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 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, in vivo angiogenesis in avian chorioallantoic membrane, and angiogenesis associated with angiogenic or inflammatory cytokine-impregnated implants in mammals. A significant addition to these models has been the use of genetic knockouts in mice to test the relevance of angiogenic genes in vivo. 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 neovascularure 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. 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.

Several important molecular profiling analyses have been performed on endothelium undergoing cytokine activation and during the angiogenic process in vitro and in vivo. These studies have used a number of techniques including differential and subtractive hybridization, differential display, GeneCalling, 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. These differential expression

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Involvement of Functional Genes in Various Stages of the Angiogenic Process

<table>
<thead>
<tr>
<th>Protein type</th>
<th>Angiogenic processes</th>
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<tbody>
<tr>
<td></td>
<td>Endothelial cell activation</td>
</tr>
<tr>
<td>Secreted proteins/lipids</td>
<td>VEGF, FGF-2, Ang-2, LPS, TNFa, HGF</td>
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<tr>
<td>Receptors</td>
<td>VEGF-R2, FGF-R, Tie-2, E-selectin, VCAM-1</td>
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<tr>
<td>Cell migration</td>
<td>Integrin αβ3, integrin α2, integrin α5, Fn, Vn, osteopontin</td>
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<tr>
<td>Extracellular matrix</td>
<td>t-PA, u-PA, MMP-2, MMP-9</td>
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<td>remodeling activators/inhibitors</td>
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<td>Cell-cell junctions</td>
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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.
survival.

from a small number of therapeutically resistant or static cells selected for self-reactivity to stable disease, 4: effective anti-angiogenic therapy to derive a balance between tumor support and apoptosis, 3: anti-angiogenic therapy alone that will benefit from 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.

ion 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, 3 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 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.

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 (R2') and the images aligned and reconstructed in 3-D. Animal on right indicates a tumor with prominent R2' 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.
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

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References

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