Abstract

Angiogenesis is essential to the development of all clinically significant tumors and has been the subject of intense scrutiny in recent years as researchers have sought to develop anti-tumor drugs that target genetically stable stromal cells within tumors. Despite the theoretical advantage of targeting such stable cells, angiogenesis inhibitors have thus far been shown to fail by-in-large as mono-therapies due to inadequate response and succession of resistant tumors. In this proposal I hypothesize that such resistance phenotypes emerge as a result of tumor modulated compensations that activate redundant, parallel, angiogenesis signaling pathways. I set forth herein four specific objectives aimed at testing this hypothesis by fleshing out cellular-level tumor and stromal responses to angiogenesis inhibitor mono-therapies and generating a comprehensive model of tumor-specific angiogenesis signaling pathways. With my latter two aims I propose to evaluate these nascent models by testing their ability to guide clinical reasoning in the approach to delivering angiogenesis-targeting therapeutics.

Hypothesis

Extensive redundancy and cross-communication exists in anti- and pro-angiogenic signaling. This gives rise to a complex combinatorial system of angiogenic signaling. This system is driven out of its normal homeostatic balance in favor of pro-angiogenesis in the context of a growing solid tumor. This results in the hasty formation of tortuous, leaky vessels that characterize solid tumor vasculature and elevation of tumor interstitial fluid pressures, ultimately compromising perfusion of the tumor and limiting
delivery of chemotherapeutics and oxygen necessary for radiation treatments. On account of the redundancy in angiogenic signaling, anti-angiogenic mono-therapies are not capable of swaying the balance of this signaling back from a pathological pro-angiogenic state, through a normal homeostatic window, and into an anti-angiogenic state – as would be necessary to yield tumor regression. Rather, anti-angiogenic mono-therapies are at best capable of transitioning most tumor endothelial microenvironments from a pathological pro-angiogenic state back into a homeostatic window of balanced pro- and anti-angiogenic signaling. When this occurs it results in a clinically observed pruning and normalization of tumor vasculature that improves tumor perfusion but typically does not go so far as to eliminate tumor vasculature and elicit an observable decrease in tumor size (Jain, 1989; Jain 2005). This vascular normalizing response is transient and “resistance” is eventually observed as tumors adapt to exploit alternative pro-angiogenic pathways not targeted by a given mono-therapy.

As a consequence of this hypothetical model, one can surmise that multi-drug combination anti-angiogenic treatment regimens may be necessitated to achieve observable clinical responses in the absence of other cytotoxic treatments. Such treatments, however, may risk catastrophic adverse vascular events in normal tissues as endothelial cells outside of the tumor environment may be moved outside of their homeostatic window to a state where they are unable to respond appropriately to the stresses of daily fluctuations in hemodynamics and instead undergo apoptosis, trigger immune response, and/or initiate proteolytic hemostatic cascades.

Under the fore-mentioned hypothesis of combinatorial redundancy in angiogenic signaling, an alternative to potentially toxic multi-drug combination treatment regimens
emerges. Given a model proposing compensatory adaptations as the basis for development of resistance to angiogenesis inhibitor mono-therapy, one might reason that regular alternation between anti-angiogenic mono-therapies targeting distinct pathways may enable clinicians to avoid this “resistance” by anticipating tumor adaptations. By alternating angiogenic treatments targeting diverse pathways at regular (eg. bi-annually) intervals, one may be able to maintain long-term efficacy of anti-angiogenic treatments in the role of augmenting traditional chemotherapeutic or radiation treatments by increasing tumor perfusion through normalization of tumor vasculature. Given evidence that specific pro-angiogenic signals may be driven by oncogenes expressed in tumors (Rak, 2000; Rak 2002), it is possible that one might be able to specifically identify the most appropriate angiogenic pathway to target for a given tumor by characterizing that tumor’s reliance on specific oncogenes prior to any initiation or alternation of therapy (“angiogenic typing”). In the context of an oncogene-addicted tumor, such a tumor-specific selection of an anti-angiogenic treatment may optimize response over a given treatment interval by forcing the tumor to radically adapt and up-regulate production of alternative angiogenic signals.

