Spontaneous Reversion of the Angiogenic Phenotype to a Non-angiogenic and Dormant State in Human Tumors

Authors and affiliations:

Departments of Surgery and Anesthesia, & the Vascular Biology Program, Boston Children’s Hospital, and Harvard Medical School, Boston, MA 02115; Department of Microbiology, Haukeland University Hospital, Bergen, Norway; The Gade Institute, Section for Microbiology, Centre for Cancer Biomarkers, Section for Pathology, Institute of Internal Medicine, Section of Oncology, University of Bergen, Norway.

Corresponding author:
George N. Naumov, Merck Research Laboratories, Merck & Co., Inc., BMB-9, 33 Avenue Louis Pasteur, Boston, MA 02115, Tel. (617)992-3233, Fax (617) 992 2412, email: george_naumov@merck.com

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Abstract

The angiogenic switch, a rate-limiting step in tumor progression, has already occurred by the time most human tumors are detectable. However, despite significant study of the mechanisms controlling this switch, the kinetics and reversibility of the process have not been explored. The stability of the angiogenic phenotype was examined using an established human liposarcoma xenograft model. Non-angiogenic cells inoculated into immunocompromised mice formed microscopic tumors that remained dormant for ~125 days (vs. <40 days for angiogenic cells) whereupon the vast majority (>95%) initiated angiogenic growth with second-order kinetics. These original, clonally-derived angiogenic tumor cells were passaged through four in vivo cycles. At each cycle, a new set of single-cell clones was established from the most angiogenic clone and characterized for in vivo for tumorigenic activity. A total of 132 single-cell clones were tested in the 2nd, 3rd and 4th in vivo passage. Strikingly, at each passage, a portion of the single-cell clones formed microscopic, dormant tumors. Following dormancy, like the original cell line, these revertant tumors spontaneously switched to the angiogenic phenotype. Finally, revertant clones were transcriptionally profiles and their angiogenic output determined. Collectively, these data demonstrate that the angiogenic phenotype in tumors is malleable and can spontaneously revert to the non-angiogenic phenotype in a population of human tumor cells.

Implications: Leveraging the rate of reversion to the non-angiogenic phenotype and tumor dormancy may be a novel anti-cancer strategy.
Introduction

In cancer, a tumor’s switch to angiogenesis is a rate-limiting step in its progression from microscopic to macroscopic size (1). As a result, small, occult tumors are a common finding on autopsy of individuals who die of non-neoplastic causes (primary studies summarized in (2)). In contrast, the converse finding—of tumors that switch off angiogenesis and thus cease macroscopic growth—is rare (2). Nevertheless, there are a limited number of reports of tumors that cease macroscopic growth, suggesting that such a reversion of the angiogenic switch may occur (3, 4).

Regulation of angiogenesis is typically viewed as a switch whose state is governed by the relative local concentration of angiogenesis stimulators and inhibitors. The switch metaphor is common in the field because of the strong biphasic nature of angiogenesis. In experimental models of pathological conditions it is rare to observe small, stepwise accumulation of additional vessels. Rather it is common to observe that once angiogenesis has been initiated, vessel growth proceeds throughout the pathologic process. In addition, variation in intensity of angiogenesis has been observed, and appears to be a major rate-limiting factor in tumor growth. For example, substantial variation in microvessel density is observed throughout tumors, with regions exhibiting the highest density predicting the overall growth rate of a tumor, metastatic status, and patient survival (5-7).

In contrast to the tumor as a whole, we and others have shown that individual tumor cells can exhibit significant variation in their ability to induce angiogenesis. For example, when individual cell clones derived from a primary human liposarcoma are implanted in immunocompromised mice, the timing of the transition to macroscopic growth varies from 7 to >160 days (8). In addition, several commercially available tumor cell lines, originally derived from human tumors that were macroscopic in size, exhibit extended periods of pre-angiogenic growth, ranging from few weeks, to years, to the lifespan of the animal (9). However, an experimentally-induced increase in the angiogenic output of these tumors (e.g. by transfection...
with VEGF (10, 11)), results in early and rapid macroscopic growth. Similarly, alterations occurring during extended evolution of dormant tumors in mice can result in increased net angiogenic output. In at least one system, this was accompanied by a decrease in expression of angiogenic inhibitors (e.g. thrombospondin-1), rather than an increase in angiogenic stimulators (e.g. VEGF) (12). Importantly, in vitro growth rates for the angiogenic sublines, derived from the non-angiogenic parental lines, in all of these experiments did not differ significantly from the growth rate of non-angiogenic sublines. These findings exclude differences in cell division rate as a mechanism for the observed differences in macroscopic growth.

