially interference with VEGF signaling, are believed to cause the inhibition of angiogenesis (Lee et al. 2009) consistent with the mechanism of VEGF-induced angiogenesis discussed above (Fantin et al. 2010; Fantin et al. 2011; Fantin et al. 2013; Lanahan et al. 2013; Fantin et al. 2014). This appears to be the primary effect of the angiostatic agents at low doses (dose rates).

High Dose Rates of Angiostatin

On the other hand, access of exogenous angiostatin (a moderate size 38kD protein) introduced from the blood stream (Kenan and Wahl 2005) into the ECM (i.e., where the new blood vessels are being formed) is facilitated by the VEGF/VPF-induced dilation (Koshida et al. 2003) and leakage (Sima et al. 2004; Shyong et al. 2007) of the existing blood vessels. At higher

dose rates, angiostatin causes constriction of blood vessels (Koshida et al. 2003) and stops the leakage (Sima et al. 2004; Shyong et al. 2007), thus preventing the exogenous drug from getting into the ECM where tumor-associated macrophages are facilitating the completion of vascular circuits. It is also relevant that reduction in the permeability of the blood vessels ensures that the VEGF/VPF (protein 32-44 kDa) will be concentrated in the stroma rather than dissipated in the blood (Figure 1) and enhances angiogenesis. Hence, high doses (dose rates) actually reduce the effectiveness of angiostatin and produce a U-shaped dose-response curve (Figure 1). Endostatin seems to follow a similar pattern (Celik et al. 2005; Tjin Tham Sjin et al. 2006) for the same reasons (Brankin et al. 2005; Marneros et al. 2007).

While high doses of angiostatin appears to interrupt VEGF signaling by interacting with its receptors (VEGFR), a humanized monoclonal antibody has been designed to directly target VEGF (i.e., bevacizumab, Avastin). This VEGF antibody suppresses perfusion of water and docetaxel (molecular weight 808 g/mole) into tumors (Van der Veldt et al. 2012) presumably by preventing VEGF from reaching its receptors on pericytes (Greenberg et al. 2008). This observation is at odds with the hypothesis that ''normalized'' (non-leaky) blood vessels are more efficient for drug delivery to tumors (Azzi et al. 2013). It has recently been suggested (Van der Veldt et al. 2012) that introducing cytotoxic anti-cancer agents before administration of agents that suppress VEGF signaling and allows the cytotoxic agent to enter the tumor and then be trapped there (i.e., reduced clearance). Because of their size, access of blood-borne angiostatin and endostatin to the ECM may be particularly sensitive to the porosity of the blood vessels.

Summary

This U-shaped dose-response of angiostatin is consistent with the variety of high-molecular-weight drugs (i.e., that do not readily diffuse through tissues) that are known to have U-shaped dose response against angiogenesis (Slaton et al. 1999; Motegi et al. 2002; Panigrahy et al. 2002), and thus, could provide a universal explanation for this behavior (without specifically addressing molecular interactions, which are still poorly understood). One prediction of this hypothesis is that the angiogenic effects would be more effective at high doses if the drug did not depend on reaching the tumor via the blood vessels. Indeed, strategies using viruses to express the anti-angiogenic agents in the tumor itself have been developed and seem to work well (Shyong et al. 2007; Luo et al. 2011).

Declaration of Conflicting Interests

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