Specific Aims

The purpose of this proposal is to set forth experiments capable of evaluating the hypothesis that anti-angiogenic mono-therapies fail because inherent redundancy in angiogenesis signaling stabilizes tumors during pharmacological disruption of any one signaling component and eventually enables such tumors to overcome mono-therapy by exploiting parallel signaling pathways. To fulfill this purpose I set forth four principle aims for this proposal with the first two being foremost for the sake of evaluating the
proposed hypothetical model and the remaining two offering supporting evidence while seeking to unravel the implications of the proposed hypothesis for the biological and clinical sciences that have emerged around angiogenesis. The four specific aims of this proposal are:

1. Probe and observe the molecular responses of tumor cells, tumor stroma, and tumor endothelial cells during anti-angiogenesis mono-therapy. Evaluate the necessity and sufficiency of individually observed responses with respect to the development of anti-angiogenesis resistance phenotypes.

2. Generate tumor-specific models of the angiogenesis/oncogene signaling system and evaluate these models for their utility in predicting responses to modifications of the homeostatic state.

3. Evaluate our ability to predictably inhibit angiogenesis in a clinically significant manner with a sufficiently comprehensive, multi-drug combination, anti-angiogenesis therapy in accordance with models emerging from Aim 2 – recognizing such therapy may not be clinically useful because of toxicity.

4. Assess the models developed in Aim 2 by testing our ability to alternate anti-angiogenic mono-therapies in a tumor-specific manner for the purpose of prolonging the efficacy of anti-angiogenic vascular normalization as an augment to traditional chemotherapeutic and radiological treatments.
Background and Significance

The formation of clinically relevant solid tumors is critically dependent upon angiogenesis and the ability of the tumor to procure nutrients and oxygen from circulation. In the absence of vascularization, solid tumors are only capable of growing to 1-2 mm (Folkman, 1971). All detected and clinically significant solid tumors have presumably, therefore, made the “angiogenic switch” and are able to recruit new blood vessels through oncogene-driven production of pro-angiogenic growth factors such as vascular endothelial growth factor (VEGF). Yet, as solid tumors grow it is well known that they often develop a central core of necrosis secondary to ischemia. For a long time this was thought to be a result of tumors simply out-growing their blood supply. More sophisticated studies of fluid dynamics indicate, however, that central tumor ischemia is likely a result of intra tumor vessel constriction that itself is secondary to elevations in interstitial tumor pressure (Jain, 1989). This elevated pressure is likely a product of the excessive leakiness that characterizes hastily formed and tortuous tumor vasculature that results from pathologic over expression of pro-angiogenic signals by hypoxic tumor cells (Shweiki, 1992).

Increasing awareness of the critical role of vascularization in tumorigenesis has made angiogenesis an attractive therapeutic target for treatment of solid tumors. To date a number of such therapeutics have been developed and many have entered clinical trials. Most promising among these anti-angiogenic therapies has been Bevacizumab (Avastin), the first anti-angiogenesis drug approved for human use (Horwitz, 2004). Bevacizumab is a human monoclonal antibody fragment that targets VEGF and is thought to prevent it from binding its tyrosine kinase receptors and thereby prevent it from eliciting
endothelial proliferation responses that contribute to angiogenesis (Ellis, 2005). A major theoretical advantage of such an anti-angiogenic therapy is its targeting of non-cancerous endothelial cells within a tumor and therefore its decreased propensity for developing resistance – a phenomenon that is commonplace for drugs targeting genetically unstable cancer cells (Kerbel, 1991). Unfortunately, clinical and recent laboratory evidence (Klement, 2000; Yu, 2002; Casanova, 2005) suggests that resistance to anti-angiogenic mono-therapies is a reality that limits their clinical utility.