Finally, experiments in which angiogenic cells were admixed with non-angiogenic cells prior to inoculation in mice have demonstrated that even a minority of pro-angiogenic cells is sufficient to induce growth (and metastasis) in the entire tumor (11). Non-transformed (i.e. stromal) cells have also been shown to play a critical role in the induction of angiogenesis in some tumors (13). These observations lead to the notion that the angiogenic switch may be an ensemble property comprised of contributions from all the cells in the tumor, rather than an obligate property of only the tumorigenically transformed cells in a tumor (10). Therefore, it is possible that individual tumor cells, although derived from an angiogenic tumor, may not possess the angiogenesis-inducing potential of that tumor. We sought to test that possibility by serial in vivo passage, cloning, and quantitative analysis of the growth phenotypes of such individual tumor cells. In addition to assessing the stability of the switch to an angiogenic phenotype in individual cells, these experiments allowed us to assess the nature of the events that lead to the angiogenic switch, and determine that it is not comprised of a single step.
Materials and Methods

Generation of single cell clones. Angiogenic (Clone -9) and nonangiogenic (Clone -4) cell lines were established from the SW-872 cell line as previously described (8, 14). For the first in vivo passage, a new cell line (Clone -9#2) was generated following subcutaneous inoculation into a SCID mouse. Subclones with similar growth rates were prepared for subsequent in vivo passage from monolayers of the Clone -9#2 cell line following limiting dilution (average 0.2 cells/well) in 96-well plates. Each clonal population was expanded to approximately 30x10^6 cells and inoculated into 5 SCID mice. The most angiogenic clone was selected to produce single cell clones for the next in vivo selection cycle. Specifically, 31, 33, and 68 single cell clones were tested in the 2nd, 3rd, and 4th in vivo passage, respectively. In vitro growth curves were generated using a Coulter counter.

Subcutaneous tumor growth. Male severe combined immunodeficient (SCID) mice, 6-8 weeks of age (MGH, Boston, MA) were cared for in accordance with the standards of the Institutional Animal Care and Use Committee (IACUC), and used under an approved protocol. Tumors were generated by injecting 5 x 10^6 cells in 0.2 ml PBS subcutaneously into the shaved lower right flank. Injection sites were palpated weekly for tumors (typically detected at ~50 mm^3) and measured every 3-4 days thereafter, with volumes calculated using the formula 0.52W^2L. Mice were euthanized when tumor volume reached ~1000mm^3 or mice showed signs of discomfort.

Immunohistochemistry and histology. Excised tumors were rinsed in ice-cold PBS and fixed in 4% paraformaldehyde. Paraffin-embedded tissue was sectioned (4μm) and representative sections (5/tumor) were stained with H&E, PCNA (PC10, 1:150; DAKO, Carpinteria, CA), and CD31 (PECAM; 1:250; BD Biosciences, San Diego, CA).

Quantitation of angiogenesis regulators. Serum-free medium (15ml) was incubated on day-old cultures (5 x 10^6 cells/15 cm plate) a further 24 hours. Concentrations of human VEGF (R&D, Minneapolis, MN), bFGF (R&D), and Tsp-1 (CYT Immune Science, College Park, MD)
were measured by ELISA kits using manufacturers’ protocols with control values (serum free media) subtracted and results normalized per $10^4$ cells. Protein lysates from angiogenic and revertant cell clones were western blotted as previously described (13) with Tsp-1 (Ab11, LabVision, Fremont, CA), and β-actin (Abcam), using HRP-conjugated goat anti-mouse (Jackson Immunoresearch Labs, West Grove, PA) for detection.

**Gene expression analysis.** Independent angiogenic (two) and non-angiogenic (three) cell lines from the second cycle of cloning were analyzed using the Agilent Human Whole Genome (4x44k) Oligo Microarray (Agilent Technologies Inc., Palo Alto, CA) as previously described (15). Significance Analysis of Microarrays (SAM) was performed in J-Express (www.molmine.com) (16), by dividing samples into angiogenic and non-angiogenic (revertant) groups. Functional and pathway analysis of genes upregulated in revertant or angiogenic clones by 1.5-fold or more was generated using Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com) as well as GOEAST (http://omicslab.genetics.ac.cn/GOEAST) (17).

**Real-time RT-PCR.** RT-RT-PCR analysis was performed in triplicate on 20 ng cDNA prepared using Thermoscript RT-PCR system using validated Taqman gene expression assays (Applied Biosystems, Foster City, CA). Following outlier exclusion (samples with a CT standard deviation $> 0.3$), angiogenic and non-angiogenic samples were compared by $\Delta\Delta$CT using GADPH as endogenous control.

