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The tumor-endothelium interaction in pioneering studies and the revisited concept on the angiogenesis process during tumor progression and metastasis

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Abstract. The growth of solid tumors and their dissemination require the continuous formation of new capillary blood vessels. However, the association of uncontrolled growth of tumors and angiogenesis, i.e. the mechanism that allows the formation of new blood vessels starting from pre-existing vessels, dates back to 1971, in relation of Judah Folkman's works. Since then, his group and other researchers added new key results confirming the important role played by angiogenesis in tumor growth and metastasis, and multiple efforts have been made to exploit this knowledge in developing innovative anti-cancer therapies. In this article, we discuss seminal works regarding molecular mechanisms involved in aberrant tumor angiogenesis, biology of endothelial cells within extracellular matrix, function of diverse pro- and anti-angiogenic factors, roles of metalloproteinases and pro-tumor effects played by stromal and immune cells in the tumor microenvironment. Interestingly, growing evidences indicate a key role played by inflammatory and stromal cells in both tumor development and progression. The present article also aim to provide up-to-date information concerning new therapeutic concepts involving tumor vessels normalization and anti-angiogenic agents, among which inhibitors of metalloproteinases and of the main angiogenic factor, vascular endothelial growth factor (VEGF) or its receptors, and the combination of them with immune checkpoint blockade, that seem to be the most promising ones at present.

Key words: tumor angiogenesis, biology of endothelial cells, anti-angiogenic agents, tumor vessels normalization.

Introduction

Angiogenesis is defined as the formation of new blood vessels from pre-existing ones, in a tightly orchestrated balance between pro- and anti-angiogenic stimuli. All the contemporary knowledge about tumor angiogenesis is based on the pioneering works of Judah Folkman in the 70s, when he first hypothesized that tumor cells communicate with normal host cells allowing angiogenesis-dependent growth of solid tumors (1, 2).

Folkman proposed that tumors are strictly dependent in their induction and dissemination on the incessant triggering and growth of new blood vessels. He postulated that angiogenesis phenomenon functions as an active physiological feature regulated by biological factors and that tumor angiogenesis is driven by specific molecules released by tumor cells that can be potentially inhibited by new developed pharmacological agents. Such complex multistep process involves not only plasma proteins extravasation and extracellular matrix (ECM) degradation, but also endothelial cell proliferation and migration, as well as capillary tube formation.

Such process is very important in many stages of human life, such as vascular remodeling in the embryo female reproductive cycle or wound healing. In physiological conditions this phenomenon is tightly regulated since in healthy tissues angiogenesis is quiescent, due to the dominant influence of endogenous inhibitors over angiogenic stimuli (3). On the other hand, in pathological conditions (e.g. cancer development)

aberrant angiogenesis occurs disrupting the equilibrium by an increased secretion of pro-angiogenic factors and/or a down-regulation of endogenous anti-angiogenic components (3-6).

Both normal and tumor angiogenesis share some basic characteristics, as both types of new vessels formation involves the migration and invasion of both precursors and mature endothelial cells into the surrounding stroma, requiring the degradation of the existing basement membranes, as well as an active proteolytic remodeling of the resident ECM, mainly performed by a large family of enzymes, collectively named matrix metalloproteinases (MMPs). Basically, new vessels could be generated through two different mechanisms, involving or not sprouting events. In non-sprouting angiogenesis the new vessels derive from the splitting of an existing capillary in two or more tubes following resident endothelial cells proliferation. On the other hand, sprouting angiogenesis involves endothelial cells invasion of the surrounding ECM, where they re-organize to form tubular structures and recruit pericytes (7). Stimuli regulating new vessels formation are quite the same in both physiological and pathological angiogenesis and in both cases, ECM displays a pivotal role as a reservoir of regulatory factors. Among pro-angiogenic factors there are many cytokines (i.e. IL-1, IL-8) and growth factors such as VEGFs, vascular endothelial growth factor a protein family first described at the end of the last century, which earliest identified member was named VEGFA, or VEGF (8), fibroblast growth factors (FGFs), angiopoietin, transforming growth factor β (TGF-β), platelet derived growth factors (PDGFs), epidermal growth factor (EGF) secreted by inflammatory cells (e.g. mast cells and macrophages), pericytes, keratinocytes (during epidermal wound healing) or tumor cells. Both normal and tumor tissues could also produce anti-angiogenic factors (e.g. angiostatin, endostatin, thrombospondins, interferons (IFNs), vasostatin) to modulate new vessels formation locally as well as in distant sites (3, 5, 7, 9).

