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Review

Synchronizing Cancer Research Worldwide, a Way of Overcoming Resistance to Anti-angiogenic Drugs

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Abstract

Multiple biological pathways manifest and latent, meant for human survival, become a liability in cancer cure. With an increasing understanding of innumerable complex paths, cancer progression and development of resistance is no surprise. For the three “vasculature-immune-phenotypic” fundamental changes, hypoxia is the maestro orchestrating the whole gamut of changes (through the master manipulator - HIF-1 α), simultaneously transactivating hundreds of pro-angiogenic genes. Such a complex molecular bio-network begs the question, “Is our cancer research caught in such a tangled web that we have lost sight of the Spider?”. Hypoxia being this Spider weaving compensatory webs with every intervention/ obstruction. Anti-angiogenic (AAG) research has been conducted mainly in silos – exploring independent paths. This review conceptualizes a convergence of a multitude of research worldwide to a single theme of normalizing vasculature as a primary baseline for overcoming resistance to AAGs or their combinations.

Keywords: Anti-angiogenic, HIF-1 α, Normalization-window, research, hypoxia, resistance, SBRT, Immunotherapy

INTRODUCTION

Neo-angiogenesis (NAG) is an integral part of the healing mechanism in the body, which is a normally well-orchestrated series of events to provide additional oxygen and nutrients to the fast-multiplying healing cells of the body. NAG is a process well balanced between growth factors and inhibitors. The unfolding of NAG involves migration, proliferation, and differentiation of endothelial cells (ECs), leading to the formation of lumen-bearing tubes with a stable branching [1] structure covered by duly formed encasing cells - the pericytes. However, in cancer, NAG is the process that has gone haywire to a variable degree. Consequently, tumor vasculature ends up as an unstable, chaotic network with the features of tortuous tubes with varying lumen size, functionally leaky due to weak endothelial junctions, supported by a thicker basement membrane topped by deficient pericyte coverage; resulting in a vasculature that is “aberrant” structurally and functionally to a varying degree.

The introduction of the anti-angiogenic (AAG) concept was very innovative and promising; theoretically, depriving oxygen and nutrition to the cancer mass is expected to make the latter wither away dramatically. Initial work on this concept in preclinical studies raised immense expectations but generally failed in a clinical situation, with only a modest gain in clinical benefit. Although the clinical role of anti-angiogenics (AAGs) is undisputed today in selected indications, it is necessary to optimize its effects. After the initial response, almost invariably, “the wall” of resistance

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shows up after a while. The foremost reason is the development of “evasive resistance,” which refers to establishing alternative routes by cancer cells steering to progression when one pathway is blocked [2].

The general aspects of the role of AAGs vis-a-vis tumor vasculature are dealt with briefly in the published article by the present author [3]. The present paper will focus on and expand on NAG, anti-angiogenics, and proposed strategies to overcome the resistance.

Pathophysiology of tumor anti-angiogenesis response

1. Angiogenesis terminologies

Angiogenesis, a broad term, is the formation of new vasculature with a full-fledged lumen from the existing one. The organization happens either by sprouting of “tip cells” supported by the growth of “stalk cells” for elongation from the side of the blood vessel (sprouting angiogenesis) by activation of endothelial cells or by intussusceptive angiogenesis (also known as splitting angiogenesis) by splitting existing blood-vessel into two. On the other hand, vasculogenesis is the process where precursor cells differentiate to become endothelial cells. Neo-angiogenesis is the acknowledged characteristic of cancer growth and is considered “aberrant angiogenesis” given its abnormal nature. Thus, angiogenesis or neo-angiogenesis may end up as “aberrant angiogenesis” or “normal vasculature,” depending on the modulation of intricate pathways. The term neo-vascularization encompasses both neo-angiogenesis and vasculogenesis.

2. Types of NAGs

In its quest for easy access to the vasculature, cancer cells adapt to all the available methods enumerated below.

a. Vascular Co-option

Solid tumors originating growing along pre-existing vasculature is a phenomenon of “vessel co-option,” which increases its metastatic potential [4]. Vascular co-option has the advantage of immediate access to vascular supply. Vessel co-option shows inherent resistance to AAGs since co-option is independent of the angiogenic switch and angiogenic growth factors. However, vessel co-option may tend to follow rather than precede AAG. For example, using an anti-VEGF antibody in glioblastoma increased vessel co-option [5]. Yet, the co-opted vessels initiate Ang-2 mediated apoptotic cascade resulting in regression of the co-opted vessels soon followed by hypoxic tumor cells expressing VEGF, in turn giving rise to further neo-angiogenic response [6].

b. Aberrant Angiogenesis

Of the two types of angiogenesis, sprouting and splitting/ intussusceptive, the latter carries less metabolic demand since no endothelial proliferation is required, and tumors may prefer the latter during AAG therapy. Post-TKI therapy relapse may happen with extensive splitting angiogenesis [6].