**Statistical analysis.** Time to palpable tumor for the angiogenic and non-angiogenic cells was compared using the Kaplan-Meier method, with survival curves compared using log-rank test (16), and confidence intervals determined using Greenwood’s formula (15). For a subset of tumors, we compared the median time from initial palpation to a size of 500 mm$^3$ using the Mann-Whitney U-test. Two-tailed values of $P<0.05$ were considered statistically significant.
Analysis of escape kinetics. Growth curves were fit to a model of exponential growth after a variable delay using least squares fitting to the following equation: $d(t) = (d_0 \text{ if } t < t_0; d_0 10^{m(t-t_0)} \text{ if } t > t_0)$ with $m$ (growth rate), $d_0$ (dormant tumor size) and $t_0$ (length of dormant period) as free variables. The hypothesis that the data might be consistent with different growth rates (vs. tumor growth initiation times) was rejected by F-test ($p < 10^{-300}$) after holding $t_0$ to zero and again fitting $d$ and $m$ for each animal, excluding the possibility that a small number of exponentially growing tumor cells led to the delay in tumor growth observed for the “non-angiogenic” tumors.
Results

In vivo growth of angiogenic and non-angiogenic human liposarcoma single-cell derived clones.

We previously described an animal model of human liposarcoma, based on the phenomenon that all human tumors are comprised of angiogenic and non-angiogenic tumor cell populations (8). Specifically, two single cell clones were derived from the original tumor population: one displayed angiogenic properties (Clone-9) and one was non-angiogenic (Clone-4). Here, we characterize the in vivo growth potential of these clonal populations in detail using a xenograft model (Figure 1). We observed that 100% of SCID mice (n=13) inoculated with angiogenic Clone-9 cells initiated exponential tumor growth early (within one week of inoculation) and reached a mean size of 1500 mm$^3$ within one month of inoculation. In contrast, mice inoculated with the non-angiogenic Clone-4 cells developed only microscopic tumors (less than 50mm$^3$) in the first week after tumor cell inoculation. The majority (97%, 78 out of 80 mice) of Clone-4 tumors, however, spontaneously initiated exponential tumor growth at a median of 125 days ($\pm$25 days, standard deviation), a phenomenon previously described as the "angiogenic switch" (9). Remarkably 2.5% of Clone-4 tumors (2 out of 80 mice) remained at this small size for more than 300 days.

Growth escape kinetics are inconsistent with single event causation.

Biological transitions are typically thought of in terms of two different processes. In the first, a transition is regulated by a small number of discrete events such as mutations. The second is more clock-like and is often governed by accumulation of specific factors until a threshold is reached thereby triggering the phenotypic change. For the angiogenic switch, the mutation model predicts stochastic conversion of cell populations to angiogenic growth, vs. maintenance of the non-angiogenic phenotypes. In contrast, if the delay in angiogenic growth is the result of a "clock", then one might expect drift in the timing of the clock to earlier or later time...
points in various clones as a result of differences in angiogenic/anti-angiogenic factor
accumulation, but relatively stable dormant times within a given cell clone.

To better understand the nature of the process underlying the switch to the angiogenic
phenotype (and exponential tumor growth) we examined the distribution of switch times (Figure
1C-D). If the process were clock-like (e.g. results from local accumulation of a pro-angiogenic
factor to a pre-determined threshold), escape times would be distributed in a Gaussian fashion.
In contrast, if the process is governed by $n$ events (e.g. mutations or other heritable regulatory
events), then escape times will follow a multistage model curve of the form $e^{-k(t-t_o)^n}$ (18, 19).
Comparing these models using AIC (Akaike Information Criterion) shows that in our
experimental model there is greater evidence for the mutation model (evidence ratio $>10^9$).
Fitting the distribution of escape times to the multistage model equation yielded a best-fit value
of 1.8 for $n$ (with $n = 2$ fitting marginally worse; $p = 0.029$). These findings indicate that, on
average, approximately 2 changes were necessary to convert a non-angiogenic tumor to an
angiogenic tumor.

Not all clones from angiogenic tumors retain the growth phenotype of the original tumor.

