

# Report from the Society for Biological Therapy and Vascular Biology Faculty of the NCI Workshop on Angiogenesis Monitoring

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**Abstract:** The field of tumor angiogenesis has seen explosive growth over the last 5 years. Preclinical as well as early clinical evaluation of novel compounds is progressing at a rapid pace. To gain a perspective on the field and to take stock of advances in the understanding of molecular mechanisms underlying the process of tumor angiogenesis as well as ways of monitoring the activity of agents, the Society for Biologic Therapy and the National Cancer Institute's Vascular Biology Faculty convened a Workshop on Angiogenesis Monitoring in November 2002. The Workshop was composed of invited speakers and participants from academia, industry, and government. It was divided into 3 sessions, each chaired by leaders in the field. The first focused on advances in the understanding of the cellular and molecular mechanisms of angiogenesis in tumors. The second examined preclinical assay systems that are useful in vascular biology. The third addressed the translation to the clinic and monitoring of antiangiogenic activity of agents in patients and novel trial designs. What follows is a summary of the discussions and findings of each session.

**Key Words:** tumor, models, angiogenesis, therapy

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## SESSION 1: UNDERSTANDING ANGIOGENESIS THROUGH MOLECULAR MECHANISMS

### Pathways of Endothelial Cell Survival Based on Activation of Raf Kinase

David Cheresch of The Scripps Research Institute, La Jolla, began the session with a discussion of the importance of growth factor-mediated signaling in endothelial cell survival. Growth factors promote endothelial cell survival through mul-

iple mechanisms involving the activation of Raf kinase.<sup>1,2</sup> In studies of the role of integrins in endothelial cell survival during angiogenesis, Dr Cheresch reported that  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrins are up-regulated on growing blood vessels.<sup>3</sup> Inhibition of these integrins triggers apoptosis of endothelial cells, inhibits new vessel growth, and suppresses the growth of tumors.<sup>4,5</sup>

At least 25 growth factors can stimulate angiogenesis. Dr Cheresch focused on basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which have very different vascular effects. bFGF-induced angiogenesis can be blocked by inhibitors of  $\alpha_v\beta_3$  integrin, whereas VEGF-induced angiogenesis is blocked by inhibitors of  $\alpha_v\beta_5$  integrin.<sup>6,7</sup> Angiogenesis induced by IL-8 or TNF- $\alpha$  resembles that produced by bFGF in its dependency on  $\alpha_v\beta_3$  integrin. Angiogenesis mediated by TGF- $\beta$  and phorbol ester is similar to that produced by VEGF in its dependency on  $\alpha_v\beta_5$  integrin. Despite these differences, all of these factors act through intracellular signaling events involving the map kinase pathway initiated by Raf activation. According to Dr Cheresch's work, Raf kinase is a central player in angiogenesis, regardless of the stimulus, and may be the first common element of the bFGF and VEGF pathways.

Why should there be multiple pathways of angiogenesis? Perhaps different mechanisms are involved in different organs or different conditions of angiogenesis. A more complete understanding of how growth factor receptors mediate intracellular signaling events may make it possible to inhibit angiogenesis in tumors while leaving intact angiogenesis associated with wound repair or inflammation.

bFGF and VEGF activate Raf by two distinct mechanisms. bFGF activates its receptor together with  $\alpha_v\beta_3$  integrin, which in turn activates Raf in a pathway dependent on P21-activated kinase (PAK1). Specifically, bFGF stimulates Raf-dependent phosphorylation of tyrosine-338 of PAK1 through an  $\alpha_v\beta_3$  integrin-dependent mechanism. Angiogenesis mediated by bFGF depends on PAK1, but VEGF-stimulated vessel growth does not. VEGF, acting on its receptors together with  $\alpha_v\beta_5$  integrin, activates Raf through a pathway dependent on

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protein kinase C (PKC) and phosphorylation of Src kinase, not PAK1. Inhibition of PKC or Src kinase blocks Raf activation in response to VEGF but not to bFGF. Further, VEGF activation of extracellular signal-regulated protein kinase (Erk) is dependent on both PKC and Src, whereas bFGF activation of Erk is independent of both of these kinases.<sup>8,9</sup> These 2 pathways of Raf activation have different effects on endothelial cells and different consequences. These differences may explain the contrasting effects of the 2 growth factors on vascular permeability, for example, even though both are endothelial cell mitogens.

The central role of Raf in bFGF- and VEGF-mediated angiogenic pathways was shown by delivering, by retroviral transfection, an inactive mutant form of Raf to the chick chorioallantoic membrane. Raf inactivation suppressed Raf kinase activation and blocked angiogenesis produced either by bFGF or VEGF.

What are the consequences of Raf activation? Raf is known to participate not only in cell proliferation but also in cell survival and may play a central role in survival of endothelial cells. Once activated, Raf translocates to mitochondria, where it leads to phosphorylation and removal of the Bcl-2 family member Bad, inhibition of caspases, and increased cell survival. Removal of Bad from mitochondria prevents stress-induced apoptotic cell death. Consistent with this finding, Raf knockout mice die in utero with widespread vascular apoptosis.