Angiopoietins, Ang-1, Ang-2, and tyrosine kinase receptor (Tie-2) axis in association with vascular endothelial growth factor (VEGF) play a significant role in NAG. Ang-2 initiates NAG in response to hypoxia/injury. During the normal healing process, these newly formed blood vessels get “matured” and are selectively “sealed” by the Ang-1, and Ang-2 essentially gets restricted at this maturation stage [7]. In cancer, Ang-2 outstrips Ang-1 leading to deprivation of the maturation process resulting in progressively immature, thin-walled, tortuous, functionally deficient leaky blood vessels with aggregated endothelial cells covered by defective pericytes low in Ang-1 levels. Ang-2 overexpressed and Ang-1 deficient mice tended to die early of hemorrhage. Ang-2 also facilitates infiltration of

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myeloid cells, which later polarize into Tumor-Associated Macrophages (TAMS), weakening the local immunity induced through integrin pathway expression of matrix metallopeptidases promoting invasion and metastases. Hypoxia is the potent inducer of Ang-2. Soluble Tie-2, Ang-2 neutralization antibody, and small peptides have shown antitumor effectiveness offering a promising strategy [7].

After anti-VEGF bevacizumab treatment of glioblastoma, recurrence is highly infiltrative, and salvage surgical excision and chemotherapy become ineffective. A study when U87MG glioma–bearing athymic mice were treated with the anti-VEGF agent aflibercept showed that with the extended treatment of 6 weeks, there was enhanced invasion with TEMs migration, a dramatic increase in Ang-2 expression mainly in the invasive periphery, possibly due to increasing VEGF levels but not with a short treatment of 3 weeks. During this period, Ang-1 expression continued to remain low. A similar observation of increased Ang-2 was seen with bevacizumab and not with temozolomide. Further, targeting this Ang2/Tie2 axis to counter the invasive phenotype in AAG therapies prolonged survival in mice [8]. Thus, the literature indicates that role of Ang-1 is crucial in vascular normalization. However, in a study, stabilizing Ang-1 signals did not contribute to survival [9], showing that studies are required to unravel the complex interaction between Ang-1 and Ang-2.

The role of the other two angiopoietins, Ang-3 and Ang-4, is yet to be understood. In a study, Ang-3 did reduce angiogenesis and pulmonary metastases [7] and is worth exploring.

c. Vasculogenesis

It is the process of differentiation of precursor cells to endothelial cells. Contribution to vasculogenesis can come from the differentiation of hemopoietic normal stem cells or cancer stem cells by direct endothelial differentiation. The latter's role is emerging as a powerful mechanism for tumor progression. This vasculogenesis could not be affected by anti-VEGF bevacizumab but could be inhibited by tyrosine kinase inhibitor sunitinib or the anti-VEGF-receptor-2 neutralizing antibody, suggesting that vasculogenesis is a VEGF-independent mechanism [10], and target precisely.

d. Vascular mimicry

There is a process of cancer cells forming the vascular-like structures independently of neo-angiogenic blood vessels. Vascular mimicry was shown to get upregulated following treatment with bevacizumab or induction of hypoxia during the resistance phase [5]. It is proposed that vasculogenic mimicry might depend on cancer stem cells since classical angiogenics has no role in vasculogenic mimicry [6].

3. Angiogenesis pathways – "The Web."

Aberrant angiogenesis is one such intricate process involving mainly VEGF, Notch, and Ang signaling precipitated and sustained by hypoxia, orchestrated through inducible factor (HIF). The VEGF spectrum is primarily made up of VEGF A, B, C, D, and placental growth factor (PlGF), a central driver being VEGF through receptors VEGFR 1, 2, and 3 ending up with intracellular activation of PI3K, PKC, and RAS/RAF/ERK/MAPK pathways [4]. Alternative paths include cyclooxygenase-2 (COX-2) blockade, oligo¬nucleotide complementary to the miRNA competing with the mRNA target, and the inhibition of matrix metal¬loproteinases (MMPs) [4]. Additionally, 18 glycoproteins of the FGF family of growth factors interacting with four transmembrane receptors, FGFR1 to FGFR-4, is also a potent moderator of tumor angiogenesis independent of VEGF signaling [2]. Further, other complementary pathways are described, which depend on key proteins such as platelet-derived growth factor (PDGF) influencing pericyte recruitment and coverage, fibroblast growth factor (FGF), and various inflammatory mediators of angiogenesis, etc. [11]. Furthermore, there are stromal pathways where

bone marrow-derived cells (BMDCs) are a source of endothelial as well as pericyte progenitors and pro-angiogenic, tumor-infiltrating immune cells (TILs). Ang-2 increases the expression of the pro-angiogenic genetic phenotype of Tie2+ monocytes/macrophages associated with the blood vessels [11].

The voluminous literature is available about the current approach of blocking one or other multitude of pathways, and despite various combinations, AAG therapy finally ends up with resistance or unacceptable toxicities.