Since the above analysis indicates that the angiogenic switch requires two distinct
mutations, it also predicts an intermediate phenotype in which cells possess only one mutation.
The intermediate phenotype would manifest in a growth curve that displayed a delay in the
acquisition of exponential growth that was longer than the early-growing angiogenic phenotype
and shorter than the dormant phenotype. Thus, in order to prove our model, we sought to
identify such an intermediate. To that end, we passaged the original clonally-derived, early
growing, angiogenic tumor cells (Clone-9 cells) through 4 cycles in SCID mice (Figure 2), with
the idea that a fraction of such tumors would contain cells that only carried a single mutation.
After each *in vivo* cycle, the fastest growing early tumor was used to establish a cell line and produce a new set of single-cell-derived clones used for *in vivo* testing of angiogenic activity. We selected 31, 33, and 68 single cell clones in the 2\(^{nd}\), 3\(^{rd}\) and 4\(^{th}\) *in vivo* passages, respectively. These clones were chosen (from over 200 single cell clones per *in vivo* passage) under *in vitro* conditions, such that they exhibited similar proliferation rates. Thus, any differences in *in vivo* growth kinetics would not be due to intrinsic proliferation rates. Each clonally-derived tumor cell population was inoculated into 5 SCID mice for determination of *in vivo* growth phenotype. Observation of these tumors revealed that 40-70% of clones retained the growth properties of the parental line, while 20-50% displayed an intermediate phenotype (Figure 3).

Strikingly, after each *in vivo* passage, 3-6% of the single cell clones derived from the fastest -growing early-arising clone of the previous passage remained at a microscopic, dormant size for a median of 72 days. In other words, these cells reverted to a dormant phenotype (Figure 4). Following this dormancy period, most of the dormant tumors spontaneously switched to the fast -growing phenotype, a process that was regulated by the acquisition of angiogenic potential in the original Clone -9. In contrast, others reverted to the non-angiogenic Clone-4 phenotype, remaining dormant for more than 200 days (Figure 4 B, E, H). Specifically, during the 2\(^{nd}\) *in vivo* passage, 55% of the clones had an early-growth phenotype, 6% demonstrated a "revertant" phenotype (dormant period >1 month for at least 4/5 mice, i.e. *in vivo* growth similar to non-angiogenic Clone-4 cells), and 39% displayed the intermediate or “hybrid” phenotype (Figure 4). During the 3\(^{rd}\) *in vivo* passage, 76% of the clones grew early, 3% were revertant, and 21% were intermediate. As part of the 4\(^{th}\) *in vivo* passage, 43% grew early, 6% were revertant, and 51% were intermediate. The overall distribution of early-growing, hybrid, and revertant cell lines is consistent with that resulting from a 26% chance of individual reversion events in a two-event model (55%, 38%, 7%; P>0.7 by chi-squared test). The time it took for a tumor to reach 50mm\(^3\) following inoculation has been summarized in Figure 4 using
Kaplan-Meier curves for each in vivo passage. Importantly, the presence of a "revertant" group of clones, characterized with a dormancy period and delay of exponential tumor growth start was evident at each in vivo cycle.

Early-growing and revertant clones did not differ in proliferation in vitro or in vivo, but did exhibit differences in expression of angiogenic regulators.

We then sought to determine the underlying mechanism governing the in vivo properties of the early-growing and revertant clones. We first examined whether the two types of clones possessed different in vitro growth kinetics. We compared the in vitro proliferation potential of two revertant clones and three early-growing clones from the 2nd in vivo passage and generated in vitro growth curves (Figure 5). Importantly, we did not observe a significant difference in growth rates in vitro between the two types of clones. We also compared the in vivo growth rates via PCNA staining of dormant and exponentially growing tumors. While there was no significant difference in the fraction of nuclei that stained positive for PCNA (Supplemental Figure 1), we observed an increase in apoptotic cells in dormant tumors compared to exponentially expanding ones (8). Another possible explanation for the failure of the microscopic lesions formed by the revertant clones to grow macroscopically would be a genetic or epigenetic event(s) that resulted in the loss of the transformed phenotype. To test this possibility, we assayed the ability of these angiogenic-tumor-derived clonal cell lines to grow in an anchorage-independent manner. No differences were observed between early-growing and revertant cell lines.

Since we observed no differences in in vitro or in vivo proliferation rates or the ability to form colonies in semisolid media between the early-growing and revertant clones, we postulated that there may be a difference in net angiogenic output between the two clone types, as had occurred in the parental lines. In order to begin to test this hypothesis we examined these clones for expression of the angiogenic stimulator VEGF and the angiogenesis inhibitor
thrombospondin 1 (Figure 6A-C). VEGF was chosen because manipulations that induce VEGF expression have been shown to induce exponential, angiogenic growth in otherwise dormant tumors (11). Similarly, we have previously observed down-regulation of thrombospondin 1 in dormant tumors that spontaneously initiate exponential growth (20). The revertant clones (for example, clone 10) demonstrated significant (~4-fold higher levels, p=0.01, Student-t test) up-regulation of thrombospondin-1 when compared to the angiogenic clones (for example, clone 29) that grow exponentially in animals (Figure 6A and B). This difference was evident in both western blot and ELISA assays. Interestingly, a similar thrombospondin-1 regulation pattern (~3.5-fold difference between the angiogenic and non-angiogenic cell lines; p=0.03) was observed in the original, patient-derived cell lines. In contrast, while VEGF was down-regulated in the revertant clones as expected (~6-fold in revertant clone 10 vs angiogenic clone 29, p <0.0001), it was slightly (~20%) up-regulated in the original non-angiogenic Clone -4 when compared to the angiogenic cells from the same tumor (Figure 6A and C). These data indicate that thrombospondin-1 expression might play an important role in the interconversion of angiogenic and non-angiogenic cells in this model.

