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Anti-angiogenic therapy for cancer and the mechanisms of tumor resistance

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Summary. Tumor progression requires the activation of neovascularization, or angiogenesis, a process orchestrated by tumor and by stromal cells within the tumor mass. In the therapeutic targeting of angiogenesis, the aim is to inhibit tumor growth and progression. Indeed, anti-angiogenic therapy is currently used in several types of cancer. Nevertheless, both the tumor cells and the stromal components may be variably resistant to anti-angiogenic therapy, demonstrating refractoriness, or intrinsic resistance, on the one hand, and acquired resistance, gained progressively during treatment, on the other. Several strategies have been proposed to overcome both types of resistance but they remain to be tested in preclinical studies and clinical trials.

Keywords: anti-angiogenic therapy · tumor cells · stromal cells · intrinsic resistance · acquired resistance

Resum. La progressió dels tumors requereix l'activació d'una neovascularització, o angiogènesi, un procés orquestrat per les cèl·lules tumorals i per l'estroma dins la massa tumoral. La inhibició terapèutica d'aquest procés té per objectiu impedir el creixement i la progressió del tumor. Actualment la terapèutica antiangiogènica s'aplica en determinats tipus de càncer. No obstant això, hi ha cèl·lules tumorals i components estromals que poden presentar una resistència variable a la terapèutica antiangiogènica, que pot ser refractarietat o resistència intrínseca, per una banda, o resistència adquirida progressivament al llarg del tractament, per una altra. S'han proposat diverses estratègies per lluitar contra aquests dos tipus de resistència, però encara han de provar-se en estudis preclínics i en assajos clínics.

Paraules clau: teràpia antiangiogènica · cèl·lules tumorals · cèl·lules de l'estroma · resistència intrínseca · resistència adquirida

Angiogenesis in tumor development

CANCER CELLS ARE CELLS THAT have lost their capacity to divide in a controlled manner. They give rise to a neoplastic lesion that is supported by stromal cells. Both tumor cells and stromal cells contribute structurally and functionally to tumor development. Nevertheless, the tumor mass is typically limited to a size of 1–2 mm³, because further growth requires the diffusion of adequate amounts of oxygen and essential nutrients. To meet this demand, tumors induce the growth of blood vessels, a process referred to as angiogenesis, by up-regulating the expression and secretion of growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), angiopoietins (Ang), placental growth factor (PlGF), and some integrins, and concomitantly down-regulating several anti-angiogenic factors [22]. In addition, angiogenesis coincides with increased circulating tumor cells, facilitating metastatic

spread. There is also evidence that angiogenesis precedes tumor formation, which implies that it is the rate-limiting step not only for tumor growth but also much earlier, for tumor development [5].

Tumor cells cooperate with other cell types in the tumor microenvironment, such as immune cells, inflammatory cells, hematopoietic cells, and stromal fibroblasts. These cells then secrete various types of inducers, which activate endothelial cells and therefore angiogenesis [6]. In 1986, Dvorak described the inflammatory phenotype of tumors as "wounds that never heal." In the tumor mass, the balance is tipped in favor of angiogenesis, with the newly formed vasculature able to oxygenate and nourish the growing neoplasm.

The imbalance resulting in the sustained production of pro-angiogenic factors, together with the persistent lack of vascular stabilizing factors, leads to the formation of an immature and dysfunctional vascular system that cannot keep pace with the rapid growth of the tumor mass. Therefore, the vascular tree in a tumor is typically chaotic, with dead-end vessel branches and areas of inverted and intermittent blow flow, which may impair vascular function and lead to regions of lowered perfusion and hypoxia. The latter in turn causes up-regulation of the transcription factor hypoxia-inducible factor (HIF) and therefore of hypoxia-dependent genes (e.g., carbonic anhydrase and glucose transporters) [42]. For example, HIF-1 modulates the transcription of genes involved in glycolytic metabolism, oxygen consumption, survival, angiogenesis, migration and invasion. Accordingly, its stabilization has dramatic repercussions for the gene expression profile and eventually the behavior of the tumor cells [2,25]. Moreover, hypoxia actively participates in the activation of angiogenesis, by regulating the inducers and inhibitors that contribute to it. Specifically, tumor hypoxia induces the expression of molecules that disrupt endothelial and pericyte coverage, such as Ang2, which further contributes to the initiation of vascular sprouting. In addition, hypoxia results in the mobilization of multiple types of stem cells from the bone marrow and the recruitment of immune cells to the tumor microenvironment [7].

