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Review

What Do Oncogenic Mutations Have To Do with Angiogenesis/Vascular Dependence of Tumors?[1]

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Subversion of normal physiological functions of certain host cells and tissues is currently regarded as a hallmark of cancer (1). Indeed, host endothelial cells are believed to play a central role in tumor growth, progression and metastasis, acting as main building blocks of the tumor microvasculature (2). Experimental evidence uniformly points to the fact that tumor growth cannot proceed without access to, and recruitment of host blood vessels, a process broadly referred to as tumor angiogenesis (2). Nearly 30 years ago, Folkman predicted that because of this "angiogenesis dependence" of solid tumors (now extended also to leukemias and lymphomas), selective inhibition or destruction of the tumor vasculature (using antiangiogenic or antivascular treatment modalities, respectively) could trigger tumor growth inhibition, regression and/or, a state of dormancy and thereby offer a fundamentally new (host cell directed) approach to cancer treatment (3, 4). To date, preclinical studies with endostatin, angiostatin, VEGF[3] antagonists, and many other new generation angiogenesis inhibitors have convincingly validated the guiding principles of this concept (2, 3).

Unlike traditional cytoreductive anticancer therapeutics, antiangiogenic agents target activated but genetically stable endothelial cells. Therefore, it was originally felt that such host cell-directed anticancer therapy would not be significantly affected by the consequences of genetic instability of cancer cells themselves and tumor progression in general (4–7). It could be reasoned that no matter how malignant tumor cells became during the course of the disease, they would always require access to blood vessels as a source of growth factors, oxygen, and nutrients to maintain their metabolic activity and survival. In this sense, chronic administration of angiogenesis inhibitors would not be expected to lead to "drug resistance" (4–6), regardless of the type of genetic lesions (*e.g.*, mutator phenotype, inactivated tumor suppressor genes, or activated oncogenes) cancer cells may carry.

Angiogenesis Inhibition in Cancer: Hopes and New Questions. The notion of absolute and unconditional tumor "angiogenesis/vascular dependence," supported by ample preclinical evidence (2), fueled great optimism and high expectations for therapeutic efficacy of the prospective tumor angiogenesis antagonists, many of which have entered clinical trials recently.[4]

There is no doubt that, both as a group and individually, these agents (directed against the endothelial cell compartment) have shown considerable promise, as compared with various cytoreductive modalities developed to target the tumor cells directly, *e.g.*, at least some of the leading antiangiogenic agents (*e.g.*, endostatin) appear to be extremely well tolerated and able to induce tumor blood flow perturbations (8). Despite these encouraging properties, significant overall clinical responses in Phase I trials were (not unexpectedly) relatively rare. Notably, great variations in response have been observed between tumor types, patients, and tumor sites in the same patient (8). It is possible that extended observation and a larger and/or more uniform patient base may eventually reveal a more consistent pattern.

Alternatively, results such as these may suggest that our understanding of the interaction between cancer cells, their associated vasculature, and antiangiogenic agents administered to cancer patients may be far from complete. It is conceivable that, just as endothelial cell properties and molecular angiogenic pathways show a degree of tumor or site specificity (9), so could the responses to a given antiangiogenic treatment. On the other hand, it is important to keep in mind that even in well-controlled and standardized preclinical experiments, tumor relapses and quantitative variability of responses were sometimes observed, even when potent and specific angiogenesis inhibitors were used (10). Is this variability in results merely experimental "noise," or is it a manifestation of some under-appreciated aspects of malignancy?

Genetic Tumor Progression and Angiogenesis. While considering the question of variability in tumor responses to antiangiogenic treatments, it may be helpful to revisit the relationship between angiogenesis and genetic tumor progression. Many properties of cancer cells arise as a result of their genetic instability, diversity, and clonal evolution (11, 12). The sequential activation of mutant oncogenes and loss of tumor suppressor genes associated with this process (13) is thought to contribute to, and promote the acquisition of, the angiogenic phenotype by tumor cell clones, mainly through the influence of these "transforming" genes on expression of endogenous angiogenesis inhibitors (*e.g.*, thrombospondin-1) and stimulators (*e.g.*, VEGF; Refs. 14 and 15). A threshold change in the net "balance" between these stimulatory and inhibitory influences (in favor of the former) is believed to trigger the initial onset of tumor angiogenesis ("angiogenic switch"; Ref. 2). However, expression profiles of various angiogenesis regulators undoubtedly continue to evolve in parallel with genetic tumor progression (16), as do properties of tumor-associated microvascular networks (17), a process that may be globally referred to as "tumor angiogenesis progression" (18). Thus, tumor angiogenesis, although influenced by microenvironmental conditions (*e.g.*, hypoxia), can be viewed as being, to a large extent, genetically driven. As such, angiogenesis is a means by which genetic alterations in cancer cells (*e.g.*, mutant oncogenes) transcend cellular boundaries and secure an "external" (host dependent) support mechanism for tumor cell survival. This is consistent with the observation that when neovasculature is compromised, tumor cells are prone to undergo apoptosis, mainly as a result of hypoxia, and metabolic stress (2).