Types and mechanisms of resistance

Enduring responses to AAGs do not happen due to intrinsic “evasive” resistance, or the initial response ends with continued growth while still on treatment due to “acquired” resistance following mutational alteration of the gene encoding a drug target or by modifications in drug uptake and efflux [4]. Tumor growth did not slow down even with extensive pruning of vessels by anti-VEGF [9]. The AAGs, over the long term, may lead to aggravation of tumor hypoxia [12]. High Ang-2 levels correlated with resistance to anti-VEGF therapy, and Ang-2 countered the anti-VEGFR2 treatment-induced initial normalization in mice bearing gliomas [9].

Hypoxia generated ensuing angiogenesis inhibition by AAGs is a powerful trigger for setting in the alternative or latent, innumerable pathways that make tumors more aggressive, metastatic, and progressively less sensitive to AAG. The ability of normal and cancer cell biological systems to reroute through the existing local bypass, activate latent pathways, or adapt to genetic or environmental perturbations is a poorly understood process of adaptive evolution at the molecular level [13]. The development of resistance to AAG can be classified mainly as VEGF and non-VEGF dependent pathways [11]. Enhanced expression of angiogenic cytokines, such as VEGF and PlGF, or recruitment of pre-metastatic niche EPCs can be induced by AAG [6].

Epithelial-mesenchymal transition in TME also contributes to the aggressive phenotype of surviving tumor cells. The possible mechanisms are upregulation of network of alternative angiogenic factors, up-regulation of HIF-1a and over-pruning of blood vessels, TME changes due to expansion and recruitment of myeloid cells and CAFs including increased co-option/ mimicry, impediment in the delivery of AAG, etc., promoting further aberrant angiogenesis, tumor growth, EMT transition, and metastasis [5].

Tracking of Neo-Angiogenesis for clinical trials

Modern imaging techniques become essential to develop a model to answer and titrate the appropriate use of the AAG for long-lasting response in vivo since there are limitations to mice longevity. Dynamic tracking of the “window for vascular normalization” will help determine the optimization of drugs, dosages, [14] combinations, and cycles. Indirectly, the vasculature can be evaluated with dynamic contrast-enhanced perfusion magnetic resonance imaging (MRI) or perfusion computed tomography (CT) and PET or both to decide on the period of therapy and drug holidays [6]. CT perfusion study can estimate blood volume, fractional intravascular plasma volume, blood flow, and peak enhancement index. Dynamic contrast-enhanced CT (DCE CT) can evaluate intra-tumoral vascular physiological statuses such as perfusion, permeability surface area, and interstitial space. Using paramagnetic nanoparticles targeting avb3 integrin in positron emission or sonography with gas-filled microbubbles directed against a specific target and optical techniques can also track angiogenesis directly [6].

Serum level of soluble VEGFR (sFlt1) produced by ECs, Ang 1/Ang 2 ratio, hypoxia-regulated Apelin and its mRNA circulating type IV collagen, hypoxia correlating TSP-1 [14] will be convenient and can be done repetitively compared to imaging techniques.

Summary of AAG approach

In the first phase, following the evolution of the attempt to cut the blood supply to eliminate cancer, VEGF became the preferred target since it is considered the prime driver. Soon it was revealed that the initial response was followed by re-growth due to the development of numerous complementary VEGF and non-VEGF pathways angiogenic pathways [11]. Consequently, to block the vertical and horizontal parallel paths together, combination anti-angiogenics are tried. However, toxicity and subsequent development of resistance became an issue [11]. In clinical situations (unlike animal studies), bevacizumab improved survival only when administered with chemotherapy [6]. However, AAG can decrease the subsequent delivery of cytotoxic drugs by progressively reducing and cutting off the tumor's vasculature.

In the second phase, a whole new aspect opened up with the introduction of the vascular normalization concept in 2001 [15]. According to this concept, initially, VEGF inhibition altered the aberrant angiogenesis towards normality by reduced vascular permeability, decreased interstitial pressure, enhanced oxygen perfusion, etc., leading to tumor response. Increased collagenase IV activity restored a thinner basement membrane and improved vascular pericyte coverage by upregulation of Ang-1. However, with continued administration, VGFR blockade leads to Ang-2 accumulation, destabilizing blood vessels and compromising the survival benefit of VEGFR-2 inhibition due to increased vascular permeability. This action indicates the negating effects of Ang-2 on vascular normalization of the anti-VEGF blockade, aggravates hypoxia, and makes cancer cells resistant, which winds up in the selection of more resistant invasive clones [6], contrary to the initial concept of AAG. Additionally, a hypoxic volume is refractory to subsequent radiotherapy and chemotherapy and contributes to cancer cell escape to an aggressive selection of stem-cell-like tumor cells.