Recent advances in molecular biology and the study of families with hereditary renal cancer (in the setting of von Hippel-Lindau, hereditary papillary, Birt-Hogg-Dubé, and hereditary leiomyomatosis) have led to the recognition of genes and proteins involved in the pathogenesis of several tumor entities, and thus of the potential for patient-tailored targeted therapy [35,51]. In particular, inactivation of the von Hippel-Lindau tumor suppressor gene in patients with renal cell carcinoma (RCC) involves the failed degradation of HIF1α signaling, even under normoxia, and therefore the accumulation of this transcription factor, which results

in hyperactivation of the HIF signaling pathway and transcription of the genes encoding its downstream effectors [30,36]. Therefore, the therapeutic use of molecules that inhibit binding to, e.g., VEGF, glucose transporter GLUT1, transforming growth factor-α, and platelet-derived growth factor (PDGF) receptors, has been investigated in many types of tumors [43,44].

Anti-angiogenic strategies

Discovery of the dependence of tumor growth on angiogenesis and the stromal contribution to new vessel formation suggested new therapeutic targets. Targeting of the cells that support tumor growth rather than the cancer cells themselves is a relatively recent approach in cancer therapy, one that is particularly promising because these cells are genetically stable and therefore less likely to accumulate mutations that allow them to rapidly develop drug resistance. In 1971, Judah Folkman proposed the inhibition of angiogenesis as a therapeutic strategy for cancer. Subsequently, several anti-angiogenic drugs aimed at inhibiting endothelial cell growth were developed. More recently, other cell types in the tumor microenvironment, either instead of or together with endothelial cells, have been targeted. These cells include pericytes, which contribute to vascular maturation by releasing signals that maintain endothelial cell survival and structurally support the vessel wall [12] (Fig. 1).

Based on their mechanism of action, anti-angiogenic drugs can be classified in two groups. (1) Direct-acting drugs that prevent vascular endothelial cells from proliferating, migrating, or avoiding cell death in response to a spectrum of pro-angiogenic proteins, including VEGF, bFGF, interleukin-8, and PDGF. In addition to the VEGF-blocking antibody bevacizumab, for use in combination with chemotherapy in patients with metastatic colorectal cancer, metastatic breast cancer, and other cancers, the U.S. FDA has approved chemical inhibitors of VEGF receptor 2 (VEGFR2), such as sunitinib and sorafenib, as first-line monotherapy for metastatic kidney cancer. (2) Indirect-acting drugs that secondarily either prevent the expression or block the activity of tumor proteins that activate angiogenesis. The targets of the latter group are the tumor-cell signaling pathways responsible for the synthesis or secretion of pro-angiogenic molecules. A typical example is mTOR inhibitors, which act on tumor-cell survival pathways and secondarily decrease VEGF expression, thereby indirectly exerting an anti-angiogenic effect.