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; ES, embryonic stem; MVD, microvascular density.

[4] Internet addresses: http://www.angio.org and http://cancertrials.nci.nih.gov/news/angio/table.html.

Progressive Changes in Resistance of Cancer Cells to Ischemia, Hypoxia, and Apoptosis. One of the consistent correlates and, indeed, major consequences of tumor progression is the gradual reduction in the intrinsic propensity of cancer cells to undergo apoptosis under a variety of noxious conditions (19). Such is the effect of many oncogenic/transforming genetic changes, which directly or indirectly impact intracellular apoptotic/survival signaling pathways (20). In this regard cells deficient for expression of the p53 tumor suppressor gene ($p53^{-/-}$) have been recently demonstrated to possess a survival advantage under hypoxic culture conditions over their wild-type ($p53^{+/+}$) counterparts, a property that may reflect the important consequences of the frequent loss of this tumor suppressor in major human cancers (21, 22). Interestingly, *p53* gene product is involved in the regulation of hypoxia responses, in part, through its impact on the stability of the oxygen-regulated transcription factor known as HIF-1α (23). Moreover, according to a recent study, constitutive expression of this latter transcription factor in pancreatic cancer cell lines renders them resistant to apoptosis induced by hypoxia or nutrient deprivation (24). Similar changes in cellular responses to low oxygen conditions and metabolic stress have reportedly been found in other types of cells with altered expression of HIF-1α (24–26). Although both up- and down-regulation of HIF-1α have been linked to cellular resistance to hypoxia-induced growth arrest and apoptosis, all tumor cell lines, in which such resistance has been induced, exhibit a parallel increase in their aggressiveness *in vivo* (24). Again, HIF-1-dependent (and independent) pathways of cellular response to hypoxia are profoundly affected in cancer cells by altered expression of tumor suppressor genes (*p53*, *PTEN*, and *VHL*), as well as oncogenes (*ras*, *src*, and *HER-2*; Ref. 23).

Heterogeneous Vascular Dependence of Cancer Cell Subpopulations. We have postulated previously that the cumulative effect of genetic lesions involved in cancer progression could alter the pathways of hypoxia response and apoptosis in such a way as to enable more malignant cancer cells to survive even under relatively severe ischemia, *e.g.*, induced by an antiangiogenic treatment (27). Ischemic conditions are known to occur locally within tumor masses because of anatomical and functional abnormalities of the tumor microcirculation (Ref. 28; *e.g.*, abnormal vascular patterning, shunts, intravascular clotting, or emboli). Therefore, it could be expected that cancer cell subpopulations could "adapt" over time to these diverse perfusion conditions, a process that would likely lead to coexistence in the same tumor of cells either sensitive or resistant to hypoxic stress and ischemia. Those respective tumor cell subsets would likely occupy regions immediately adjacent to ("proximal"), or remote ("distal") from, the well-perfused tumor capillaries.

Precisely this pattern of perivascular tumor cell segregation was observed recently in several independent experimental systems (18, 22, 26). In these various settings, tumor cell heterogeneity was either a result of injecting artificial cell mixtures composed of cells expressing different malignant properties or occurred naturally within an established melanoma cell line (WM239) grown as tumors in immunodeficient mice (18, 22, 26). Distribution of these various tumor cell subpopulations as a function of their distance from tumor capillaries was determined by injection of the nontoxic fluorescent dye Hoechst 33342 into the circulation of tumor-bearing mice and cell sorting (18, 29). By allowing the dye to circulate for a short period of time and to form perivascular diffusion gradients within the tumor parenchyma, individual tumor cells were assigned stable fluorescent "signatures" corresponding to their distances from the nearby capillaries (29). Cells derived from tumor regions close to functional capillaries (thereby exposed to high Hoechst 33342 concentrations) could be identified subsequently by using flow cytometry because of their bright fluorescence, whereas their counterparts from hypoxic areas of the tumor (exposed to low Hoechst 33342 levels) were unstained or exhibited only weaker fluorescence (18, 26). By using this approach, it was possible to detect a nonrandom distribution of tumor cell subsets around the vasculature in several different tumor types, including (*a*) mixed tumors composed of two H-*ras*-transformed intestinal epithelial cell lines with different malignancy (RAS3 and RAS7; Ref. 18); (*b*) tumors established from mixtures of HIF-$1\alpha^{-/-}$ and HIF-$1\alpha^{+/+}$ mouse ES cells (18); (*c*) human WM239 melanoma xenografts (26); and (*d*) mixtures of $p53^{+/+}$ and $p53^{-/-}$ human colorectal cancer cells (HCT116) (22). In all cases, cells more sensitive to hypoxia (*e.g.*, HIF-$1\alpha^{+/+}$ ES cells) clustered preferentially around blood vessels, whereas their hypoxia-resistant counterparts (*e.g.*, HIF-$1\alpha^{-/-}$ ES cells) could be found throughout the tumor (26). Some of these results have more recently been confirmed by using noninvasive multiphoton laser scanning microscopy to image *in situ* ES tumors heterogeneous for HIF-1α expression (7). Collectively, these observations suggest that cancer cells with elevated resistance to hypoxia and metabolic stress are capable of growth and survival at increased distances from perfused tumor vasculature compared to their hypoxia-sensitive counterparts. The properties of the former cells can be regarded as tantamount to *reduced angiogenesis/vascular dependence*.