However, there is evidence that long-term AAGs, even with continued pruning of vasculature, produce a small survival benefit beyond the progression [6], supporting the original concept of cutting off vasculature for tumor control. A third observation from the literature emerges that the tumor vasculature normalization phenotype becomes re-established on discontinuation of anti-VEGF-targeted therapy [15]. Putting the reports of the benefit of long-term administration of AAGs and cyclicity together, it can be reasonably hypothesized that, by strategically giving "drug holidays" in-between, the usefulness of AAGs can be prolonged. Tracking the "normalization period" and "pruning period" with various methods enumerated above would help in properly synchronizing the extended "cyclical" AAG therapy.

Proposed Strategies for Overcoming Resistance

This section is predominantly about the authors' commentary based on the literature evidence. The literature review summary is juxtaposed where relevant with the commentary as points of substantiation.

Three primary and critical changes happen in cancer- Vascular, Immunological and Phenotypic- the vascular component being the crucial change. For retracing of immunological escape of cancer cells and for reversal of phenotypic changes in the tumor and its microenvironment (TME), vascular normalization remains a desideratum [16] when discussing the resistance to AAGs.

Proposed Terminology: The present author has conceptualized the term eu-angiogenesis to include all the processes of "supportive" (beneficial) angiogenesis changes for cancer treatment. The terminology encompasses; a) Angiogenesis initiated by Ang-2 before the process turns "aberrant" by lack of Ang-1. b) The theoretical possibility of angiogenesis initiated by Ang-2 leads to mature, well-formed, thinner vessels in cancer if Ang-1 accumulation can be timed for the maturity of vessels. c) The reversal of aberrant vasculature to normal vasculature after AAGs, commonly termed "vascular normalization." The term aberrant angiogenesis is often used interchangeably with neo-angiogenesis in the description of cancer vasculature, making it difficult to interpret the underlying pathophysiological process at any given time when planning for therapeutic intervention. Any normal (eu) angiogenesis element within the cancer mass is expected to be more effective than without. Therefore, the term eu-angiogenesis is proposed to indicate the process of intervention sustaining normal angiogenesis to prevent them from becoming aberrant or reversal to normalization from aberrant angiogenesis or a range of combinations thereof and to strategize the synchronization of anti-angiogenic treatment approaches in combinatorial therapies envisaged in the "implementation of strategies" section.

1. Criticality of Hypoxia, HIF-1α, and "Tangled Web"

Tumor cell hypoxia is a key driver of angiogenesis primarily through stabilization of HIF-1 α as and when the tumor growth outstrips the blood supply of oxygen. From then on, HIF -1 α transactivates hundreds of pro-angiogenic genes, including growth factors, inhibits the production of anti-angiogenic factors, and intensifies angiogenesis [1]. Therefore, upregulation of hypoxia-induced HIF-1 α transcription factor is central in the cellular response having multiple downstream effects on VEGFA, VEGFRs, PlGF, Ang-1/2, and PDGF, TGF-β, etc., which are intimately involved in promoting cell survival, endothelial cell migration, anaerobic metabolism, and metastasis [11]. In AAG therapy, aggravation of intratumoral hypoxia during the vascular regression phase leading to a concomitant increase in HIF-1 α followed by up-regulation of VEGF plays a crucial role in resistance. This upregulation, in turn, results in tumors acquiring more angiogenic and invasive potential, local and metastatic, after the initial response. AAG-induced hypoxia also leads to downstream changes of over-expression of the tyrosine-protein kinase c-MET, stimulates β1 integrin expression (which in turn interacts with c-MET), decreases adherens junction protein expression affecting pericyte coverage favoring increased invasiveness and colonization [5]. In addition, tumor hypoxia favors angiogenic dormancy, tumor augmentation autophagy (a process of sequestration of dysfunctional cell components and their degradation), glycolysis-induced lactate production in TME aggravating the acidification, cancer stem cell proliferation, lymph-angiogenesis promoting metastases, etc., all ensuring AAG therapy resistance [1].

Implication: The presence of oxygen is required to enhance the sensitivity of cancer cell lysis and fix the partial damage to the cancer cells by anticancer therapies. The absence of oxygen encourages the recovery of cancer cells from partial damage with more resistant mutations. In cancer initiation and progression, "all roads lead to and are from hypoxia." First and foremost, fixing the hypoxia dismantles the resistance. The following proposed strategies either effectively target hypoxia or its main downstream effects.

2. Criticality of Ang-1, 2 - Tie2 axis and Ang-1/Ang-2 ratio

While inhibition of Ang-1 had minimal effect on tumor vasculature, the combination of Ang-1 and Ang-2 blockade trims tumor blood vessels without normalization. Contrarily, inhibition of Ang-2 can enhance the interaction of Ang-1/Tie-2. Simultaneous activation of Tie-2 and blockade of Ang-2 in mice with some cancers resulted in more efficient normalization, improved blood perfusion, and

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reduced lactate acidosis, limiting tumor growth and metastasis. Another study in the mice model demonstrated that dual inhibition of VEGFRs and ANG-2 was more effective than single inhibition due to widened normalization window [17]. Targeting Ang-1/2 and Tie-2 receptor axis with trebananib, a peptide-Fc fusion protein, in ovarian xenografts tumors reduced angiogenesis and growth. It improved PFS but not OS in a phase III trial in recurrent ovarian cancer [18], indicating that blanket Ang-1/2 and Tie2 axis block did not give the survival benefit.