The expression changes in VEGF and Tsp-1 between the angiogenic cell lines and their revertants suggest that the cause of the revertant phenotype is a decrease in net angiogenic activity. However, expression data alone cannot firmly establish that the change in tumor growth kinetics results from lower angiogenesis-inducing activity in the revertant clones than the early-growing “angiogenic” cell lines. Therefore, to determine whether the clones actually exhibited different angiogenic output, which could account for their differential growth properties in vivo, we performed the endothelial migration assay, a well-validated surrogate for the angiogenic response (21). This allowed us to directly measure the net angiogenic output of the revertant and “angiogenic” clones. The ability of two early-growing (9-1 and 9-29) and two revertant clones (9-2 and 9-10) to induce the migration of human microvascular endothelial cells (HMVECs) were compared in a modified Boyden chamber assay. We observed the early-
growing “angiogenic” clones had induced 3-4-fold more migration than the revertant clones (P<0.05 by ANOVA, Figure 6D). Taken together with the expression data and the previous findings that Clone -9 was more angiogenic than Clone -4, the data indicate that reversion from the early-growing, angiogenic phenotype to dormancy is mediated by a decrease in net angiogenic output.

Differences in in vitro gene expression were observed between angiogenic and non-angiogenic clones.

In order to obtain more comprehensive insight into the molecular differences between angiogenic and revertant cell lines, we performed global gene expression profiling. Two independent angiogenic and three independent non-angiogenic cell clones from the second cycle of cloning (clones 9-2, 9-10 vs. 9-28, 9-29, 9-37, respectively) were selected and expression of RNA transcripts was measured using Agilent microarrays. Differences in expression of the top 31 genes were confirmed by real-time RT-PCR (Figure 7). Clustering of upregulated genes from either the angiogenic clone dataset or the revertant clone dataset around distinct chromosomal locations was not observed (see Supplemental Figure 2).

Of the genes upregulated in angiogenic clones, the top canonical pathways represented as determined by Ingenuity Pathway Analysis were acute response phase signaling, ephrin receptor signaling, axonal guidance signaling, the complement system and relaxin signaling (Supplemental Figure 3A). Other pathways significantly overrepresented included PPAR signaling, molecular mechanisms of cancer, and sphingolipid metabolism. In revertant clones the top canonical pathways represented were antigen presentation pathway, dendritic cell maturation, autoimmune thyroid disease signaling and allograft rejection signaling (Supplemental Figure 3B). Other pathways significantly overrepresented included semaphorin signaling in neurons, NFκB signaling and death receptor signaling.
Gene Ontology analysis using GOEAST software identified extracellular matrix structural constituent (P-value=8.38e-6) as the top molecular function and proteinaceous extracellular matrix (P-value=2.46e-11) as the top cellular constituent among genes upregulated in angiogenic clones in comparison with revertant clones. Receptor binding (P-value=8.01e-8) was a significantly overrepresented molecular function in revertant clones and MHC class II protein complex (P-value=8.71e-7) was one of the most significantly overrepresented cellular constituents in revertant clones. Immune response was a significantly overrepresented (P-value=6.3e-7) biological process in the revertant liposarcoma clones. Finally, axon guidance was a significantly overrepresented biological process in angiogenic clones (P-value=6.89e-5).

Eph receptor A4 (1.76 fold) and Eph receptor B3 (1.52 fold) were expressed in angiogenic clones at higher levels than in revertant clones. Eph receptors and ephrins regulate cell movements during neural crest cell migration, gastrulation, neuronal axon guidance and angiogenesis. Significantly, a number of downstream effectors of ephrin receptors including syndecan 2 (SDC2), actin related protein 2/3 complex (ARP2/3) and Ephexin were also elevated in expression in angiogenic clones compared to revertant clones. Previous studies have demonstrated that blocking EphA class receptor activation inhibits angiogenesis in mouse tumor models (22), and the level of expression of EphA2 can be correlated with a more aggressive phenotype in prostate cancer (23). Overexpression of ephrin B3 in a mouse tumor xenograft model has been demonstrated to reduce microvessel density and tumor growth (24). In a corneal angiogenesis model stimulation of Eph B receptors enhanced FGF induced blood vessel formation (25).