In all experimental settings examined to date, the relative diminution of vascular dependence of cancer cells appears to cosegregate with features of increased malignancy (18, 25, 26), *e.g.*, the "distal" cell subpopulation isolated (by a "vascular selection" protocol; Ref. 26) from hypoxic regions of human melanoma (WM239a) xenografts was found to form more aggressive tumors in mice than a corresponding cell line derived from areas "proximal" to tumor capillaries (26). Of note, growth of the former (more aggressive) type of tumor was associated with a lower blood vessel density or MVD, as compared with their less aggressive counterparts (originating from the "proximal" WM239 cells; Ref. 26). On the surface, this may seem counterintuitive given the established prognostic value of MVD in various types of human cancers (30, 31). However, the increase in intervascular distances can be easily understood, if not only angiogenic activity but also the impact of hypoxia resistance on tumor cell-blood vessel geometry is taken into consideration (32). In a recent study, a decrease rather than an increase in MVD was found to correlate with unfavorable prognosis in patients with late stage colorectal carcinoma (33).

It could be hypothesized that, whereas at the earlier stages of tumor progression an increase in vascular supply through angiogenesis may represent a prevailing mode of satisfying metabolic demands of growing tumor masses, later on, selection may favor a decrease in vascular demand, through reduction of tumor cell sensitivity to hypoxia (also lowered dependence on anabolites and growth factors) which would result in at least partial, diminution in vascular dependence of such cancer cells possibly coupled with lowered vascular density (Fig. 1). Such decreased vascular demand could also lead to a diminished sensitivity to antiangiogenic treatment (27), a notion that was recently substantiated experimentally (22).

Oncogenes and Vascular Dependence of Cancer Cells: Therapeutic Implications. The molecular mechanisms responsible for changes in vascular dependence that might accompany tumor progression are not always clear. However, in at least one of the three aforementioned experimental systems (*i.e.*, RAS-3/RAS-7 tumors), reduced dependence on proximity to blood vessels was found in connection with a transforming effect of the H-Ras oncoprotein (18). In this context, it is not unreasonable to ask whether *ras* and other oncogenes can contribute to changes in vascular dependence of various types of cancer cells, in addition to their known influence on promoting tumor angiogenesis. The former, hitherto unsuspected, potential role of oncogenes may be of particular interest in cases where there is an evident connection between the mechanism of