In this review we discuss direct-acting anti-angiogenic drugs, which for the most part inhibit pro-angiogenic signaling pathways. Consistent with its role as the main promoter of angiogenesis, VEGF is the primary target of the currently approved anti-angiogenic drugs, which include monoclonal antibodies and selective inhibitors of kinase activity [22].
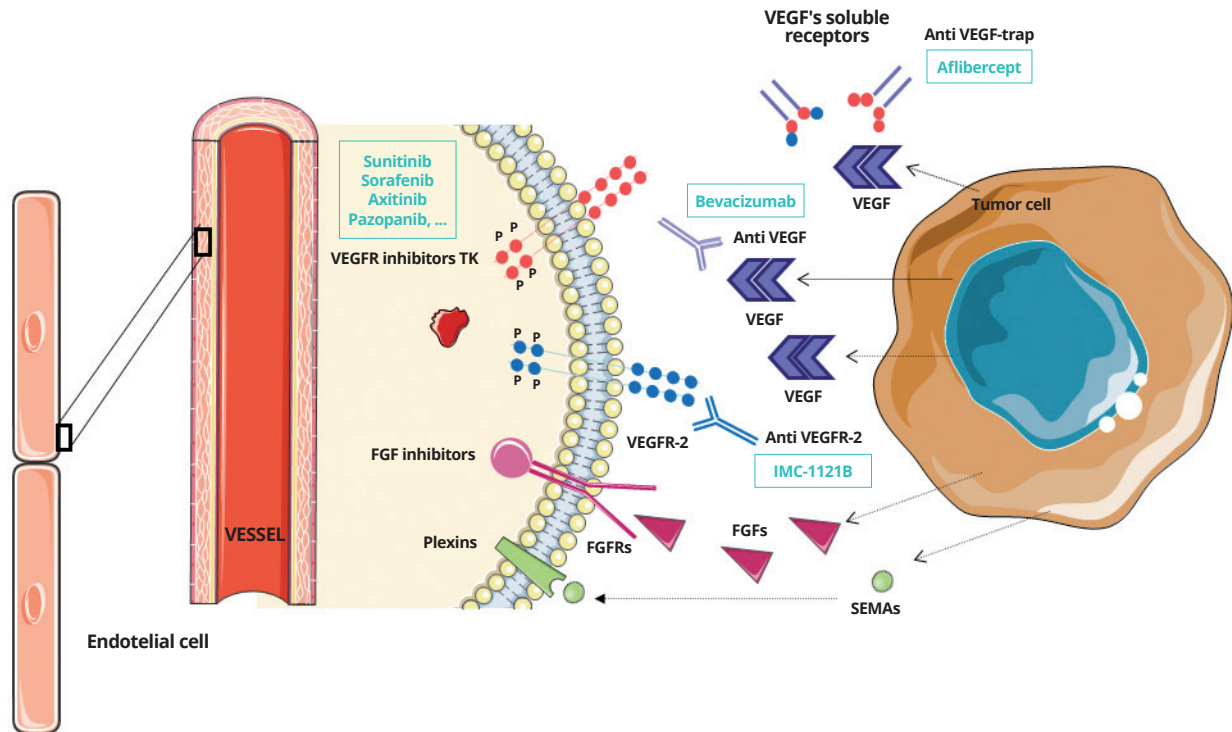
Fig. 1. Therapeutic targets and their angiogenic inhibitors currently used in preclinical and clinical studies. (See text for details). VEGF, vascular endothelial growth factor; anti VEFG-Trap, antiangiogenic VEGF-Trap; VEGFR-2, VEGF receptor-2; FGF, fibroblast growth factor; FGFRs, FGFreceptors; SEMAs, sema-phorins. (See text for details).

VEGF as a prototypical anti-angiogenesis target

Monoclonal antibodies may be direct or indirect acting. Their direct action is to block VEGF signaling by blocking the li-gand or its receptors (VEGFRs) whereas their indirect ac-tion is mediated by the immune system (complement sys-tem activation, cytotoxic lymphocytes, and macrophages), resulting in tumor-cell destruction. Monoclonal antibodies were the first anti-angiogenic drug to demonstrate a clear clinical effect, increasing the survival of patients with meta-static colorectal cancer. The most well known example of these drugs is bevacizumab, an antibody against human VEGF ligand [28] (Fig. 1).

Selective inhibitors of kinase activity compete with ATP for binding to the catalytic domain of the protein, thereby block-ing kinase activity including that of VEGFRs. Initial tests of these drugs as anti-proliferative agents for tumor endothelial cells resulted in the development of a large number of inhibi-tors acting on different pathways and different cell types apart from those expressing VEGFRs (promiscuous tyrosine-kinase inhibition profile). Currently, sunitinib and sorafenib are the most widely used drugs of this class since they dem-onstrate the best anti-angiogenic activity [1] (Fig. 1).