Semaphorin 7A and a number of its effectors including FES (feline sarcoma oncogene) and LIM kinase, an inhibitor of cofilin were upregulated in revertant clones in comparison with angiogenic clones. Semaphorins can have pro- or anti- tumorigenic effects depending on the semaphorin expressed and the celltype where it is expressed within the tumor. Anti-angiogenic effects of semaphorin 3A and semaphorin 3F have been described (26-29), whereas
semaphorin 3C has been shown to induce endothelial cell growth and migration (30). The role of semaphorin 7A has not been extensively studied in carcinogenesis or angiogenesis, but there is some evidence that expression of its receptor Plexin C1 is inversely correlated with melanoma cell invasiveness (31). Expression of the plexin B3 receptor was also increased 1.6-fold in revertant clones in comparison with angiogenic clones in the current study. In contrast, semaphorin 5B was upregulated 2.23-fold in angiogenic clones and this altered expression was further confirmed by real-time PCR analysis. Genome wide expression analysis of renal cell carcinoma has identified Semaphorin 5B as being upregulated in tumor tissue in comparison to the normal surrounding tissue, and a pro-tumorigenic role for this semaphorin has been demonstrated via promotion of renal carcinoma cell survival (32).

Finally, ROBO1, which was upregulated by 1.33 fold in angiogenic clones on the microarray and 1.13 fold by real-time RT-PCR, is also involved in axonal guidance and is a positive regulator of angiogenesis when expressed on endothelial cells (33). PPARγ was also confirmed as an upregulated gene (1.32 fold) in angiogenic clones by real-time PCR analysis while other genes that influence PPARγ signaling such as retinoid X receptor alpha (RXRA, 1.59-fold), nuclear receptor interacting protein 1 (NRIP1, 2.39-fold), and c-Fos (1.70-fold) were upregulated on the microarray.

Taken together these findings strongly support the conclusion that the observed increase in a tumors net angiogenic output is due to genetic mutations. Further, at different times in tumor development there are transition states regulated by heterogeneous populations of tumor cells with varying degrees of angiogenic output. Based on these findings it is reasonable to assume that if tumors could be detected early enough, it may be possible to prevent the acquisition of the angiogenic phenotype and maintain dormant lesions for extended periods of time.
Discussion

Our studies provide clear evidence that the angiogenic switch is reversible at the cellular level. While the findings in this report are specific to the liposarcoma cell lines used, there is reason to believe that dormancy and angiogenesis may also exhibit the same plasticity in other tumor types though not necessarily by the same mechanisms (34). Specifically, we demonstrate the striking finding that a single cell clone that forms angiogenic tumors can also give rise to cells that are incapable of inducing angiogenesis. Importantly, this indicates that even in actively growing tumors, a significant fraction (perhaps a majority) of tumor cells are non-angiogenic. Importantly, we observed no difference in growth rate or anchorage-independent growth in angiogenic vs. non-angiogenic tumors. These findings demonstrate that pro-angiogenic events can exist independent of changes that affect cell growth rate, differentiation, and the transformed state. Further, the delay in growth for non-angiogenic tumors demonstrates that pro-angiogenic events are required for macroscopic tumor growth, and the kinetics of that delay suggest that two such changes were necessary in this model. This notion is bolstered by the observation that a significant fraction (21%-52%) of clones from angiogenic tumors exhibited an intermediate phenotype in which a fraction of tumors exhibited growth indistinguishable from angiogenic clones and a fraction exhibited growth indistinguishable from non-angiogenic clones. One possible explanation for such behavior would be that the initiating cell clone had lost one, but not all of the changes necessary to become angiogenic. As this clone was then expanded, additional changes would result in some subpopulations that were angiogenic and some that were non-angiogenic. This phenotype was then manifest when these cell populations were injected in vivo.

While there are inherent limitations to studying expression changes in cell culture to explain complex in vivo phenomena, the fact that our analysis identified several genes previously demonstrated to be critically involved in regulating angiogenesis indicates that at least a portion of these changes also occur in vivo. In this study we observed several
differences in gene expression in cultured cells that later proved to have reverted to the non-angiogenic phenotype. Marked among these was the up-regulation of the anti-angiogenic protein thrombospondin-1. Interestingly, we did not observe a difference in VEGF expression that could account for the differences in growth that we noted. However, one caveat to these studies is that VEGF expression that we observed in vitro may not completely reflect VEGF expression in the tumor implant. For example, VEGF might be upregulated by the hypoxic environment in the nascent tumor or stimulated in the tumor microenvironment. Importantly, however, this effect is likely to be similar for both the angiogenic and nonangiogenic tumors, and may even be accentuated by the extended period of hypoxia experienced by the nonangiogenic tumors. In our broader examination of the transcriptome of angiogenic and nonangiogenic cell types, additional differences in gene expression were observed. Many of the most highly upregulated genes in revertant clones have been previously implicated in tumor progression and/or metastasis (35-43) This might suggest that upregulation of these genes in revertant clones is a secondary effect of the overall decrease in angiogenesis induced by these cells and that the primary determinants of the reduced angiogenic stimulus remain to be determined.