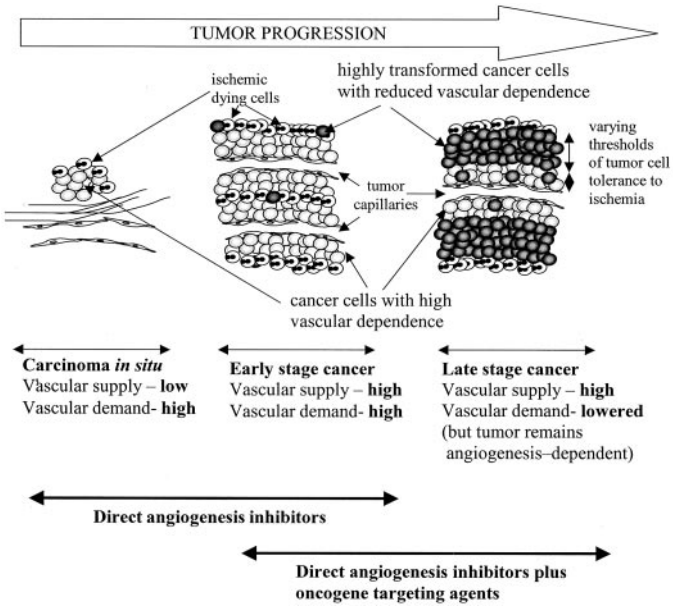

Fig. 1. Changing relationships between cancer cells and the vasculature during tumor progression. Cancer cells must mount a sufficient angiogenic response for tumor expansion to occur. Therefore, early stage cancers can be characterized as having high vascular demand and on "angiogenic switch" also having high vascular supply. Because of developments of apoptosis resistance, and as a result of cancer-associated genetic alterations (activation of oncogenes and loss of tumor suppressors), at later stages of the disease, cancer cells can withstand considerably greater ischemic stress than their less malignant predecessors. This diminution of relative vascular dependence (or vascular demand) is partial but may nevertheless permit tumor growth under lower vascular density conditions and suboptimal doses/regimens of antiangiogenic treatment. It is hypothesized that signal transduction inhibitors could counteract the loss of vascular dependence and thereby "sensitize" tumor cells to direct angiogenesis inhibition.

oncogene-dependent cellular transformation and deregulation of cellular responses to hypoxia (*e.g.*, in the presence of activated *ras*, *src*, or *HER-2*; Ref. 23).

There is an important corollary to this possible link between oncogenes and the vascular dependence of cancer cells. Because of the progress in our understanding of the molecular nature of cellular transformation and tumor progression (1), it has been possible in recent years to develop a new class of prospective anticancer agents targeted at defined transforming oncogenes believed to play a pathogenetic role in several types of human cancers (8). A striking example of the therapeutic potential associated with such agents is the rather unprecedented clinical efficacy of Gleevec/STI571, an antagonist of the *bcr-abl* oncogene expressed in chronic myelogenous leukemia (34). Indeed, a large panel of oncogene-directed signal transduction inhibitors is currently being tested in clinical trials (8), including farnesyl transferase inhibitors aimed at small transforming GTPases (*e.g.*, Ras and/or Rho; Ref. 35), as well as various inhibitors of oncogenic kinases (*e.g.*, Herceptin, Cetuximab, Iressa, Gleevec, and several others; Ref. 8). It is intriguing that, whereas most of these agents possess an appreciable growth inhibitory activity against their target tumor cells in culture, their activity *in vivo*, at least in animal models, is often much greater and cytotoxic rather than cytostatic in nature (36, 37). This puzzling property could be explained, at least in part, by the fact that such signal transduction inhibitors are capable of obliterating the oncogene-dependent angiogenic phenotype of cancer cells, *i.e.*, can act as indirect angiogenesis inhibitors (38). Such activity could only be detected *in vivo* (*i.e.*, under angiogenesis-dependent growth conditions; Refs. 15 and 37).

Inhibition of angiogenesis by oncogene antagonists is a likely but perhaps incomplete explanation of their heightened *in vivo* activity. Indeed, it is possible that in addition to their antiangiogenic, antimitogenic, and many other effects, various signal transduction inhibitors could reverse, at least to some degree, the negative impact of their target oncoproteins (pathways) on vascular dependence of cancer cells, *i.e.*, render such cells more susceptible to ischemia-induced growth arrest and apoptosis. If this were in fact the case, oncogene-directed therapies could represent an ideal addition to direct angiogenesis inhibitors (*e.g.*, endostatin or VEGF inhibitors) by acting as 'drug sensitizers' (Fig. 1).

There are several considerations that may prove useful in further development of angiogenesis inhibitors as anticancer agents: (*a*) the genetic profile of cancer cells may be a significant determinant of therapeutic effects associated with antiangiogenic therapy because of its influence on both the angiogenic phenotype and level of vascular dependence of cancer cells; (*b*) site-dependent differences in efficacy of antiangiogenic agents may be related to organ-specific properties of the respective vascular beds, local differences in expression of endogenous angiogenesis inhibitors and stimulators (against which the therapy must act), and/or differential vascular dependence of tumor cell clones/metastases spread into various organs; interindividual variability in angiogenic responses is also worth considering (39); (*c*) antiangiogenic agents may sometimes improve tumor perfusion by 'normalizing' the structure of the tumor microvasculature (28); however, certain tumors may also have an angiogenic reserve, *i.e.*, excess of vascular supply as compared with their actual (lowered) perfusion requirements (for such tumors, more rapid/complete obliteration of the neovasculature may be needed to achieve tumor stasis or regression); and (*d*) oncogene-directed signal transduction inhibitors may be valuable additions to direct-acting antiangiogenics, as they may, at least in theory, render aggressive cancer cells more vascular dependent and vulnerable. Such drugs can, at the same time, act as indirect angiogenesis inhibitors and further potentiate the effect of therapy (38).

Angiogenesis is a *sine qua non* of tumor growth and progression. However, it is conceivable that the vascular dependence of various tumor cell populations may be heterogeneous, variable, and quantitative rather than absolute and qualitative in nature. It may be worthwhile to consider this aspect in the process of design and interpretation of clinical protocols aimed at destruction of the tumor vascular compartment.

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