Other recently proposed novel targets

The *fibroblast growth factor/fibroblast growth factor receptor* (FGF/FGFR) signaling axis plays an important role in nor-mal organ, vascular, and skeletal development. The deregu-lation of FGF/FGFR signaling through the genetic modifi-cation or œverexpression of either component has been observed in numerous tumors, consistent with the key role of FGF/FGFR axis in driving tumor angiogenesis. Preclini-cal data showed that the inhibition of FGFR signaling causes antiproliferative and/or pro-apoptotic effects, both in vitro and in vivo, thus confirming the validity of the FGF/FGFR axis as a potential therapeutic target [10]. Ac-cordingly, several drugs against different pro-angiogenic targets have been developed for testing [22].

Semaphorins (SEMAs) are a superfamily of secreted or membrane-associated glycoproteins that have been impli-cated in the control of axonal wiring. They are also known to be involved in angiogenesis and cancer progression. SEMAs positively or negatively modulate many intrinsic properties of tumor cells, such as proliferation, cell survival, cell adhesion, and tumor invasiveness, but they also act on stromal properties, including endothelial cell migration

and survival [11,48]. Thus, the overexpression of Sema3E reduces tumor burden by counteracting angiogenesis, but it also increases metastatic spread of the tumor. Casazza et al explored the pleiotropic therapeutic activities associated with an uncleavable Sema3E isoform (Uncl-Sema3E) [16], which retains the anti-angiogenic activity of endogenous p61-Sema3Ebut also has anti-invasive and anti-metastatic effects on the tumor. Similar to p61-Sema3E, Uncl-Sema3E binds to PlxnD1 in endothelial cells and induces the expected SEMA-driven anti-angiogenic collapse of the tumor. Furthermore, in tumor cells, the Uncl-Sema3E-PlxnD-1complex fails to elicit the ErbB2-mediated pro-invasive and pro-metastatic pathway. With these results the authors proposed Uncl-Sema3E as a novel anti-angiogenic and anti-metastatic therapeutic approach.

Angiopoietins are growth factors that promote angiogenesis and help stabilize the formation of blood vessels from pre-existing ones. Ang1 and Ang2 are required for vessel maturation, as demonstrated by knock-out studies in mice [52]. Moreover, Ang2 is critically associated with tumor angiogenesis and progression, cooperating with VEGF and Ang1 through Tie2-dependent pathways. In addition, Ang2 stimulates tumor angiogenesis, invasion, and metastasis through Tie2-independent pathways involving integrin-mediated signaling. Therefore, Ang2 is also an attractive therapeutic target, as corroborated in recent studies using a neutralizing anti-Ang2 antibody [27].

