American Journal of Pathology, Vol. 161, No. 1, July 2002

Commentary

Molecular Profiling of Angiogenic Markers

A Step Towards Interpretive Analysis of a Complex Biological Function

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Gene expression profiling is now being used routinely to define complex biological events. The profiling of a large array of genes expressed in the progression of a biological response opens the door to our understanding the unique relationships between genes and their functions. Angiogenesis, the process of new blood vessel development, is a necessary component of both normal and pathological physiology. In this issue of *The American Journal of Pathology*, Shih and colleagues[1] have used quantitative molecular profiling of angiogenic-related factors to define some of the elements required for angiogenic profiling. Although presented as a technical advance, the basic concept of this work is that the use of quantitative molecular profiling of gene expression gives additional insight into functional interrelationships between the genes expressed during the angiogenic process. This approach can be applied in large array format as a diagnostic tool for experimental systems and pathological samples.

During the last two decades, an explosion in our understanding of angiogenesis at the molecular level has occurred. These advances have been facilitated by effective angiogenic models consisting of endothelial capillary tube-like formation *in vitro*,[2–7] *in vivo* angiogenesis in avian chorioallantoic membrane,[2,8,9] and angiogenesis associated with angiogenic or inflammatory cytokine-impregnated implants in mammals.[10–16] A significant addition to these models has been the use of genetic knockouts in mice to test the relevance of angiogenic genes *in vivo*.[17–21] Defects that occur, if any, in the developing vascular tree would indicate that the null gene is required for vasculogenesis or angiogenic events that occur during pre- and early postnatal development. From these and other functional models, the molecules involved in select events required for the angiogenic process to occur, including endothelial cell-specific proliferation, migration, cell-cell association, and vessel morphogenesis, have been partially defined.

Angiogenic cytokines and their receptors have been identified as key regulators of the angiogenic process. Central to the activation and maintenance of the neovasculature are members of the vascular endothelial growth factor family, VEGF-A, -B, -C, -D, and placental growth factor (PLGF), and the VEGF receptors, VEGF-R1, -R2, -R3, and R4.[7,22–26] In addition, the secreted angiopoietin, Ang-1 acts on the Tie-2 receptor to stabilize the vascular structure. Antagonism of the Tie-2 activity by the cytokine-induced Ang-2 is indicative of vascular destabilization and may be a early priming step in the angiogenic pathway.[5,27–29]

Several important molecular profiling analyses have been performed on endothelium undergoing cytokine activation and during the angiogenic process *in vitro* and *in vivo*.[6,30–36] These studies have used a number of techniques including differential and subtractive hybridization, differential display, GeneCalling,[37] serial analysis of gene expression (SAGE), and cDNA arrays and microarrays. These studies have defined differentially expressed genes that are most likely to play a role in the angiogenic process. The genes identified fall into a number of protein subclasses such as secreted proteins, extracellular matrix, metalloproteinase, receptors, junctional molecules, protease inhibitors, transporters/channels, and miscellaneous cell surface proteins as was comprehensively presented by Peale et al.[34] These differential expression

Supported by National Institutes of Health:National Cancer Institute grant CA64436.

Accepted for publication May 6, 2002.

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Table 1. Involvement of Functional Genes in Various Stages of the Angiogenic Process

	Angiogenic processes			
Protein type	Endothelial cell activation	Stromal cell/matrix modulation	Proliferative expansion	Vascular morphogenesis/ maturation
Secreted proteins/ lipids	VEGF, FGF-2, Ang-2, LPS, TNFa, HGF	IL-8, Gro-Ia, IL-2, MCP-1, TGFb, HGF, CTGF, MIP-1a	VEGF, FGF-2, HGF, PLGF, jagged	Ang-1, S-1-P, BMP-6, VEGF, ephrins
Receptors	VEGF-R2, FGF-R, Tie-2, E-selectin, VCAM-1	FGF-R (flg) FGF-R (bek) CXCR4	VEGF-R2 VEGF-R1, Tie-1, notch	VEGF-R1 Tie-1, Edg-1, Eph B4, PDGF-R
Cell migration	Integrin aVb3, integrin a2, integrin a5, Fn, Vn, osteopontin	Collagen I, III, VI	Integrin aVb3, Fn, Vn, denatured collagen	SPARC, hevin, TSP-1,-2
Extracellular matrix remodeling activators/inhibitors	t-PA, u-PA, MMP-2, MMP-9	MMP-3, MMP-2, MMP-9, MMP-11, ADAMTS-1,-4	u-PA, UPAR, Pai-1	Laminin, collagen IV, TSP-1,-2, Pai-1, TFPI-1,-2
Cell-cell junctions			PECAM-1 (CD31)	VE-cadherin, connexin 37, connexin 45