Implication: Theoretically, a stratagem of maintaining the Ang-2: Ang-1 optimum balance favoring synchronization of accumulation of Ang-1 in the later phase of neo-angiogenesis complimenting the initiation of neo-angiogenesis by Ang-2 would be eu-angiogenesis optimization. These harmonized events are the foundation for forming normalized vasculature with a thinner diameter with effective "tip cells," leading to improved tumor perfusion, reduced interstitial pressure, increased drug delivery, etc.

3. Importance of Pericytes

The role of pericyte in angiogenesis is very complex, manifested by inadequate coverage at the start of AAG therapy and excessive pericyte coverage later coinciding with the time of resistance. Studies have shown double blockade with VGFR (endothelial cell targeting) and PDGFR (pericyte targeting) shows improved efficacy of AAG therapy. Another study showed that targeting Ang-2 along with pericyte depletion restored vascular stability. In yet another study, tumor vasculature was enhanced in a breast carcinoma xenograft model using the TGF-β-fibronectin axis, which improved pericyte-endothelium association. Overall, pericyte depletion disrupts vascular integrity [19].

Implication: There appears to be a delicate balance between initial deficient coverage and later excessive coverage of pericytes during AAG therapy (the latter coinciding with the phase of the resistance). This observation specifies a need for the proper sequencing of AAG initiation followed by pericyte targeting to reduce its thickening after about four weeks of AAG therapy, which can theoretically extend the normalization window.

4. Importance of TME volume & Interstitial Pressure

Tumor volume/TME comprises a heterogeneous group of cancer cells, vasculature, lymphatics, stromal fibroblasts later transformed to cancer-associated fibroblasts (CAFs), increasingly acidic interstitial fluid, immune modifying cell infiltrates, etc. As the cell mass and interstitial pressure increase, hypoxia is amplified due to vasculature compression and increased blood viscosity, creating a vicious cycle. Enhanced cancer cell kills leads (e.g., by use of non-disruptive Stereotactic radiotherapy dose in the range of 6 Gy to 10 Gy per fraction [20], or timing of chemotherapy/ immunotherapy/nanoparticle dugs in the vascular normalization phase of AAG therapy) to decreased interstitial pressure (IP) followed by decompression of vasculature which sets-up a virtues cycle locally.

Implication: One way of further improving the normalization effect of AAG is complimenting it with successful concurrent cancer cell kill. This association could be the one reason for positive results when AAG is combined with chemotherapy.

5. Importance of Cytokines, Chemokines, and Interferon-gamma (IFN-ϒ)

Immune cell effects: Innate immune cells like mature dendritic cells (mDCs) and M1-like TAMs produce various cytokines such as IFN-α, IL-12, IL-18, TNF, and chemokines such as CXCL9, CXCL10, CCL21 which influences the phenotypic and functional features of tumor vasculature. Adaptive

immune cells secrete cytokine IFN-γ, which induces TME vascular normalization. Additionally, mDC, CD8, and TH1 immune cells direct macrophage polarization from M2 to M1 phenotype [21].

Endothelial cell effects: Interestingly, CD8+ CTLs and CD4+ T helper 1 (TH1) cells assist in tumor vessel normalization with vessel phenotype representing high-plump endothelial cells (HEVs) functionally specialized in lymphocyte extravasations by producing IFN-γ in the TME. LTβR signaling pathway generates HEVs in TV with anti- VEGFR2 and anti-PD-L1 combined blockade. STING activation, simulator of IFN genes demonstrated synergism with anti-VEGFR2 and ICIs (either anti-PD-1 or anti-CTLA-4) through type-I IFN signaling activation and the upregulation of genes leading to endothelial-CD8+ CTLs interaction with complete regression of tumors resistant to either of the mono-therapies [21].

Pericyte effects: Depleting CD4+ TH1 cells decreases pericyte coverage and increases distorted vessels. IFN-γ signaling downregulates VEGF-A and simultaneously upregulates CXCL9, CXCL10, CXCL11, etc., with resultant pericyte recruitment along with ECs, finally leading to vascular maturation [21].

Immuno-modulatory effects: Immunomodulation is observed in mouse ECs in vitro induced by Interferon-γ (IFN-γ) and TNF in antigen uptake, processing, and presentation process. Also, IFN-γ brings about antigen degradation and antigen loading via 'immunoproteasome' [22].

Implication: Combination therapies to encourage IFN-γ and other cytokine/chemokine accumulation open up a whole vista of possibilities in targeting hypoxia with added specific immune-modulation features.