Pathway and gene ontological analysis of differentially expressed genes in angiogenic and revertant clones highlighted a number of possible mechanisms for the differential ability of these cells to stimulate tumor growth beyond a particular size. A number of genes first identified for their roles in neuronal axon-guidance signaling, including ephrins and their receptors, semaphorins and plexin receptors were differentially regulated in the revertant and angiogenic clones. These signals for axon guidance have since been demonstrated to have important roles in the regulation of angiogenesis. In addition, extracellular matrix genes such as thrombospondin 2, fibronectin 1, laminin alpha IV, collagen type V alpha 1, and ADAMTS9 were differentially expressed in angiogenic liposarcoma clones in comparison with revertant clones. Finally, revertant clones had an elevated expression of class II major histocompatibility molecules in comparison with angiogenic clones, resulting in an overrepresentation of many
canonical pathways related to immune responses. Increased expression of these genes in revertant clones may indicate that a more efficacious presentation of tumor antigens to immune effector cells was in operation in this model. Subsequently, a more effective immune response in comparison with the angiogenic clones may have occurred when implanted in vivo, resulting in retardation of revertant clone growth. However, as these experiments were conducted in SCID mice, which lack B and T lymphocytes, natural killer cells would be the effectors of any immune response in this model (44).

These studies raise an important question: If tumor growth initiates when a bare minimum of tumor cells exhibit a pro-angiogenic phenotype and tumor cells are constantly reverting to the non-angiogenic phenotype, why do so few tumors cease growth and/or spontaneously regress? Our results suggest that there must be some hysteresis in the angiogenic switch. At least two processes may underly this property of the angiogenic switch. First, there are indications that the threshold for the initiation of angiogenesis is higher than that for its continuation. Processes such as pericyte dissociation and degradation of basement membrane (with its associated inhibitors) once accomplished need not be repeated for vessels to be extended. Second, once a tumor has begun to expand there is more (angiogenic factor producing) tumor mass available to supply angiogenic factors (such as VEGF, HGF etc.) to a given region of the tumor periphery. Thus for a tumor whose radius has expanded 2-fold, the fraction of angiogenic cells can be reduced by a similar amount without compromising the ability of the tumor to induce blood vessels at its periphery. Indeed, once established, a tumor can expand even if a fraction of the periphery of the tumor has become net non-angiogenic. In that case, the tumor will not expand in all directions, but will become irregular in shape as only the angiogenic portion of the tumor expands.

The specific molecular basis for the change of a tumor cell from the angiogenic to non-angiogenic phenotype and its subsequent reversal is currently unclear. There is evidence in the literature that the angiogenic phenotype can be regulated by mutations to specific oncogenes
and tumor suppressors (45, 46), however these mutations are typically associated with substantial changes in cell growth and/or survival that we do not observe in our clones. There are multiple possible mechanistic explanations for the observed reversion in phenotype. For example, an epigenetic event, such as methylation of the tsp-1 promoter, which gave rise to a pro-angiogenic phenotype could be lost, restoring expression. Also, an increase in copy number of a gene encoding a pro-angiogenic gene could be lost or an increase in copy number of gene encoding an anti-angiogenic gene could be acquired in the revertant cell line. Finally, a mutation could arise downstream of a gene in a pathway that drives angiogenesis and results in the overexpression of an angiogenic factor or loss of an anti-angiogenic factor (47). The identification of the specific events that drive reversion to the dormant phenotype is an active area of investigation for us, as the precise understanding of this process may have clinical implications. If angiogenic tumor cells can be induced to exhibit a non-angiogenic phenotype, or if non-angiogenic clones can be given a selective advantage, then it may be possible to reverse the angiogenic switch in a given tumor. Alternatively, if the reversion to the dormant phenotype is governed by a genetically-driven increase in the expression of a secreted anti-angiogenic protein, such as Tsp-1, then tumors could be reverted to dormancy by stimulating that pathway. The conversion of an actively growing tumor to the dormant phenotype would thus render that tumor incapable of further growth and harmless to cancer patients, significantly extending their lives.