Matrix description of how genes involved in endothelial cell regulation would contribute to various functions over the course of angiogenic events ranging from early endothelial cell activation, proliferative expansion, and maturation. The stromal/inflammatory mediators will likely promote angiogenesis at any stage and thus are placed in the middle.

studies have provided an essential first step in defining the boundaries for the molecular characterization of the angiogenic process beginning from the initial activation events to vascular maturation and stabilization.

There are important distinctions, however, between genes induced in the process and how these genes might interact with each other to modulate function. For example, in the molecular profiling of angiogenic markers by Shih et al,[1] an important step toward defining the relationship between genes involved in the angiogenic process has been achieved. Taqman analysis was used for quantification, using standards that quantify the actual copy number of the target gene, compared to cyclophilin, an internal control. Data from this method allows for the direct comparison of the mRNA levels for several genes in the same sample. This can have important implications in the interpretation of the role for these genes in the angiogenic process. For example, a direct comparison of the expression of competing ligands for the same receptor, as is the case for Ang-1 and Ang-2, can be determined. One interesting observation from this study of two different prostatic tumors from the transgenic adenocarcinoma of mouse prostate (TRAMP) model has revealed an inverse relationship between Ang-2 and Tie-2 expression. The larger tumor had higher Ang-2 and less Tie-2 than the tumor of roughly half its size. Overexpression of both VEGF and Ang-2 also correlated with higher levels of the endothelial markers, kinase-domain receptor (KDR) (VEGF-R2), fems-like tyrosine kinase-1 (Flt-1) (VEGF-R1), VE-cadherin, platelet endothelial cell adhesion molecule-1 (PECAM-1), and Tie-1. Future studies such as these will likely provide the quantitative data for which mathematical algorithms may be designed to form a molecular profile for different stages in the tumor angiogenic process.

To date, most molecular profiling approaches have taken a differential expression approach, ie, comparing the relative expression patterns of genes induced or repressed genes under different physiological circumstances. Several groups have utilized computerized database extraction and functional assignment of genes found to be differentially expressed in the angiogenic process.[34,38,39] However, without quantitative analysis of each of these genes, it is difficult to interpret the stages in the angiogenic process. To capture the true state of a complex biological process with quantitative gene expression data, the interrelationship of genes expressed at any one time will have to be critically modeled and tested in experimental systems. Thus, differential expression combined with how the differentially expressed genes might interact together to influence a complex process such as angiogenesis will be required for effective molecular profiling. An example of the complexity presented by this approach is given in Table 1, where the expression of genes that might be involved in the various stages of angiogenesis is tabulated as functional protein classes compared to the stages or targets in the angiogenic process. For example, during initial endothelial cell activation, the levels of angiogenic cytokines, such as VEGF, fibroblast growth factor (FGF)-2, and Ang-2, requires the action of VEGF-R, FGF-2, and Tie-2 on susceptible local microvasculature. The cytokine activation of local vasculature would then induce endothelial cell adhesion molecules such as E- and P-selectin and vascular cell adhe-

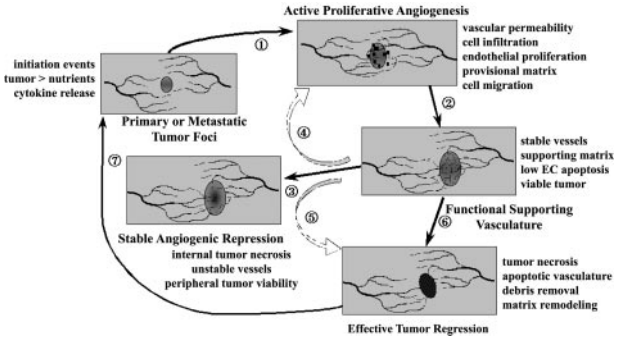

Figure 1. Model of tumor angiogenic stages in relation to therapeutic intervention. Representation of the angiogenic stages in the initiation and expansion of tumor as well as the possible result for therapeutic intervention. Text indicates angiogenic events, cell types, and changes in microenvironment for each stage. Transitions between stages are indicated as **1:** active and proliferative angiogenic response, **2:** maturation into functional tumor vasculature, **3:** effective anti-angiogenic therapy to derive a balance between tumor support and apoptosis, **4:** re-entry of treated tumor into active angiogenesis due to increased expression of angiogenic stimuli from affected tumor or therapeutic relapse, **5:** anti-angiogenic therapy alone that will benefit from self-reactivity to stable disease, **6:** effective multi-modal therapeutics effective for blocking both angiogenic and tumor growth events, **7:** long-term relapse from a small number of therapeutically resistant or static cells selected for survival.