6. Importance of "Normalization Window"

Cediranib caused a consistent and dramatic reduction in tumor enhancement within 24 h of therapy along with a decrease in vascular permeability, indicating the start of the normalization window conforms with the onset of normalization following blockade of the VEGF/VEGFR2 pathway in preclinical studies. This vascular normalization "time window" persisted for at least 28 days, and although relative tumor vessel size was reversed by day 56, interestingly, vascular permeability remained low even till day 112 in the clinical study, not seen in animal studies due to short survival time in mice. Outstandingly, in patients who required "drug holidays" because of toxicity, the reversibility of vascular normalization was also demonstrated in this study with the reversal to normalization phenotype on MRI. Additionally, the cycle gets repeated upon recommencement of therapy [15].

Upregulation of tumor Ang-1 gene expression typically produced by perivascular cells (PVCs) is the crucial pathway during NW through binding and activating the Tie-2/TEK receptor on ECs. Reorganization of the basement membrane of vessels, which is usually haphazard and thick, is restored to a thinner and more closely associated one during NW commencing in 48–72 hours [15].

One possible mediator of the escape from the normalization window includes hypoxia-driven bFGF. Temporal changes in circulating FGF2 levels with the VEGF axis inhibition are linked to disease progression seen in glioblastoma [11]. In the U87 xenograft model, one week of short-term VEGF blockade did not increase bFGF levels with a significant reduction of microvessel density, yet in 7 weeks of continued VEGF blockade, bFGF levels increased with microvessel density and tumor cell proliferation [2]. The literature indicates that the normalization process has 3 phases during AAG therapy.

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The first normalization phase starts immediately and is known to last about four weeks, bringing down hypoxia with ensuing improved cancer cell kill. Simultaneously, decreased interstitial pressure further improves the oxygenation, reflecting on cascading effects of TME hostile to the cancer cells. This scenario enhances the effectiveness of combinatorial radiation therapy and Chemotherapy/ immunotherapy [15].

In the following second phase, due to vascular pruning and shrinking of the vasculature, hypoxia rebounds with resistant phenotypic changes, which in turn increases the invasiveness of cancer, creating an AAG treatment resistance milieu. The data about subsequent changes are restricted given the short life span of mice. Most reports show that continuing AAG therapy in this stage does not confer significant benefit, letting down the original concept of AAG therapy (that cutting off blood supply would be a strategy to eliminate cancer). The time between initiation of AAG and the onset of the second phase is the "normalization window" described by Jain RK et al. [23].

The third phase is substantiated by anecdotal literature. When AAG therapy is discontinued, there will be the return of normalization susceptible phenotypes, and reintroduction of AAG can set in the process of 2nd "normalization window." The present author believes that the study of the process of 2nd and possibly further normalization windows are essential to adopt the "cyclical" treatment concept for AAGs. The evidence for restoration of normalization phenotypes comes from MRI studies in patients who were given drug holidays due to toxicity [15].

Implications: Tumor vasculature is quite diverse. The angiopoietins, Ang-1 and Ang-2, ligands of the Tie-2, are known to play an indispensable role in angiogenesis for recovery from injury. However, their role in the pathophysiology of diseases is wide apart. Primary requirements for any effective drug action are that it should be distributed uniformly, have a high concentration relative to the blood, and be retained for a longer duration in the cancer mass. However, vascular pruning action of AAG at the later phase heralds very antithesis of effective action.

Implementation of Proposed Strategies

1. Managing hypoxia

The prerequisite for any strategy is to overcome hypoxia and HIF-1 α stability. Therefore, the logical approach would be to effectively counter hypoxia by normalizing perfusion, facilitating the immediate degradation of HIF-1 α on its formation. The other line of attack would be to target HIF-1 α. There have been efforts to develop HIF inhibitors, e.g., by dissociating HIF2α and HIF1β dimers, leading to inhibition of transcriptional activation of HIF2. The treatment was successfully tried in multi-treated RCC patients and can be combined with classical anti-angiogenic drugs or immunotherapies [1].

2. Fortification and extension of vascular normalization window

Several avenues exist for enhancing and extending vascular normalization to overcome AAG resistance. Some of them are a). The use of angiostatic factors such as tumor necrosis factor α (TNFa), thrombospondin-1 (TSP-1), and endostatin to improve vascular perfusion; b). Injection of tumor-infiltrating lymphocytes (TILS) and TNF-α to enhance in-vivo vaccination effect; c). LIGHT (also known as herpesvirus entry mediator ligand), activating various intermediaries, can repair abnormal tumor vasculature; d). ABT-510, one of the mimics of TSP-1, promotes normalization and immune-modulation without reducing the vascular density; e). Recombinant human endostatin, an angiogenesis inhibitor, possibly to restore vascular homeostasis, etc. [14].

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The other potential pathways to focus on are to target deranged EC metabolism to counter VEGF effects or excessive acidification of glycolysis in TME or by targeting FOXO1.