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References:


Figure Legends

Figure 1. Growth and survival of angiogenic vs. non-angiogenic liposarcoma xenografts and analysis of escape kinetics. A) Size of subcutaneous xenografts of $5 \times 10^6$ liposarcoma cells from an angiogenic (red) and non-angiogenic (black) clone originating from the same human tumor. B) Freedom from palpable tumors of mice bearing these tumors. C) The time of escape for each of 80 mice is plotted (◊) along with the fraction of mice that have not yet had their tumor escape from dormancy. Best fit 1 and 2 event models are indicated by the dashed and solid curves, respectively. Residual fraction remaining for a 2-event model is also indicated (-). D) Tails and residuals of the curve fit highlighting differences in the model fits.

Figure 2. Scheme for serial cloning, expansion, and phenotypic analysis of human liposarcoma cells.

Figure 3. Growth pattern of clones derived from highly angiogenic tumor cell lines. Left: examples of growth patterns observed for the 2$^{nd}$ (top), 3$^{rd}$ (middle), and 4$^{th}$ (bottom) $in vivo$ passage of the most angiogenic clone from the previous cycle. Middle: The fraction of clones exhibiting each of the phenotypes shown on the left. Right: Tumor-free survival for clones exhibiting each of the three phenotypes shown on the left.

Figure 4. Gross appearance of tumors in mice. A) Mice bearing an angiogenic clone. B) Time-matched mice bearing a revertant clone. C) Appearance of non-angiogenic clone in the subcutaneous space.
Figure 5. Cell division rate of angiogenic and non-angiogenic liposarcoma clones. Left: 
*In vitro* proliferation of angiogenic (gray) and non-angiogenic (black) clones in cell culture as 
measured by cell counting.

Figure 6. Expression of known angiogenesis modulators in angiogenic and revertant cell 
lines. A) Western blot detection of thrombospondin-1 (TSP-1) and VEGF expression in the 
indicated angiogenic and revertant clones, with beta-actin as loading control. B) ELISA 
measurement of thrombospondin-1 production comparing the original angiogenic and non- 
angiogenic cell line pair vs. representative angiogenic (clone 29) and revertant (clone 10) 
clones, derived in passage two. C) ELISA measurement of VEGF production in the indicated 
cell lines, as outlined in panel B. Bars represent mean values with SEM. Student-t test was used 
to compare various groups. D) HMVEC-d migration to wells containing angiogenic (Clone 1, 
29) and revertant (Clone 2, 10) cell lines cultured in serum-free media. Differences between 
each angiogenic line and each revertant line were significant (P<0.05 by ANOVA).

Figure 7. Real-time RT-PCR analysis of genes identified as differentially regulated in 
angiogenic and revertant clones. Expression of genes identified by microarray as 
differentially regulated was assessed by real-time polymerase chain reaction following reverse- 
transcription using Taqman probes. Bars represent the ratio of gene expression, non- 
angiogenic/angiogenic (left of vertical axis), or angiogenic/non-angiogenic (right).
Figure 1
Figure 2

Human liposarcoma angiogenic clone

1st in vivo passage

Single cell clones are created and expanded in vitro

Each clone is tested in vivo for angiogenic potential

Select most angiogenic clone

Non-angiogenic clone?

2nd in vivo passage

3rd in vivo passage

4th in vivo passage

?
Examples of clonal phenotypes
(1 clone = 5 mice)

Angiogenic
Revertant
Hybrid

Angiogenic
Hybrid
Revertant

Angiogenic
Mixed
Revertant

Angiogenic
Hybrid
Revertant

Angiogenic
Hybrid
Revertant

Angiogenic
Hybrid
Revertant

Angiogenic
Hybrid
Revertant

Angiogenic
Hybrid
Revertant

Angiogenic
Hybrid
Revertant

Angiogenic
Hybrid
Revertant

Figure 3
<table>
<thead>
<tr>
<th>Angiogenic clone</th>
<th>Angiogenic clone which reverted to the nonangiogenic phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
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</table>

Figure 4
Figure 6
Up-regulated in Revertant clones

CXCR4
MIA
Pleiotrophin
ADAM23
TNF
ADAM20
ROBO1
CD33
MMP9
TSP-1
Spondin-2
TNFR-1
ADAMTS9
NF-κB
BRCA-1
CYP26A
TP53
IL12A
TRAF1
LCP1
HSPB2

CD95/Fas
VEGF
DAXX
ID2
PPARγ
RARRES2
STAT4
SEMA5B
PDGFb
IL12B

Up-regulated in Angiogenic clones

Angiogenic/revertant fold difference in cDNA
Molecular Cancer Research

Spontaneous Reversion of the Angiogenic Phenotype to a Non-angiogenic and Dormant State in Human Tumors

Michael S. Rogers, Katherine Novak, David Zurakowski, et al.

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