The precise mechanism by which tumors evade anti-angiogenesis mono-therapies remains unclear although various theories have been proffered. The work of Yu et. al, comparing the effects of anti-angiogenesis treatments on cell lines with different p53 competence, has suggested to some that certain cell lineages within tumors may be less dependent on oxygen and circulation derived nutrients and these cells undergo clonal expansion in the presence of anti-angiogenic treatments, ultimately forming resistant tumors (2002). Others have hypothesized that anti-angiogenic therapies favor the remodeling of vessel to more “mature” forms that are by their nature less responsive to anti-angiogenic therapy regimens (Glade Bender, 2004). Such a theory is supported to a degree by experimental and clinical observation that the overall effect of anti-angiogenic therapies is not to eliminate vascular supply to tumors but to normalize this supply into less leaky, less tortuous, more recognizably mature networks (Jain, 2005). Equally supported by such clinical and experimental observation is a growing theory that the true basis for resistance to anti-angiogenic mono-therapies is the inherent redundancy of angiogenic signaling pathways and the ability of tumors to induce vascularization in the absence of any single pathway that has been shut down pharmacologically (Kerbel,
This theory has derived recent support from the experimental work of Mizukami \textit{et al.} (2005) and Casanova \textit{et al.} (2005), both suggesting that there are likely many alternative pathways that arise to stimulate angiogenesis in tumors where one particular pathway has been targeted. It is from this recent experimental evidence that I derive my hypothesis in this current proposal.

\section*{Research Design and Methods}

\textit{Specific Aim 1}

\textit{General Approach}

To begin evaluating the hypothesis that anti-angiogenic mono-therapies fail because inherent redundancy in angiogenesis signaling stabilizes tumors during pharmacological compromise of any one signaling component, \textit{I will probe and observe the molecular responses of tumor cells, tumor stroma, and tumor endothelial cells during anti-angiogenesis mono-therapy}. To start, I will follow the basic experimental design of Casanova \textit{et al.} (2005) to profile gene expression in treated versus untreated mouse tumors. Essentially these experiments will involve long-term (4 + weeks) treatment of various types of solid mouse tumors with an angiogenesis inhibitor and observation of treatment responses. Upon observing tumor relapse and absence of further response to the given angiogenesis inhibitor (generally at some time point after the typical 10 day treatment course for mice), tumors will be profiled for alterations in angiogenesis signaling pathways relative to the initial untreated tumor and the responsive early-treatment tumor. This profiling will seek to elucidate alterations at the level of expression (transcriptional and translational), sub-cellular localization, post-translational
modification, and concentration of secreted products. Following initial studies conducted after development of resistance to angiogenesis inhibitor treatment, a time-course analysis will be conducted in order to characterize the steps by which resistance comes about following onset of treatment. Subsequently, I will evaluate the necessity and sufficiency of individually observed responses with respect to the development of anti-angiogenesis resistance phenotypes. Observed changes, which are correlated to drug exposure and development of resistance, will be characterized to define their precise role in angiogenesis and to confirm the necessity and sufficiency of said role in development of resistance phenotypes. In order to produce a model with sufficient predictive capabilities to warrant clinical application, these experiments will need to be comprehensive in nature, particularly with respect to tumor type, tumor stage, mono-therapy given, and type of responses considered. Moreover, they will need to differentiate responses of tumor cells from those of tumor stroma and endothelium.