The other approach with excellent future potential is to use miRNAs, which can restore the integrity of vasculature, e.g., the use of miR-20b to inhibit the nuclear aggregation of HIF-1α and activation of Transcription 3 (STAT3). Another group evaluated the suppression of miRNA-153 in preclinical breast cancer studies for HIF-1α & Ang-1 targeting [19]. Studies elucidate the impact of miRNA-140-5p silencing VEGF-A; miRNA-29b inhibiting all steps of angiogenesis, impacting proliferation, migration, and tube formation by targeting Akt and down-regulating VEGF and c-Myc; overexpression of miRNA-497 reducing VEGF and HIF-1α, etc. [19]. Yet another approach is to normalize the extracellular matrix (ECM), especially CAFs, to make them supple [14], which will facilitate unhindered vascular perfusion.

3. Synchronization

By the timing of chemotherapy/ immunotherapy, the normalization window of AAG efficacy can be enhanced [15] due to the more efficient distribution of the drug, increased cancer cell lysis, and improved immunological reaction (local as well as distant). Theoretically, SBRT with a vascular non-disruptive range (6 to 10 Gy per fraction) can be timed during the normalization window period of AAG for oligometastases and as a boost in residual disease in advanced malignancies [3], keeping in mind the possibility of reported increased intra-tumoral bleeding episodes near the critical structures.

4. Cyclicity:

Stopping the AAG at the expected onset of vascular regression and continuing with chemo/ targeted/ immunotherapy is expected to improve cancer cell lysis. Subsequently, waiting for the normalization phenotype to return, as evaluated indirectly by imaging studies, will facilitate cyclic administration of AAG. Modern imaging and other techniques for tracking the vascular changes enumerated above will be essential for the trials.

5. Moderate/lower dose therapy

The use of moderate doses is another approach to optimally normalize aberrant tumor microvessels and increase blood perfusion [24]. Excessive pruning by high dose AAG results in exaggerated hypoxia, and infiltration by immunosuppressive cells is observed after the normalization window period [17]. Short-term high dose sunitinib (120 mg/kg per day) increased tumor growth, and low-dosage sunitinib (30 and 60 mg/kg per day) did not stimulate metastasis in mice models [5]. In glioblastoma, low-dose bevacizumab gave better results. Amassed data from other studies also show that low and not high doses facilitate tumor vessel normalization [17]. Ramjiawan et al. showed that lower doses of VEGFR-2 blockade led to increased perfusion and shift to M1-type TAMS and infiltration of CD8+ T-cells, in contrast, to reverse effects with expanded Tregs of a higher dose in breast cancer. In addition, added cancer vaccine increased IFN-γ+CD8+T-cells and decreased Tregs, resulting in improved overall survival in mice with spontaneous MMTV-PyVT breast cancer [18]. It is not yet clear which dose level is optimal for normalization lower dose of 5 mg/kg of bevacizumab when used in colorectal cancer or 15 mg/kg in lung cancer, warranting further study [21]. All these studies indicate the potential of combining low-dose AAGs in combinatorial therapies combining the effect of cell kill with eu-angiogenesis.

6. Reinforcement of eu-angiogenesis

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A combination of AAG and vascular normalizing drugs can extend the former's effectiveness and the NW's period. Theoretically, one such combination that has the potential to be explored is a combination of AAG along with appropriately timed Ang-1 to produce more viable vasculature during the window period. Even with prolonged treatment of pancreatic tumors in transgenic mouse models with anti-VEGFR2 antibody, a delay in growth and modest survival benefit was observed due to increased expression of the pro-angiogenic growth factors, Ang-1, Ephrin-A1, Ephrin-A2, and FGF1, FGF2a. Ang-1-Tie pathway results in the maturation or stabilization of blood vessels, which is blocked by accumulated Ang-2 [5]. Increased expression of factors like Ang-1 antibody immunotherapy indicates the role of appropriately timed vascular maturation agents to reduce the hypoxia to be focused on along with synchronized blocking of both VEGF and Ang-2.

7. TME Immune modulation:

Normalization facilitates the effectiveness of immunotherapy, immunoadjuvants, immunogenic schedule of SBRT, and newer nano-technological targeting, etc., with the added possibility of in vivo therapeutic vaccine generation.

Infiltrating immune cells, stromal cells, and abnormal vascular and lymphatic vessels are the main components of TME, immunologically compromised by hypoxia, with high interstitial pressure and a low pH [17]. Immunotherapy agents do influence vascular normalization furthering their effectiveness. The NW period improves the immunological milieu of the tumor. Also, cancer cell phenotypic changes to sensitive types can take place. For example, PDL-1 expression was upregulated in tumor endothelial cells after AAG treatment along with increased infiltration of CD4+ and CD8+ T cells resulting in improved tumor control with immunotherapy [17]. A combination of AAG and immunotherapy agents like bevacizumab, atezolizumab, and apatinib with an anti-PD-L1 antibody could normalize the TME [14]. IFN-Ɣ secreted by Th1 cells had a positive association with vessel normalization. A virtuous cycle sets in when immunotherapy improves the vasculature, facilitating immune cells' further infiltration.