Therefore here, together with the aim to discuss and to highlight subtle interactions between various and complex signals involved in the initiation and triggering of tumor angiogenesis during tumor progression and metastasis, we also have the purpose to point out the relevance of innovative therapeutic strategies associated to inhibition of this process, such as those involving tumor vessels normalization and combination therapies using anti-angiogenic agents and immune checkpoint blockade, that seem to be the most promising ones at present.

Tumor angiogenesis

Folkman's hypotheses were based on the evidence that in physiological conditions vascular endothelium is a relatively quiescent tissue that can be activated to a rapid proliferation phase by appropriated stimulatory signals (5, 10, 11). According to Folkman's theory about tumor angiogenesis, solid tumor development could be separated into two stages, whose main difference is represented by vascularization.

In 1984 Folkman's group discovered a tumor growth factor named fibroblast growth factor 2, also known as basic fibroblast growth factor, which is endowed with angiogenesis inducing capacity (12). Two years later, in 1986, Harold F. Dvorak published an interesting review, based on his previous experimental studies on vascular permeability and angiogenesis in tumors, discussing the similarities between solid tumor stroma generation and wound healing, defining the solid tumor a wound that does not heal (13). Both stromal tumor and cutaneous wounds are characterized by a fibrin clot, which provides a scaffold for the migration of different biological elements, including new formed blood vessels, macrophages, neutrophils, lymphocytes, fibroblasts and myofibroblasts. Dvorak pointed out that wounds, similar to tumors, secrete a vascular factor and this event induces the release of fibrinogen from the blood vessels, causing blood vessels sprouting and providing a matrix through which they can spread.

This important result was the basis for other successes such as the cloning of the most powerful angiogenic protein, acting as a highly specific mitogen for endothelial cells: the VEGF discovered by Napoleone Ferrara's team (8). This was the same vascular permeability factor (VPF) that Donald R. Senger and colleagues from Harold Dvorak's group, identified in 1983 in culture supernatants of guinea pig tumor cells, then widely known as VEGF (14, 15). VEGF is a potent vascular permeabilizing agent, being effective

within one-two minutes from injection into normal skin or other tissues of experimental animals.
The intensive studies by H.F. Dvorak's group, which led to the evidence that cancer cells secrete VPF/VEGF, showed that its activity was not inhibited by anti-histaminases and other classic inhibitors of vascular permeability (16). Furthermore, unlike wounds, where VEGF production is stopped after healing, in tumors, there is no extinction of its production, and this phenomenon is at the base of the continuous process of tumor vascular neoformation and spread.
In 1990, Noel P. Bouck's team reported the identification of another inhibitor of angiogenesis: the protein thrombospondin-1 (17) and later Folkman's group discovered two important endogen anti-angiogenic factors: angiostatin, a fragment derived from plasminogen, in 1994 (18) and endostatin, derived from type XVIII collagen, in 1997 (19). The idea that endothelial cells may switch from a resting state to a rapid angiogenic growth phase was postulated by Douglas Hanahan and Folkman in 1991 (20) and further detailed by subsequent investigations (21).

Overall, most solid tumors before reaching few millimeters in diameter, seem to be able to survive thanks to oxygen and nutrients derived by simple diffusion, but after exceeding a critical diameter, they need blood supply by new vessels in order to expand (5, 6, 9, 22). To achieve a progressive increase in size, an essential requirement for solid tumor expansion lies is triggering the angiogenic switch through pro-angiogenic stimuli predominance over inhibitory factors. Given that tumors cannot make capillaries on their own, they must recruit them from the host, and it is currently well accepted that tumor blood vessel formation is a complex process involving many stages where the activated endothelial cells sprouting from pre-existing vessels is essential for angiogenesis.