Clinical results and remaining challenges

Preclinical studies often report positive results regarding the efficacy of anti-VEGF therapy, but the results of clinical trials vary depending on the cancer type and anti-angiogenic therapy used. Phase III studies have indeed shown the benefits of bevacizumab, sunitinib, and other VEGF-targeted therapies, either as single agents or in combination with chemotherapy. Blocking the formation of new blood vessels with anti-angiogenic therapy is currently used to treat certain types of cancers, including metastatic RCC [43,44]. Several clinical trials have confirmed the positive impact of anti-angiogenic therapies in controlling the growth of this typically highly-VEGF secreting tumor [19,39,45,46]. Nevertheless, many authors have found that anti-angiogenic treatments are more effective in in-
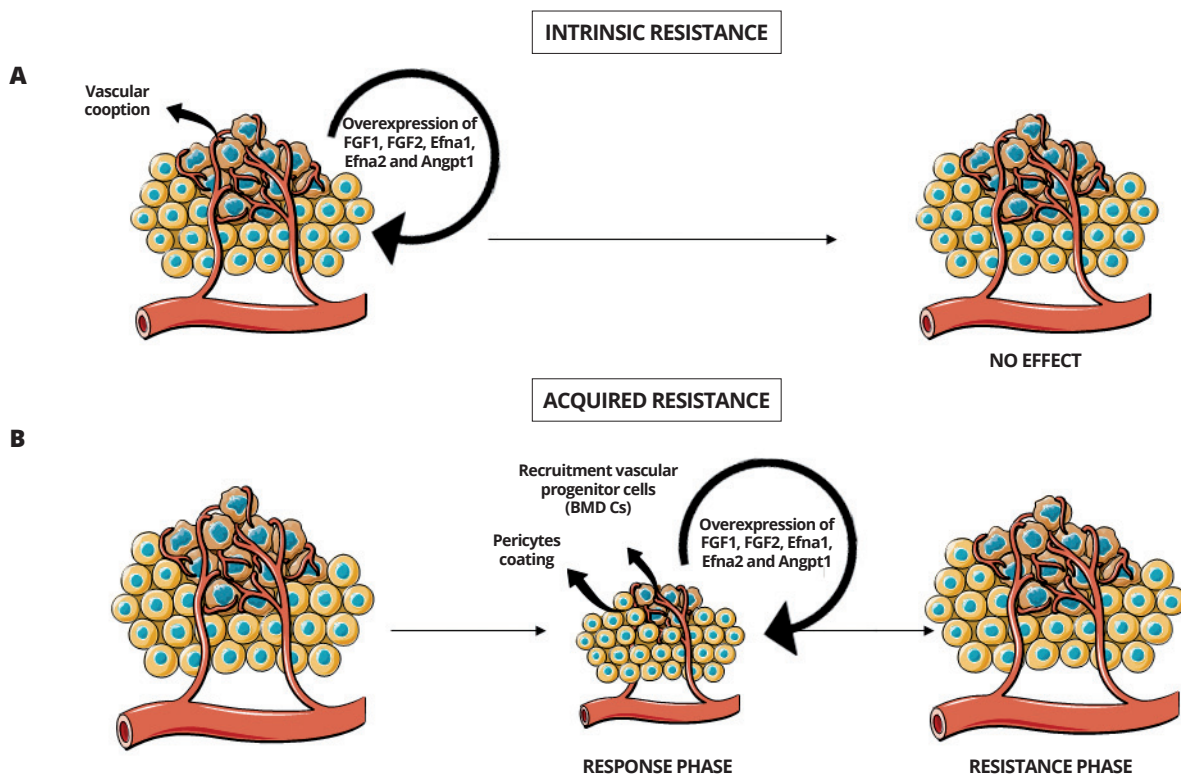
INTRINSIC RESISTANCE

A

Vascular cooption

Overexpression of FGF1, FGF2, Efna1, Efna2 and Angpt1

NO EFFECT

ACQUIRED RESISTANCE

B

Recruitment vascular progenitor cells (BMD Cs)

Pericytes coating

Overexpression of FGF1, FGF2, Efna1, Efna2 and Angpt1

RESPONSE PHASE

RESISTANCE PHASE

Fig. 2. Mechanisms of resistance to anti-angiogenic therapy: (**A**) intrinsic resistance (or refractoriness); (**B**) acquired resistance. Mechanisms of intrinsic resistance include the multiplicity of pro-angiogenic factors produced by tumor or stromal cells within the tumor mass and vascular co-option. Mechanisms of acquired resistance include the overexpression of pro-angiogenic factors, the recruitment of vascular progenitor cells (BMDCs), and an increase in pericyte coverage. Together, they allow for revascularization despite therapeutic inhibition, and thus tumor regrowth and disease progression.

creasing progression-free survival (PFS) than in prolonging overall survival (OS). However, based on the clear clinical benefits, with a remarkable increase in PFS, despite the absence of a robust, statistically significant increase in OS, VEGF pathway inhibitors are the mainstay of therapy in RCC and have been approved by the US Food and Drug Administration (FDA) [20,26].

The discrepancy between PFS and OS has fueled the controversy of how to best measure the clinical benefits of treatment, because the effects of anti-angiogenic therapies typically include increased tumor necrosis, as observed in imaging studies. Thus, cavitation and the loss of viable tumor mass ascribed to anti-VEGF agents may indeed translate into an impact on tumor growth but without significant alteration of the tumor dimensions, as required by RECIST (Response Evaluation Criteria in Solid Tumors) guidelines [18,47].