sion molecule-1 (VCAM-1), thus promoting inflammatory cell infiltration (ie, monocytes and macrophage). Thus, the ratio of activators to inhibitors for functional events will help to identify the total angiogenic potential in the sample. It is anticipated that further definition of the interrelationship of a number of gene classes might define the angiogenic process, with respect to its physiological or pathophysiological mechanism, can be achieved.

Validation of the interrelationship models that might be developed from quantitative molecular profiling is not as easy as might be assumed. The molecular profiling of mRNA levels by arrays or quantitative analyses still does not define variations in protein synthesis and post-translational modifications that might affect their function. This is especially relevant for signal transduction kinases that are in a state of constant flux due to cellular signaling from the microenvironment and cytokine activation. Also, many proteins such as metalloproteinases are expressed in pro-forms, and thus the mRNA levels do not necessarily relate to their active levels. It is clear that validation of functional gene expression by direct *in situ* methods such as immunohistochemistry and activity assays will define cell-type specificity for key proteins involved in one or more elements of the angiogenic process and which of these may be appropriate markers for molecular profiling.

An additional complication with the analysis of total gene profiles in complicated biological processes such as angiogenesis is the diversity of cellular populations that are present within the tissue. For example, during exponential or late stage tumor growth, the types of endothelial markers would include those for infiltrating precursors from the circulation,[40,41] proliferating endothelial cells (ECs), newly formed tube structures, mature tube structures, and even vessels undergoing apoptosis. Thus, the total EC-specific gene expression profile would be a mixture of all of the endothelial pools that are likely to have very different gene expression ratios at any one time. The data presented by Shih et al,[1] give some insight into this possibility. Analysis of the two different sized prostatic tumors showed some similarities, but demonstrated hypoxic-induced VEGF in the larger tumor and the coordinate expression of the higher levels of Ang-2 and somewhat repressed Tie-2, compared to the smaller tumor.

A model of the stages in tumor angiogenesis and the implications for a tumor undergoing therapies directed at the angiogenic process alone or combined therapeutic targets is given in Figure 1. The result of a partially effective anti-angiogenic therapy is likely to induce some tumor hypoxia and may potentiate tumor re-entry into an active angiogenic response by the up-regulation of VEGF and/or FGF-2. Alternatively, effective combination therapies directed toward angiogenic and tumor growth processes could lead to tumor regression which has remained an elusive goal with the anti-angiogenic strategies. The molecular profiles that are obtained from these different stages of tumor growth or treatment will be important for establishing the usefulness of this approach for evaluating the therapeu-