Compared to normal vessels, new endothelial structures demonstrate a great functional as well as anatomical heterogeneity, appearing to be immature, irregular in shape and branching, with little and fragmented basement membrane and fewer intracellular junctions, making them highly permeable, allowing tumor cells to easily enter in the blood flow and metastasize in even distant regions.

Moreover, it is well accepted that there is a continuous crosstalk between the tumor and its microenvironment, including innate immune and stromal cells, such as tumor-associated macrophages natural killer (NK) cells and other cellular components (Figure 1) (23-26). Growing evidences suggest a crucial role played by inflammatory cells within the tumor microenvironment (TME) for both tumor development and progression (27). Among the host features representing tumor hallmarks (28), there are evading immune destruction and tumor-promoting inflammation, which, together with the immune cell-based induction of angiogenesis, underline the fundamental impact of innate immune cells in cancer (23, 25).

Among immune cells, NK cells are effector lymphocytes involved in tumor immunosurveillance upon interaction with tumor cells, and though they can control tumor growth by their cytotoxic activity (29) they can also acquire altered functions, ranging from attenuation of their killing activity, to tolerogenic behavior and acquisition of pro-angiogenic activities (30-33). Two main cell subtyes of peripheral blood NK cells have been identified in humans: the $CD56^{dim}CD16^{+}$ and the $CD56^{bright}CD16^{-}$ NK cell subset, representing about 90–95% of NK cells and 5–10% of peripheral blood NK cells, respectively. The TME-dependent unfavorable feature of NK cells depends on the expansion and function modifications of the $CD56^{bright}CD16^{-}$ NK cell subset. However, the $CD56^{bright}CD16^{-}$ NK cells, being

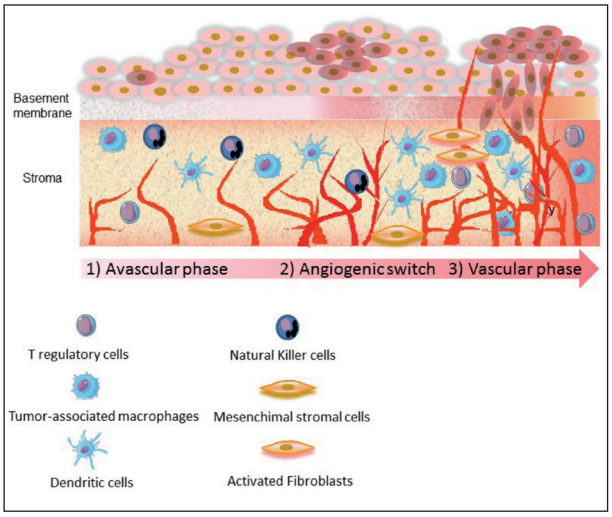

Figure 1. Tumorigenesis and invasion strongly depend on angiogenesis.

poorly cytotoxic, can release several cytokines, including IFNγ, GM-CSF, and TNFα and this latter could exert a potent stimulatory effect on endothelial cells, resulting also in normalization of tumor blood vessels, as well as on activation of innate and adaptive immune responses, reverting the microenvironment anergy (34). Targeting TNFα to tumor vessels in combination with chemotherapy is an interesting novel anti-tumor strategy (35). For these reasons, NK cells could become a suitable therapeutic target to modulate the immunosuppressive and pro-angiogenic TME and possibly become powerful cytotoxic anti-tumor effectors (33, 36). Furthermore, from a therapeutic point of view, the emerging concept of normalization of tumor blood vessels, introduced by Rakesh K. Jain in 2001, in particular for combined anti-angiogenic therapies (37) is of great importance. Indeed, tumor-associated vasculature consist of an abnormal leaky and immature irregular vessel network in close contact with cancer cells, fibroblasts and inflammatory immune cells and abundant ECM molecules such as collagen and hyaluronan. Tumor vessels normalization consists of vessel with a mature phenotype, fortified with perivascular cell coverage, and a more organized and uniform distribution of the vasculature throughout the tumor tissue. One useful approach to reach vascular normalization is the inhibition of VEGF or its receptors through anti-angiogenic agents, such as bevacizumab, and of note, the resulting normalized vessel structure induced intra-tumor high endothelial venules and reinforced tumor perfusion, thus favoring more homogeneous delivery of drugs, oxygen, and enhanced immune cells infiltration and in particular antitumor cytotoxic T lymphocytes (38-40). However, it has also been reported that a high and too prolonged infusion of anti-angiogenic drugs can definitely reach an opposite effect, thereby inducing hypoxia, immunosuppression, tumor progression, and treatment resistance (41). Moreover, the tight interconnection between tumor angiogenesis and metastatic potential is recognized as a prognostic indicator, as increased angiogenesis correlates with worse prognosis in different types of human cancers, among which gastric cancers (5), non-small cell lung cancers (NSCLC) (6), melanoma (42), and renal cell carcinoma (RCC) (43).