**In vivo experiments**

To begin such experiments I will utilize qRT-PCR to create an mRNA expression profile of known hypoxia response and pro-angiogenesis genes over the course of single drug treatments with various angiogenesis inhibitors. Unlike Cassanova et al. (2005), I will conduct such analyses not simply on whole tumor products but will attempt to isolate, either grossly or by cell sorting, the tumor, stromal, and endothelial cell components of tumors for separate analysis. By doing this I hope to better delineate and localize cause and effect relationships in angiogenesis signaling within tumors. Further, as previously mentioned I will not simply conduct this analysis at a single time point when resistance has developed, but will do so at regular intervals between onset of
treatment and observation of resistance. Control comparisons will be made between non-
tumor and non-treated tumor isolates. Through these efforts I hope to elicit a more
precise picture of the adaptations that give rise to resistant phenotypes. Initially I will
conduct such experiments on the same pancreatic islet cell mouse tumors utilized by
Cassanova et al. (2005) in order to provide external verification of validity to
experimental findings. I will then expand this analysis, in subsequent experiments to
other mouse models of solid tumors and xenographs of human tumors.

To search for additional unexpected transcription-level adaptations in response to
anti-angiogenesis mono-therapies, I will also utilize microarray hybridization studies to
compare genomic expression levels between treated and untreated tumor, stromal, and
endothelial tumor components. As a caveat for this overall approach to screening for
transcription-level alterations one should note that the initial qRT-PCR screen will only
be useful in probing components of known angiogenesis signaling pathways. Yet
because the hypothesis of this proposal relies on redundancy and complexity in such
signaling pathways, it is not wise to rule out the possibility of unknown pathways and
components leading to angiogenesis. While a microarray screen for genome-wide
transcription level changes will enable one to look for such changes, it is likely that a
wide variety of changes will be noted in such arrays due to the diversity of responses
(specific and non-specific) to any drug therapy. While these changes will correlate with
exposure to the administered pharmacological agent and resistance to anti-angiogenesis
mono-therapy, no causative or other relationship can be inferred without further
investigating these altered genes individually. This follow-up investigation will
necessitate mutant, knock-out, and knock-down analyses probing the necessity of
identified transcripts in development of resistance. It will also require over-expression or complementation studies to confirm sufficiency. Such a task may necessitate years of research as one is essentially looking to uncover the transcription level responses to a drug therapy and then determine whether and how each response contributes to the development of resistance to that drug or not. This is no small task indeed and for the near-term such analysis remains a useful objective but will necessarily be incomplete with only exemplary gene hits being initially followed-up with for illustrative purposes.

In addition to these analyses of transcription-level adaptations from \textit{in vivo} tumors; translational effects, protein stability, and post-translational modifications in these tumors will be assayed by mass spectrometry. Such analysis will utilize immunoprecipitation and subsequent mass spectrometry of known pro-angiogenic factors from cell lysates (controlled for total protein content) in order to roughly quantify protein expression levels and characterize any changes in phosphorylation or other post-translational modifications. Immunohistochemical fluorescence microscopy of tumor sections will also be utilized to examine changes in localization and co-localization between treated and untreated tumors. Unfortunately, as was the case with qRT-PCR, these experiments will be limited initially to the known pro-angiogenic and hypoxia-induced proteins. Eventually, as a better picture of angiogenesis signaling redundancy emerges from microarray follow-up studies on the transcription-level responses to drug therapy and from \textit{in vitro} proteomic analyses described later in this manuscript, this investigation of \textit{in vivo} protein-level responses will need to be extended to include any new gene products suspected of influencing angiogenesis. Collectively, these \textit{in vivo}
experiments will provide an initial characterization of the molecular responses of tumor, stromal, and endothelial tumor components to anti-angiogenesis mono-therapy.