The pattern of growth and the modifications induced at the site of tumor development are strongly dependent on the tumor type and, in particular, its angiogenic features and the pro-angiogenic capacity arising from tumor-stroma interactions. This is a crucial consideration, because in certain cancers, such as RCC and hepatocellular carcinoma (HCC), single-agent VEGF-targeted therapy has demonstrated significant activity whereas in other tumors, such as colorectal cancer (CRC), considerably fewer clinical benefits have been obtained and VEGF-targeted therapy is therefore administered in combination with chemotherapy. In RCC, angiogenesis is presumably highly VEGF-dependent, in part because of its high frequency of inactivation of the von Hippel-Landau tumor suppressor gene [53]. The same dependence on angiogenesis is presumably the key to the efficacy of anti-angiogenic therapy in HCC. These tumors are highly angiogenic in the liver and their growth displaces the normal parenchyma. This pattern is in contrast to metastatic foci of CRC in the liver, in which case the tumors often replace rather than displace the liver parenchyma, by the FAS-ligand-induced death of hepatocytes. This leads to the co-option of existing blood vessels rather than a dependence on sprouting angiogenesis [9,18].

Resistance to anti-angiogenic therapy

It was initially assumed that anti-angiogenic therapy does not induce resistance, because of its specific targeting of endothelial cells, which do not exhibit genetic instability [8]. However, experimental and clinical evidence has shown that the benefits of anti-angiogenic therapy are mild and transient [44] and that, as in classical chemotherapy and radiation, tumor adaptability is also a challenge [14,15]. Among tumor responses to therapy, it is essential to distinguish between refractoriness, sometimes called *intrinsic resistance* (IR), and *acquired resistance* (AR) [18].

Intrinsic resistance to anti-angiogenic therapy is defined as a total lack of response to the therapy; it is characterized by tumor indifference, as there is no response to treatment (Fig. 2A). IR has been described in patients treated with bevacizumab, sorafenib, and sunitinib, as determined by the continued growth of their tumors [3,34]. Acquired resistance refers to the adaptive capacity of tumors that allows them to evade continued therapeutic inhibition of their growth after an initial phase of effectiveness. In fact, anti-angiogenic drugs achieve clinical efficacy in many patients, but these clinical benefits are overshadowed by an apparent acquired resistance. Moreover, some patients do not respond at all to anti-angiogenic therapy, indicative of intrinsic resistance.

It has been shown that, from the beginning of their progression, tumors are capable of expressing multiple pro-angiogenic factors, which limits the efficacy of anti-VEGF therapy since in these cases angiogenesis is only partially blocked [23]. Another molecular mechanism that may be involved in IR is the de-regulation of the HIF pathway. HIF-activated tumors, such as renal tumors, express high levels of genes encoding pro-angiogenic molecules controlled by this pathway, thereby reducing the effect of anti-angiogenic therapy [43,44]. Other potential mechanisms supporting tumor growth include an independence from angiogenesis, including the co-option of pre-existing vessels, vasculogenic mimicry, mosaic vessels, and the mobilization of latent vessels [50].

Could the specific angiogenic features of each tumor determine their upfront sensitivity or resistance to anti-angiogenic therapy? Interestingly, in astrocytomas, a group of highly oxygen-dependent brain tumors, there is a change in the mechanism by which they acquire their blood supply. Thus, low-grade astrocytomas grow by co-opting pre-existing, normal brain vessels whereas in the progression from grade III to grade IV, in so-called glioblastoma multiforme (GBM), the enhanced demand for oxygen and nutrients activates an angiogenic program [40]. Recently, the FDA approved bevacizumab for the treatment of recurrent GBM based on several studies demonstrating efficacy in terms of increased PFS and OS when the drug is combined with conventional chemotherapy. Unfortunately, tumor resistance occurs with new distant foci of progression or diffuse in situ infiltration associated or not with local tumor recurrence, as shown by fluid-attenuated inversion recovery and magnetic resonance imaging analyses [38,54].