Folkman defined the concept of tumor dormancy, the condition related to a steady state in which competent transformed tumor cells do not develop into a clinically detectable cancer (44) until endothelial cells of host vessels are activated from their physiologic latent status to a rapid growing status by soluble molecules released by tumors or by signals delivered by tumor-conditioned innate immune cells (Figure 1). This is probably due to the absence of stimulatory signals, or to inhibitory mechanisms or a combination of these. This phenomenon appears to be present in the early stages of primary cancers, or to the remains of primary tumors as is the case for undetectable disease recurrences and micrometastases that could subsequently reactivate after a latency period and evolve in a clinically detectable disease (45-47).

Although tumor dissemination and metastases strictly depend on neoangiogenesis in response to tumor-mediated release of stimulating factors from the surrounding ECM (e.g. FGF2, VEGF, IL-8), it has to be highlighted that tumor cells are able to escape from the primary neoplasm, to invade blood and lymphatic vessels, but the growth of a new cancer lesion represents only a very small subpopulation of those cells forming the primary bulk tumor (7, 22).

Pro- and anti-angiogenic factors

Tumor angiogenesis depends on pro-angiogenic stimuli produced by both tumor cells and immune cells present in the surrounding microenvironment, such as macrophages, mast cells and lymphocytes attracted to the tumor site. Among the most important pro-angiogenic factors involved in tumor metastases there are many growth factors, such as VEGF, placental growth factor, FGF, PDGF, and interleukins, such as IL-1, and IL-8, without forgetting ECM degrading enzymes. Many of these pro-angiogenic factors act directly, typically VEGF and angiopoietins, while others show an indirect action as FGF, PDGF and ILs. As stated before, tumor angiogenesis also involves a disregulation of the normally occurring anti-angiogenetic equilibrium that normally occurs in physiological state. Among the natural inhibitors of angiogenesis, the most important are trombospondin-1 and -2, IFNs, angiostatin, endostatin, vasostatin (3, 5, 9, 18, 19).

Among tumor promoting agents, the plays a pivotal role. VEGF is a homodimeric 40-45 kDa heparin-binding glycoprotein, acting as endothelial specific mitogen which exerts pro-survival and anti-apoptotic activities. VEGF represents the major promoting factor of tumor angiogenesis since it facilitates tumor growth, dissemination and metastasis, as demonstrated by its overexpression, along with VEGF receptor, in the majority of tumor cells and in tumor-associated blood vessels (4). Interestingly, VEGF could also induce fenestrations in small vessels even in tissues where micro-vascularization is not normally fenestrated, thus accounting, at least in part, for the high permeability of tumor vessels (3, 5, 22).

Furthermore, a key anti-angiogenic role is played by IFNs. IFNs activity is critical in angiogenesis as they inhibit capillary endothelial cells migration by blocking both the production and the efficacy of tumor pro-angiogenic factors (9).

The recombinant humanized anti-VEGF monoclonal antibody bevacizumab (Avastin), developed by N. Ferrara and colleagues (Genentech, San Francisco Inc.), recognizes all VEGF isoforms and blocks binding to the VEGF receptor (48). This prototypical anti-angiogenic agent has been used in eleven important trials and more than two million patients affected by advanced solid tumors, comprising metastatic colorectal, non-small cell lung, ovarian, renal and cervical cancers. Phase III clinical trials demonstrated a significant advantage in objective response rate (ORR), overall survival (OS) or progression-free survival (PFS) in patients treated with bevacizumab in combination with chemotherapy (41-43). In contrast, its use in metastatic breast cancer was discontinued due to the lack of efficacy and a low safety profile. Disappointedly, resistance to bevacizumab is apparently acquired by angiogenesis inhibition itself, which exacerbates the tumor hypoxic microenvironment, with consequent stabilization of the hypoxia inducible factor 1 and 2 (HIF-1, HIF-2) and HIF-dependent genes (41). This condition, in turn, leads to the activation of a compensatory pro-angiogenic program, which represents a critical issue, still requiring further investigations. In this context, stromal and immune cells, which play a crucial role in supporting tumor dysmorphic neo vascularization by unbalanced release of growth factors and cytokines with pro-angiogenic activity (FGF, PDGF), deserve great attention as potential targets of therapy (43).

