

Development of the Relationship between Angiogenesis and Tumor Dormancy

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ABSTRACT Tumor dormancy, a complex and still poorly understood phenomenon, has been defined by the long-term persistence of occult cancer cells during tumor progression. Recurrence and metastasis may occur just because of an activation of a small portion of the tumor cells. In our view, sustained angiogenesis is considered essential in triggering invasive tumor growth. Here we analyze the correlation between angiogenesis and tumor dormancy, the establishment of tumor dormancy models, the imaging strategies and the new biomarkers for detecting microscopic tumors before or during the angiogenic switch. It is imperative to understand the role of angiogenesis in tumor dormancy, as this will accelerate the development of anti-angiogenesis techniques to induce dormancy and/or eradicate dormant disease.

KEYWORDS: tumor dormancy, angiogenic switch, tumor dormancy therapy.

Tumor progression depends on sequential events, including genetic, epigenetic alterations and an angiogenic switch. For a tumor to develop a highly malignant and lethal phenotype, it must first recruit and sustain its own blood supply, a process called "tumor angiogenesis". It is well established that tumor growth beyond the size of 1~2 mm is angiogenesis-dependent. Clinical and experimental evidence suggests that failure of a switch to the angiogenic phenotype may lead to tumors persisting in a "quiescent state" for long periods as solitary tumor cells, or limited in size to less than 1 mm in diameter, which is called "tumor dormancy". Recurrence and metastasis may occur just because of a small portion of the tumor cells. An angiogenic switch often marks interruption of a dormant state, thus triggering invasive tumor growth. It is imperative to understand the role of angiogenesis in tumor dormancy, as this will accelerate the development of anti-angiogenesis therapy to induce dormancy and/or eradicate dormant disease.

The conception of angiogenesis and tumor dormancy

Tumor dormancy has been proposed to exist either at the solitary cellular level, where tumor cells enter a growth arrest or as small masses of tumor cells that are unable to grow beyond a certain size. These concepts have been developed as follows: (1) the dormancy of solitary tumor cells that enter a growth arrest. Dormant tumor cells have been found to be in a G0/G1 arrest which was linked to be associated with negative staining for proliferation markers (e.g. PCNA); (2) at the population level, microscopic

tumors were not able to grow beyond a threshold size due to a balance between proliferative and apoptotic rates, and therefore the cells are not quiescent. In human tumor dormancy models, tumor cell proliferation in these tumors was approximately 12%, and tumor cell apoptosis varied between 4% and 7.5%.

In detail, dormant tumors are defined as^[1]: (1) remain harmless to the host until they switch to an angiogenic phenotype to induce angiogenic activity; (2) express equal or more anti-angiogenic compared to angiogenic factors; (3) grow to approximately 1mm in diameter or less in vivo, at which time further expansion ceases—dormant tumors are only visible by a hand lens or dissecting microscope (5~10 × magnification) or white/transparent by gross examination; (4) never metastasize spontaneously from the microscopic dormant state; (5) show active tumor cell proliferation in mice, and remain metabolically active during the dormancy period; and (6) non-angiogenic tumor cells can be cloned from a human angiogenic tumor, because human tumors are heterogeneous and contain mixture of non-angiogenic and angiogenic tumor cells.

Angiogenic switch and tumor dormancy

Angiogenic switch

Folkman described tumor growth as depending on an “angiogenic switch”. Tumor progression can be divided into two phases based on angiogenesis: (1) an avascular phase, with limited oxygen and nutrition leading to a tumor “quiescent state”; (2) a vascular phase of angiogenesis leading to tumor growth burst and metastasis. The angiogenic switch interrupts the dormant state, thus triggering invasive tumor growth. The switch is on related to these events, (1) there are more angiogenic factors than anti-angiogenic factors triggered by various signals including genetic, mutations, hypoxia and other metabolic stresses, and an immune/inflammatory response; (2) there is an increase of angiogenic factor expression by stromal cells (such as fibroblasts), that is induced by the tumor cells; (3) there is a decrease of anti-angiogenic factor expression by the tumor cells and fibroblasts; and (4) some tumors can recruit an endothelial progenitor cell like substance. New blood vessels provide adequate perfusion supports, and deliver an activation signal to the tumor cells, which can foster their proliferation or survival in vivo^[2].

Angiogenic spike hypothesis

Indraccolo et al.^[2] hypothesized that an induction threshold had to be overcome to start the process of neoangiogenesis in the tumor microenvironment.