8. Differential protection of endothelial cells (normalization) and sensitization of cancer cells:

Gene modification appears to have a lot of potential in sustaining NW. When disrupted, G-protein signaling 5 (Rgs5) could enhance the infiltration of effector immune cells into the tumor parenchyma, resulting in prolonged survival of tumor-bearing mice by an unknown mechanism [17]. The other approach would be to adopt Dual Recombinase Technology to preferentially radiosensitize tumor cells and protect the endothelium [25].

9. Optimizing vascular-immune-phenotypic (VIP) changes and innovations

Exploiting the VIP interdependence is essential to improving the tumor response, both morphological and immunological, along with the possible abscopal effect. Focus on combinatorial therapy on the component that reduces hypoxia primarily, with the added enhancement of immunological interaction along with a top-up effect on phenotypically sensitized cancer cell lysis, would fulfill the requirements of the VIP model. For example, VEGFA and ANGPT2 blockade with added bispecific antibody A2 V resulted in enhanced infiltration of CD8+ T cells leading to increased tumor antigen presentation and promoted perivascular T cell accumulation, and increased the tumor necrosis with normalized residual blood vessels [17]. Some molecular networks and potential pathways are not dependent on the HIF-1 α pathway. For example, the lack of the HIF-1 in the VEGF-B promoter site makes the latter not amenable to hypoxia-induced expression. VEGF-B has a role in inflammatory angiogenesis and arteriogenesis, leading to the regeneration of coronary collaterals [5]. Ziv-aflibercept is known to bind to VEGFB, improving survival in oxaliplatin-resistant colorectal

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cancer when given along with FOLFERI. However, there was no benefit when combined with docetaxel in metastatic non-small cell cancer, indicating the complexity of the mechanism involved.

A sufficient presence of Ang-1 leads to maturation of vessels with robust “tip cells,” thinner vasculature penetrating deeper into the hypovascular/avascular part of the tumor. Theoretically, along with restriction of Ang-2 at the maturation stage of the vasculature, Ang-1 would produce the eu-angiogenesis overcoming the “trimming effect.” Timing of Ang-2 blockade and Ang-1 promotion along with AAG would again be an example of an innovative initiative.

Sunitinib short-term treatment before intravenous inoculation of breast and melanoma cells accelerating metastasis resulting in short survival, despite cessation of therapy, is documented. Sunitinib increases metastases in orthotopic mouse models of breast and colon cancer but not in lung cancer [6], indicating the dependency of metastatic potential in a particular type of tumor.

With immunotherapy dramatically changing the cancer treatment landscape, albeit long-term responses are restricted to a minority, clinical trials in various cancers continue to evaluate combinations of immunotherapy with AAG. One such combination is PD-1/PD-L1 antibody with an anti-VEGF, with positive studies in several phase III studies. The first objective set in these studies is to circumvent VEGF-mediated immunosuppression by combined VEGF and PD-(L)1 blockade by overcoming inhibition of DC maturation which in turn facilitates recruitment of T cells to TME with improved perfusion [26].

10. AAG Convergence Research

To a variable degree, the cancer mass is a mix of all abnormal angiogenesis components (aberrant-angiogenesis, vasculogenesis, and vascular mimicry). Even aberrant-angiogenesis is initiated as eu-angiogenesis; however, normal vascular formation falters in the final steps due to the deficient availability of Ang-1 relative to Ang-2. Resistance to AAGs appears inevitable over a variable period due to compensatory pathways opening up. Overcoming this resistance requires a convergent approach to maximizing eu-angiogenesis in the tumor mass by innovative strategies.

The present article is not about simplifying the most complex multistep process of cancer. The theme is about the concept that would be a meeting ground for the multitude of research going on worldwide. The optimization steps are a) First, to decide on the logical combination AAGs with other therapy/therapies supported by the available literature (Figure 1). b) Optimize the schedule, especially concerning the dose. c) Improvise on the way by dropping or adding known effective therapies (Multi Arm- Multistep, MAMS methodology) (Figure 2). d) Expand based on the results of other teams amid vascular normalization as a common factor (Figure 3). The objective is appropriate utilization of resources, prodigious optimization of time, and amalgamation of scientific effort to impact the outcome of cancer therapy dramatically.

CONCLUSION

Future research should focus on the combinatorial approach of enhanced eu-angiogenesis, instant degradation of HIF-1 alpha on its formation, enhancement of Ang-1 pairing with Ang-2, stimulation of IFN gamma pathway, modulation of miRNAs, etc., all with the explicit purpose of improving and extending normalization of the vasculature to improve the Vascular-Immune-Phenotype cross talk. This approach envisions blocking all the downstream effects of hypoxia, obviating the need for correcting a plethora of not-so-important drivers of AAG resistance.

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