Tumor angiogenesis and extracellular matrix remodeling: a potential therapeutic target

Tumor invasiveness relies both on active cell migration and the ability to degrade to a limited extent the surrounding ECM in order to achieve tumor invasion. ECM degradation is mainly accomplished by MMPs, a wide family of Zn^{++} and Ca^{++} dependent proteases, working at neutral pH. MMPs are present in approximately all human cancers, as they can be produced by both surrounding stromal cells and tumor cells: in the last case, enzymes are generally sequestered on the cell surface and concentrated at the leading edge of tumor migrating cells. Due to their ubiquitous presence in the tumor environment, they could affect tumor spread in many different ways (e.g. by promoting tumor angiogenesis of metastases dissemination). However, the ECM degrading activity of MMPs is counterbalanced by a naturally occurring family of inhibitors called TIMPs (tissue inhibitors of metalloproteinases), which are able to inhibit angiogenesis as well as tumor growth and metastasis (7, 9, 49-54).

MMPs, as an enzyme family, are known to directly influence the angiogenetic process by either degrading the basement membrane by the direct cleavage of matrix components, or by cross-activating each other, thus allowing endothelial cells invasion, or by cleaving pro-angiogenic factors (e.g. cytokines as well as growth factors) in order to maintain the angiogenic phenotype. Among all the MMPs' family members, MMP-2 and MMP-9 (known as gelatinases) play a pivotal role in driving angiogenic processes both in physiological and pathological conditions by cleaving basement membrane components as well as through the modulation of angiogenic regulators such as IL-8, platelet factor 4 (MMP-9) and FGF receptor 1 (MMP-2). The first evidence of the role of ECM degradation in tumor dissemination dates back to early 1980s, when L. Liotta and coworkers recognized the involvement of basement membrane degradation in tumor metastasis. Liotta and colleagues' studies resulted in the identifica-

tion of MMP-2, a degrading enzyme of type IV collagen, the major component of the basement membrane (51, 52). Following such studies, the MMPs family rapidly expanded to include more than 20 different enzymes, many of which were first identified by their overexpression in tumor cells. Scientists' understanding of tumor environment remodeling rapidly grown up and actually it is known that gelatinases are not the only MMPs involved in tumor angiogenesis, but also MMP-1 and MMP-14 (also known as MT-MMP-1) play a role (7, 53, 54). As ECM degradation is strictly associated with tumor progression as well as neovasculature spreading, many studies focused on MMPs inhibition with the aim of blocking tumor dissemination. Considering that MMPs and TIMPs expression in physiological conditions and in the TME is different and undergoes specific regulatory patterns, starting from 1990s nearly every pharmaceutical company developed its MMPs inhibition research program. For these reasons both *in vitro* and *in vivo* studies focusing on MMPs inhibition using natural (TIMPs) and synthetic compounds to block tumor dissemination, began and even reached the clinical trial stage (53-55).