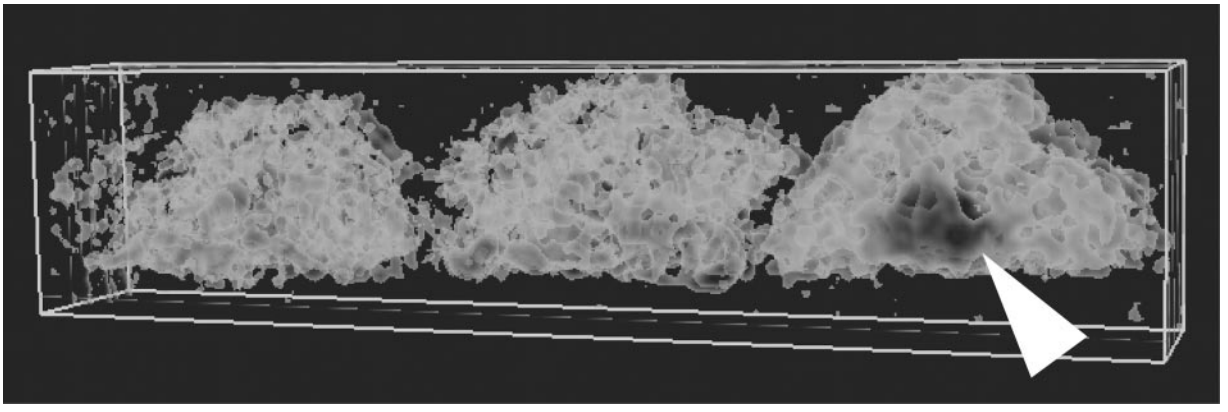

Figure 2. Diagnostic three-dimensional functional MRI identification of angiogenic prostate tumor in mice. Three mice were scanned without contrast agent in a 1.5 Tesla MR using a peripheral coil across the lower abdomen for development of orthotopically injected PC-3 prostate tumors. Functional MRI parameters were computed for the inverse transition rate (R_2') and the images aligned and reconstructed in 3-D. Animal on right indicates a tumor with prominent R_2' signal (gray, **arrow**) on the periphery of a defined tumor later confirmed by dissection and histological analysis. Note the differential levels of gray signal ranging from light to dark foci. The normal tissues appear as mosaic white/light gray.

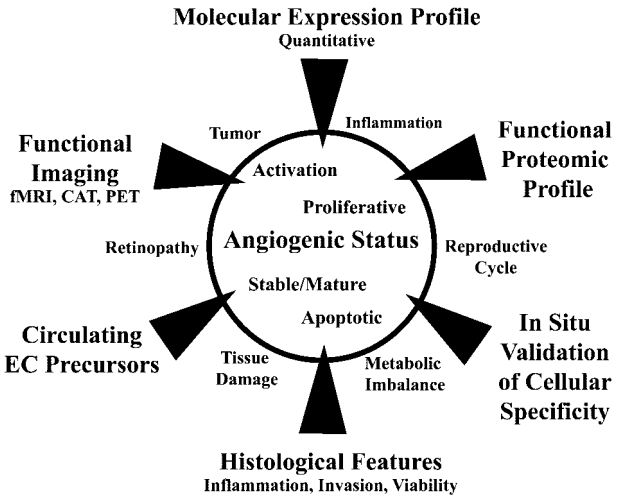

Figure 3. A multiplex approach to profiling the complex angiogenic process. Analytical and quantitative profiling methodologies are defined on the spokes around the central angiogenic processes. Given the variability in physiological and pathophysiological angiogenic activators, some of which are indicated around the central process, the analysis of the angiogenic responses in each condition may define specific molecular profiles for each.

tic effectiveness of anti-angiogenic therapies, either alone or in combination with other tumor-targeted approaches.

Combined interrelational algorithms applied to static molecular quantitative gene expression data will still have significant voids with regard to the variability in time and space within each sample. The future for resolving these kinds of temporal and spatial distinctions will likely be the combination of molecular profiling with a direct analysis of biological function. New methods for non-invasive imaging technologies that define functional vasculature and its relation to the level of tumor viability and growth are currently being developed.[42–46] The application of functional magnetic resonance imaging (MRI) has been a promising avenue to examine the angiogenic process. An example of one such application is shown in Figure 2, where the use of a functional MRI screen based on the magnetic inhomogeneity related to the oxy/deoxy-hemoglobin conversion which can delineate differences between an animal with a prostatic tumor from a normal one. This non-invasive method does not require contrast agents and easily thresholds the angiogenic tumor-derived signals from that of surrounding normal tissue. These types of imaging technologies define areas where there is significant blood flow and utilization by the expanding tumor (delineated by dark gray in the tumor), and tumor areas that are not metabolically active or necrotic. Functional imaging combined with molecular and proteomic profiling analyses might provide a better overall interpretation of the angiogenic status of complex tumor physiology.

The future of molecular profiling will likely use detailed information from advanced imaging, molecular and proteomic profiling, and *in situ* analyses to define interpretive models that are predictive of biological behavior. Multiplex analysis of the angiogenic process is presented in Figure 3, and implies that analyses of a complex biological process will likely require a multi-modal approach. Integration of data from multiple methodologies by advanced computational models will likely be needed to validate a smaller number of key molecular and proteomic profile gene targets necessary to interpret the angiogenic status of any particular sample. Although many of these candidate genes might be known at the current time, the actual expression ratio between each of them or between different functional classes combined with their positive or negative effects on angiogenic events remain to be determined. In short, the ability to provide quantitative data with respect to both molecular and proteomic profiling of angiogenic markers is essential to the interpretation of this complex biological process.

Acknowledgments

I thank Ia Gomez-Garcia, Jason Kirk, and Lahmer Lynds for graphic and technical assistance.

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