This threshold may be physically represented by the amount of angiogenic factors required to turn quiescent endothelial cells into proliferating cells, which is conceivably higher than levels required to keep them proliferating, which defined the maintenance threshold. In this regard, different scenarios can be predicted. In some instances, tumor cells may lack angiogenic potential, and remain dormant. In the large majority of cancers, however, the induction threshold is overcome by the sustained production of angiogenic factors by the tumor cells themselves, and progressive tumor growth occurs. Alternatively, cancer cells may lack sufficient angiogenic potential, and a short-term exogenous “spike” of angiogenic factors within the tumor microenvironment could suffice to start the process of angiogenesis. Although both a transient and a stable angiogenic switch are capable of triggering tumor growth, in the latter case lesions may be larger in size compared with those grown in the presence of low levels of angiogenic factors, as also observed in their dormancy model. Indraccolo et al.^[2] also speculated that there was a role for inflammation in the promotion of escape from tumor dormancy.

Angiogenic related factors and tumor dormancy

There are four main steps of angiogenesis: activation, proliferation and migration of vascular endothelial cells, and finally, formation of the vascular lumen. It is a complex process modulated by a balance between angiogenic factors and anti-angiogenic factors. Angiogenic factors include VEGF, FGF, TGF and PDGF etc., and anti-angiogenic factors such as TBS, angiostatin and endostatin. The expression of angiogenic factors and anti-angiogenic factors can be triggered by various signals including genetic, mutations, hypoxia and other metabolic stresses, and an immune/inflammatory response. Angiostatin and endostatin are endogenous angiogenic inhibitors, which selectively target at endothelial cell proliferation and migration^[3]. Dormant tumor cells which express a high level of TSP-1 and endostatin inhibited the release of circulation endothelial progenitor cells^[1,4].

Animal models of angiogenic switch and tumor dormancy

The establishment of animal models is not only a way to study the mechanism of tumor dormancy, but also an evidence of the existence of dormancy. Folkman developed the “angiogenic switch” concept in a transgenic mouse tumor dormancy model. Indraccolo et al.^[5] recently reported that poorly angiogenic human T acute lymphoblastic leukemia cells (MOLT-3) failed to form tumors in NOD/SCID mice. Microscopic or

small, dormant tumors containing viable cancer cells were found to persist *in vivo* in about 40% of the inoculation sites. They found that providing a local temporarily-limited angiogenic burst would interrupt the state of MOLT-3 cell dormancy, and sufficed to allow progressive tumor growth, which is conceivably lower than levels required to trigger their proliferation.

Naumov et al.^[1] reported on a study of SCID mice that were inoculated with 15 human “non-tumorigenic” tumor cell lines from the American Type Culture Collection (ATCC). Seven of the test human tumors did not form palpable or microscopic tumors for more than 500 days, and in some instances, for the life of the animal. Such cell lines were truly non-tumorigenic. However, half of the tested human tumor cell lines spontaneously formed palpable tumors after a dormancy period that varied from months to more than a year, depending on the cancer type. Once palpable, these tumors expanded in mass, becoming angiogenic and lethal.

Method for detection of tumor dormancy

Three non-invasive and reliable methods can be employed to detect microscopic tumors: stable staining of tumor cells with green fluorescent protein (GFP), red fluorescent protein (RFP), or luciferase.

Fluorescent protein labeling of tumor cells

Fluorescent protein-expressing (such as GFP, RFP) tumors can be easily localized even if they contain only a few tumor cells. Moreover, new blood vessels appear dark on the background of a fluorescent tumor, allowing for tumor associated vascular visualization and quantification. Udagawa et al.^[6] investigated a small number of dormant tumor cells at an avascular phase using GFP-labeled tumor cells in an osteosarcoma (MG-63 and SAOS-2) and gastric (ST-2) dormancy models. Fluorescent-labeled human tumor cells can be easily observed non-invasively in superficial organs (such as the skin and mammary fat pad). However, if tumor growth occurs in internal organs, such observations are difficult and in most cases impossible to detect. The only way to visualize internal GFP-labeled tumors (especially at the microscopic size) is to kill and dissect the animal.

Luciferase labeling of dormant tumor cells

Internal organ and brain tumors can be reliably detected *in vivo* using a luciferase reporter gene. This

imaging modality can be used for non-invasive, reliable and sequential detection of microscopic tumors in mice, even if the tumor is smaller than 1 mm in diameter^[7]. Although the luciferase signal intensity is directly correlated with the tumor size, this technique also has two disadvantages: (1) imaging modality does not provide a clear tumor boundary or a definite anatomical location of the geometry of a microscopic tumor; (2) the enzymatic activity of luciferase is rapid and transient, and can be detected only following intravenous injection of the substrate, and only viable and metabolically active tumor cells can be detected. Therefore, this method allows for real-time monitoring of tumor cell presence and viability during the dormancy period as well as throughout the angiogenic switch. The presence of a luciferase signal during the dormancy period of microscopic human tumors confirms that dormancy does not result from tumor cell cycle arrest or eradication.

High frequency three-dimensional ultrasonic and small animal MRI imaging can also be used to detect microscopic tumors and solitary tumor cells in the circulation, and also be used in combination with luciferase to provide non-invasive, reliable and sequential detection of the growth of solitary tumor cells in an avascular phase^[8].