In vitro experiments

In an effort to guide, extend, and validate these in vivo analyses, I will emulate the in vitro analysis of Mizukami et al. (2005) by using stable siRNA knock-down of specific pro-angiogenic pathway components in cell lines appropriate for analyzing the effects of targeted pharmacological angiogenesis inhibition in solid tumor and stromal cell lines. These studies will be critical in unraveling the combinatorial redundancy of overlapping angiogenic signaling pathways by allowing gene-specific targeting in an inducible manner. Following knock-down initiation, effects on non-targeted pro-angiogenesis gene expression will be monitored at specific time points under normoxic and hypoxic conditions by qRT-PCR and microarray gene chip hybridization studies. This, in effect, allows one to mimic pharmacologically targeted disruption of a known pro-angiogenesis signaling pathways without the additional unknown non-specific effects of long-term drug therapy. That having been said, one would certainly need to evaluate the specificity of siRNA targeting for each such experiment. Any transcripts identified in these hybridization screens would again only correlate to knock-down of an angiogenic signaling pathway component. To tease out the specificity of such responses and precise cause and effect relationships, various null mutant siRNA and empty vector controls will be needed, as will follow-up characterization of any target-specific transcription-level responses. Such follow up will include complementation studies and in vivo confirmation of observed responses with inducible knock-outs or knock-downs. In vivo investigation would also then be extended to probing functionality by mutagenesis,
double inducible knock-outs, immunoprecipitation-mass spectrometry, and
immunohistochemical fluorescence microscopy.

These experiments will prove a useful counter for in vivo studies by affording
greater control and easier analysis of the cellular responses and adaptations to
compromise of an angiogenesis signaling pathway. It is unclear however that angiogenic
signaling in immortalized culture cells, even under hypoxic conditions, will accurately
mimic in vivo responses. Presumably such cells maintain the same signaling pathways as
in vivo cells but this is a presumption and it could just as well be the case that cell
adhesion or other aspect of multi-cellularity is necessary for some aspects of angiogenesis
signaling. If indeed one observed this to be the case by comparison of in vitro and in vivo
findings, one might try to work around this problem by using primary culture tumor cells
with transiently transfected siRNAs. Though not ideal because of the greater variability
with primary culture cells and transient transfection, such a scenario might still afford a
window into the cellular responses to a compromise of an angiogenesis signaling
pathway. If stably transfected siRNA knock-down cells were found to be reasonable
models for cellular responses to angiogenesis signal disruption, they could then also be
utilized for mouse xenograft studies and comparisons of transcription levels could be
made between grafts in which knock-down was induced or not induced.

Mass spectrometry experiments could similarly be done on induced and non-
induced siRNA-knock down tumors in the same manner as was done for drug treated
versus non-treated tumors in order to elucidate post-translational modifications.
Moreover, with in vitro cell cultures of inducible knock-downs, proteomic-wide analysis
of protein expression levels, protein stability, and post-translational modifications could
be attempted using stable isotope labeling by amino acids in cell culture (SILAC) (Ong et al., 2002) on sub-cellular fractionation products from induced and non-induced culture cells as well as those grown in normoxic and hypoxic conditions\(^1\). One would need to be careful, in such experiments, about disrupting any post-translational modifications of proteins in the process of adequately fractionating cell lysates for mass spectrometry analysis. To complement such experiments, immunohistochemical fluorescence microscopy could be done on inducible knock-down cells to look for differences in protein level expression, sub-cellular localization and co-localization in a similar manner to that described for drug treated tumors \textit{in vivo}.

\textit{Specific Aim 2}

By analyzing results of the above experiments from diverse tumor backgrounds, one can \textit{generate tumor-specific models of the angiogenesis/oncogene signaling system and evaluate these models for their utility in predicting responses to modifications of the homeostatic state}. These models can then be evaluated for their ability to predict the responses of various tumors to different angiogenesis inhibitors and the mechanism by which resistance phenotypes will emerge. This will entail utilizing results from the experiments described above for Specific Aim 1 and extending these methods of experimental analysis to a diverse array of different tumor cell types in order to correlate patterns of response to angiogenesis inhibitors and exploitation of alternate resistance pathways with oncogene and perhaps also tumor suppressor gene expression profiles. In

\footnote{\textit{In this technique one utilizes two nearly identical cell cultures; one grown in the presence of a radio-labeled essential amino acid and the other in identical media with non-radio labeled nutrients. These cultures can then be exposed to a single differentiating variable – knock-down induction and/or hypoxia for the purposes of this study. This stimulus can then be correlated to quantitative differences observed in the amount of radio-labeled versus non-labeled protein species detected by mass spectrometry in sub-cellular fractions from these two cultures.}}
essence then, this will be an effort to define tumor markers that one can utilize to define a tumor’s “angiogenic type” and to predict tumor response to a given angiogenesis inhibitor. This angiogenic typing will define the reliance of different tumor types on specific angiogenic pathways and characterize how these tumors adapt and become resistant when this pathway is pharmacologically targeted.