The first MMPs inhibitor to be developed and clinically tested was batimastat, a broad spectrum injectable competitive peptidomimentic drug. Such compound was an efficient inhibitor of the main MMPs involved in sustaining tumor angiogenesis but, due to its poor solubility and very low oral bioavailability, along with a high toxicity profile with severe systemic side effects, its development was stopped in phase III clinical trial. Researchers thus developed a new and more bioavailable analogue, marimastat (53-55). Marimastat is known to act as a potent MMPs inhibitor acting as a competitive inhibitor that mimics enzymes' substrate. Even if such compound is a strong tumor angiogenesis inhibitor, its low cytotoxicity is not sufficient to efficiently suppress tumor cells growth and proliferation. Also in this case, clinical trials did not revealed a significant improvement in patients' OS and considering the severe systemic side effects (mainly represented by musculoskeletal pain and inflammation) its development was discontinued (53-55). In the attempt to overcome the adverse side effects linked to peptidomimetic drugs, pharmaceutical research pointed to the development of MMPs inhibitors based on small chemical molecules. The first product of this new research branch was CGS27023A (Novartis®) a chemical inhibitor specifically targeting gelatinases and acting as a Zn^{++} chelating compound. Such new drug showed a great potential in reducing tumor angiogenesis, but due to its low anti-proliferative effects along with the poor tolerability, its development was abandoned (53). Prinomastat was then developed as an optimized version of CGS27023A and entered clinical trials as anti-angiogenic drug. Unfortunately, also in this case, the ongoing phase III clinical trials were withdrawn before completion due to the lack of efficacy in patients with advanced disease (53). To date there is only one approved drug inhibiting MMPs: Periostat, a chemically modified doxycycline approved for periodontal diseases. Such drug inhibits MMPs by chelating their structural cations, thus showing an additional way of action unrelated to its well-known antimicrobial power (7, 53, 54).

Even if Big Pharma interests in developing MMPs inhibitors to be used as powerful cancer therapeutics rapidly fall down, the knowledge about ECM remodeling and MMPs role in sustaining tumor angiogenesis continues to accumulate. Nowadays, it is well accepted that MMPs still represent an interesting target for anti-cancer drugs development. In the light of the currently available scientific knowledge, it is clear that the above-mentioned clinical trials display a great drawback: they enrolled patients with cancers at different stages and were designed to evaluate OS. Currently, it is generally recognized that gelatinases play a pivotal role in the angiogenic switch at early stages after tumor neovascularization: in light of these considerations, these studies might have been more successful if they were conducted with patients with early stage cancers, or to test their efficacy as preventive agents for patients undergoing surgical resection of primary tumors. According to the latest information available, MMP inhibitors development focuses on the design of highly potent and selective compounds and/or on innovative delivery systems assuring preferential drug accumulation in the TME in order to overcome severe systemic side effects (7, 51). Moreover, the anti-angiogenesis drugs can be combined with immunotherapies, in particular the combination with immune checkpoint blockades (ICB), consisting of monoclonal

antibodies directed to PD-1, PD-L1 and CTLA-4, aimed at the blockade of inhibitory pathways on tumor-infiltrating lymphocytes (56). Indeed, targeting the tumor vessel compartment for example, could lead to local endothelial cell triggering that can increase the T-cell homing necessary in the involvement of anti-tumor T effector cells (57).

Combining tumor anti-angiogenic agents and immune checkpoint blockade

The use of the combination of anti-angiogenic drugs and ICBs as initial hypothesis concept has become a solid rationale for many new clinical tests currently underway, in particular for advanced melanoma, NSCLC and RCC (58). In a phase III trial the combination of an anti-PD-1 antibody (pembrolizumab) with a tyrosine kinase inhibitor of VEGF receptors (axitinib) resulted in improved OS, PFS and OS rates in comparison to the standard of care of patients with advanced or metastatic RCC (59). Other studies showed synergistic effects between bevacizumab and ICB treatment by enhancing antitumor immune activation in the TME as well as systemically in both RCC metastatic patients in combination with anti-PD-L1 antibody (atezolizumab) in a phase Ib trial, and in melanoma patients in combination with anti-CTLA-4 antibody (ipilimumab) in a phase I trial (60,61).

Tumor progression is a multi-step process in which developing tumors incorporate a series of genetic and molecular alterations, up to reach about 1-2 mm in diameter, until they switch to the angiogenic phenotype. The angiogenesis phenomenon is responsible for a faster tumor progression and invasion and is carried out by tumor cells, activated fibroblasts, tumor-associated macrophages and NK cells. This complex process consists of numerous interactions between tumor, endothelial, stromal and inflammatory cells, with also important effects played by various soluble pro-angiogenic factors, among which in particular different types of MMPs that are involved in the initial phase of degradation of basement membrane of the ECM and in the regulation of the angiogenic process.

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