In addition to the traditional forms of resistance to some drugs, which are acquired by mutations that affect the drug target of drugs or entry mechanisms [24], acquired resistance (AR) to anti-angiogenic therapies is both indirect and evasive. Typically, alternative mechanisms are created that lead to the activation of angiogenesis even when the drug target remains inhibited [33]. Tumors have remarkable plasticity

and adaptability to classical chemotherapy and radiation, which contribute to their resistance to anti-angiogenic therapy [6,13,32]. However, the specific mechanisms of AR to anti-angiogenic therapies are unique, and many of them are reversible after anti-angiogenic therapy has been stopped (Paez-Ribes and Casanovas, unpublished observations). This suggests that these forms of resistance reflect adaptations to therapy instead of the accumulation of gene mutations or amplifications that characterizes the AR seen in other therapeutic strategies. Clinical evidence of AR reversibility has been described in metastatic RCC treated repeatedly with VEGFR inhibitors [44].

Several different mechanisms of AR to anti-angiogenic therapy have been described, among which are (Fig. 2):

(i) Overexpression of alternative pro-angiogenic factors. These were initially described in a transgenic mouse model of neuroendocrine tumors (RIP-Tag2). After the mice received anti-VEGFR2 therapy, there was a reduction of angiogenesis followed by initial tumor regrowth and the reinduction of induced angiogenesis. The latter was promoted by the overexpression of VEGF-independent pro-angiogenic factors, such as FGF1, FGF2, ephrin A1 and A2, and Ang1 [15,21].

(ii) Recruitment of stromal pro-angiogenic cells. The hypoxic conditions induced by anti-angiogenic treatment promote the recruitment at the tumor boundaries of large numbers of bone-marrow-derived cells and thus tumor revascularization [49].

(iii) Vessel coverage by pericytes. Pre-existing tumor vessels with a large number of surface coverage by pericytes remain functional and do not regress [5, 29,31,37]. This suggests that endothelial cells are able to recruit pericytes, which then secrete VEGF and other factors promoting their survival [4,5,17].

(iv) Vascular mimicry. Microvascular channels that allow the transport of oxygen and nutrients are formed by the aggressive tumor cells themselves [50].

Although there are some similarities between the mechanisms that lead to IR and AR, there are also several differences. In AR, the molecular changes that lead to tumor resistance develop progressively whereas in IR the tumors are immune to therapy from the beginning, as they overexpress the factors that confer resistance. In anti-angiogenic therapies, resistance involves both the tumor cells and the stromal components, but their relative contributions differ according to each cancer subtype. Thus, the interplay between tumor cells and the tumor microenvironment is crucial not only for the development of the neoplastic lesion but also for the response of the tumor to therapeutic inhibition of the VEGF pathway. Together with the characteristics of the tumor cells, the stroma contributes to both IR and AR. Indeed, many of the tumor-cell-dependent mechanisms of resistance are implemented through stromal modification, e.g., the recruitment of infiltrating cells, such as cancer-associated fibroblasts and tumor-associated macrophages, or the production of alternative pro-angiogenic factors. One of the main modifications induced by anti-angiogenic therapy in tumors is the above-described increase in hypoxia and HIF-1 stabilization. However, neoplastic cells can become tolerant of hypoxia, and thus acquire therapeutic resistance, by modifying their metabolic characteristics. Alternatively, they can escape the hypoxic conditions, either alone or sustained by their stromal neighbors.

Perspectives

Overcoming resistance is a crucial step in the development of anti-angiogenic therapies. Among the strategies proposed thus far is the use of multi-pathway inhibitors. Moreover, given the plasticity of the response to treatment, observed in preclinical studies, a sequential approach in which an anti-angiogenic drug is followed by a non-anti-angiogenic drug (whether another targeted therapy or chemotherapy) may resensitize patients to a third-line anti-angiogenic agent. Obviously, many studies will be needed to identify the therapeutic approach that results in maximum clinical benefit for patients. Furthermore, the role of the stroma, in addition to that of the tumor cells, in the emergence of resistance to anti-angiogenic therapies has important clinical implications and suggests innovative treatment perspectives.

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