Specific Aims 3 and 4

These “angiogenic type” models of pathway reliance, drug susceptibility, and adaptation to drug treatment will then be the tested by pharmacological methods using combination and sequential treatment regimens to elicit improved treatment efficacy and lengthened duration of response. The use of combination therapies will be used to evaluate our ability to predictably inhibit angiogenesis with a sufficiently comprehensive, multi-drug combination, anti-angiogenesis therapy in accordance with models emerging from Aim 2 – recognizing such therapy may not be clinically useful because of toxicity.

Such combination treatments allow one to target not only the predicted primary angiogenic pathway for a specific tumor but they also confer the ability to preemptively strike down the pathways that our nascent models predict the tumor will up-regulate or alter in response to pharmacological targeting of its primary angiogenic pathway. By local and/or systemic administration in the context of diverse human tumors xenografted into nude mice, combination therapies will be tested for their ability to inhibit and perhaps even reverse tumor growth. Concomitantly such testing will provide feedback as to the accuracy and limitations of models developed in Aims 1 and 2. Because such combination therapies may present toxicity concerns that prevent their clinical use and/or reliable interpretation of observed responses, I also intend to assess the models developed
in Aim 2 by testing our ability to alternate anti-angiogenic mono-therapies in a tumor-specific manner for the purpose of prolonging the efficacy of anti-angiogenic vascular normalization as an augment to traditional chemotherapeutic and radiological treatments. Such an assessment will test the accuracy of our experimentally-derived models of redundant angiogenesis signaling by evaluating their utility in rationally generating sequential, multi-drug, mono-therapy treatment regimens that initially target the angiogenic pathway upon which a tumor has relied to make the angiogenic switch and subsequently target alternative individual pathways that are predicted to be characteristically altered in adaptive resistance-phenotype responses to the initial mono-therapy. Intra-vital microscopy on various tumors xenografted into mice will be used to evaluate both the combinatorial and sequential pharmacological tests of angiogenic type models for all tumor types considered. Ability to normalize or possibly eliminate tumor vasculature over a long-term treatment course (4+ weeks) will provide the positive endpoint for such evaluation. The kinetics of tumor adaptations will need to be determined in order to decide the appropriate timing for cycling inhibitors. Nevertheless, because the target of these therapies is genetically stable stromal and endothelial cells, such alternation of mono-therapies should challenge the adaptability of tumors. As a caveat, one might imagine that tumors would be able to develop overlapping resistance to these sequential mono-therapies if they were able to breakdown or sequester the different drugs by a common pathway. With this in mind, care should be taken to use structurally diverse pharmacological agents and not similar small molecules or antibody fragments that could be mutually redressed by desperate tumor cells.
With the conclusion of these studies, a much better picture of angiogenesis signaling pathways and homeostatic maintenance will have emerged. This picture should afford a clear verdict on the hypothesis that anti-angiogenesis mono-therapies fail because redundancy in parallel signaling pathways enables adaptive compensation and eventually development of resistance phenotypes. As this picture emerges however, one must be careful not to overstate the implications of results. Whether parallel signaling pathways do or do not contribute to resistance phenotypes, from these experiments one cannot rule out competing hypotheses that resistance emerges in some contexts from the clonal expansion of cells with decreased oxygen dependence or from the formation of a more mature vasculature that is no longer sensitive to angiogenesis signaling pathways.
References (in order of citation)


“Why anti-angiogenic treatments fail as mono-therapies”
Zachary S. Morris