Separation of angiogenic and non-angiogenic tumor cells

Tumors are heterogeneous according to their angiogenic ability, containing both non-angiogenic and angiogenic tumor cell populations, which can be separated *in vitro* or *in vivo*. Achilles et al.^[9] demonstrated that “non-tumorigenic” tumor cell lines from the ATCC can be divided into three kinds according to their angiogenic character: (1) highly angiogenic tumors; (2) weakly angiogenic or slow growing tumors, and (3) non-angiogenic tumors. The single cell clones were separated according to the highest and lowest proliferation *in vitro*. It was found that the single cell-derived clones with the highest proliferation rate were very angiogenic *in vivo* and formed large tumors within a relatively short time. In contrast, the clones with the lowest *in vitro* proliferation rate remained microscopic in size for a much longer time. After an angiogenic switch, spontaneous tumors formed.

Naumov et al.^[10] isolated non-angiogenic and angiogenic human tumor cell populations that did not differ in proliferation rates, and were not molecularly or genetically modified. Udagawa et al.^[6] also reported that stable transfected dormant human tumor cell lines with either VEGF165 or activated c-Ha-ras induced loss of dormancy, thus providing evidence for escape from the angiogenic phenotype.

Angiogenic switch-related biomarkers for detection of tumor dormancy

Even with recent advances in clinical detection of human cancer, a tumor that is microscopic in size (<1mm in diameter) remains undetectable. A panel of angiogenic switch-related biomarkers has been developed using human tumor animal models. These biomarkers^[1] include circulatory endothelial cells (CECs), circulatory endothelial progenitor cells (CEPs) and blood platelets, as well as matrix metalloproteinases (MMPs) in the urine. The detection of a single microscopic human tumor in existing animal models can be achieved using each one of these biomarkers alone or as a panel^[1,4]. Using flow cytometry and an angiogenic proteome, the accumulation and reduction in angiogenic-related proteins can be quantitatively followed throughout the angiogenic switch, and can detect the presence of microscopic human tumors in mice^[1]. These angiogenic-based biomarkers, used as tumor predictor, are biomarkers currently under investigation.

Moreover, these angiogenic-related biomarker panels may prove to be a useful diagnostic method for detecting microscopic cancers at primary and metastatic sites long before their discovery by conventional methods. These assays may be feasible for a patient who is at risk for a cancer recurrence, and possible for guiding anti-angiogenic therapy.

Tumor dormancy therapy

There is abundant evidence demonstrating that the induction and maintenance of a dormant state will prolong tumor inactivity and progression, and thus improve the quality of life and longevity. This is also called "tumor dormancy therapy (TDT)". Although TDT can not be as effective as surgery in early stage patients, it is a feasible therapeutic choice for an advanced or recurrent tumor, especially for older patients. Angiogenesis is vital for the overall process of proliferation, metastasis, and recurrence, and is therefore a prime target for TDT development.

There is substantial evidence from pre-clinical studies that anti-angiogenic therapy using different approaches induces tumor dormancy^[3,11]. The first approach is to block the effect of angiogenic factors involved in tumor cell angiogenesis, such as inhibitors of VEGF receptors PTK 787^[12], Avastin^[13], and Vandetanib(ZD6474; ZACTIMA, AstraZeneca)^[14] etc. A second approach of antiangiogenic therapy is to use drugs that directly inhibit the proliferation of endothelial cells, such as TNP-470(AGM-1470)^[12], and MMPs^[15] etc. Endostatin is an endogenous angiogenic inhibitor. In vitro and in vivo, endostatin

specifically^[3] (1) inhibited the proliferation and migration of capillary endothelial cells, (2) inhibited the growth of a wide variety of human and primary and metastatic tumors, (3) could induce apoptosis of proliferating endothelial cells, and (4) induced a virtually complete blockade of tumor angiogenesis and caused established tumors to regress to microscopic lesions.

Conclusions

An angiogenic switch plays an important role in the aggressive biological behavior of tumor progression. Understanding the mechanisms that induce dormancy would allow development at least two strategies to improve treatment: (1) there are therapies aimed at inducing dormancy which may help overcome conventional drug resistance. These therapies are based on the ability of drugs to inhibit angiogenesis; (2) reprogramming malignant cells into a growth arrest would allow converting the disease that would be otherwise untreatable, into a chronic asymptomatic condition. Although there has been encouraging results from animal models and pre-clinical studies, when it comes to clinical use the results have not met our expectations. There are still so many questions to be answered: (1) is tumor dormancy a completely quiescent state or a balance between proliferation and apoptosis, or a mixture of both of them? (2) further studies are needed to elucidate the mechanisms involved in regulation of the angiogenic switch so it may be turned off; (3) there is a relative deficiency in the methods to detect the angiogenic switch; (4) there are insufficient tumor dormancy models; (5) the ongoing clinical anti-angiogenic patient trials still have not shown definite information regarding effective dosage, period of treatment, and most importance, the outcome.

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