Dr Cheresch found that bFGF, but not VEGF, drives Raf into the mitochondria of endothelial cells. Mutation of Raf at the PAK1 site that blocks bFGF-mediated Raf activation completely abolishes Raf translocation to mitochondria. By comparison, mutation of the Src site that blocks VEGF-mediated activation of Raf does not block entry of Raf into mitochondria. bFGF-mediated endothelial cell survival and protection against stress are thus dependent on Raf translocation to mitochondria. bFGF is better than VEGF at protecting endothelial cells against stress-mediated death associated with starvation, reduced nutrients, hypoxia, certain drugs, and radiation. In contrast, VEGF is more effective than bFGF at protecting endothelial cells against apoptosis mediated by Fas ligand or inflammatory mediators. VEGF-mediated protection is dependent on the Src activation site of Raf.

In summary, both bFGF and VEGF can activate angiogenesis, promote blood vessel growth, and favor endothelial cell survival through Raf activation. However, the growth factors have different effects on endothelial cells and provide different kinds of protection depending on the death-inducing stimulus because different receptors, integrins, and differential activation of Raf are involved.

### Recruitment of Bone Marrow Stem Cells to Tumor Vessels

Shahin Rafii of Cornell University Medical College, New York, discussed the origin of endothelial cells in growing

blood vessels in tumors, with a focus on the contribution of bone marrow stem cells.<sup>10</sup> The traditional view is that angiogenic factors released from tumors stimulate the growth of new blood vessels from existing blood vessels. Tumors may also coopt normal blood vessels for their own use. Yet, recent studies suggest that some endothelial cells in tumor vessels derive from circulating stem cells rather than from endothelial sprouts or coopted vessels. According to this concept, chemical signals from tumors mobilize stem cells in bone marrow and recruit them to sites of angiogenesis in tumors.

Many types of bone marrow-derived stem cells have been identified, but it is unclear how these cells are mobilized, recruited, and integrated into their target tissues. In the bone marrow, most stem cells are close to and receive survival signals from stromal cells.<sup>10</sup> After mobilization, stem cells lose these signals and undergo apoptosis unless exposed to new survival signals.

To determine whether stem cells contribute to tumor blood vessels and, if so, how they are recruited, Dr Rafii used a novel model involving Id1<sup>+/+</sup>Id3<sup>-/-</sup> double-knockout mice developed in conjunction with Drs Benezra and Lyden at Memorial Sloan-Kettering Cancer Center in New York.<sup>11</sup> These mice appear reasonably normal but resist the development of tumors because of a defect in angiogenesis. Experiments with this model show that transplantation of bone marrow stem cells can restore angiogenesis and tumor growth in these mice, apparently because stem cells become incorporated into the tumor vasculature.<sup>11</sup>

Lymphomas and thymomas in mice have been found to recruit stem cells to the vasculature during the initial phase of tumor growth. The stem cells uniformly express the receptor tyrosine kinase VEGFR-2 (VEGF receptor 2; human homologue KDR; mouse homologue Flk1). In lymphomas, 95% of the vessels contain stem cells initially, but the proportion falls to 75% at 10 days after implantation. By comparison, only 25–30% of vessels in thymomas recruit stem cells to the vasculature. In breast cancer the number is even smaller, and many other tumors do not recruit any stem cells to their vasculature. These tumor-related differences suggest that chemokines such as VEGF released from tumors recruit stem cells to growing vasculature.

The defect in angiogenesis in Id1<sup>+/+</sup>Id3<sup>-/-</sup> mice results from the impairment of VEGF-driven mobilization of stem cells from bone marrow.<sup>11</sup> Two types of stem cells are necessary for angiogenesis in lymphomas:<sup>1</sup> stem cells that express VEGFR-2 differentiate into endothelial cells; and stem cells that express VEGFR-1 (VEGF receptor 1; Flt1) serve as hematopoietic support cells for angiogenesis.<sup>2</sup> VEGFR-2 signaling is well known to mediate the proliferation, migration, and survival of endothelial cells, but less well understood is the role of VEGFR-1, which is expressed on endothelial cells and hematopoietic cells but is not essential for endothelial cell proliferation.<sup>12</sup>

Although VEGF binds to VEGFR-1 and VEGFR-2, placental growth factor (PlGF) binds only to VEGFR-1. This property of PlGF provides a strategy for selectively activating this receptor in endothelial cells and hematopoietic cells. PlGF recruits hematopoietic VEGFR-1<sup>+</sup>/CD11B<sup>+</sup> stem cells to tumor vessels, where they interact with but do not differentiate into endothelial cells. Inhibition of VEGFR-1 blocks this recruitment and delays tumor growth. Inhibition of VEGFR-2 suppresses tumor angiogenesis and reduces tumor growth. Inhibition of both VEGFR-1 and VEGFR-2 completely stops tumor growth. Therefore, in Id1<sup>+/+</sup>Id3<sup>-/-</sup> mice, recruitment of these two types of VEGF-responsive stem cells is necessary and sufficient for tumor angiogenesis.

One explanation of these results is that angiogenesis in certain tumors depends on hematopoiesis. Cross talk between VEGFR-1<sup>+</sup> hematopoietic stem cells and VEGFR-2<sup>+</sup> endothelial progenitor cells that are comobilized to tumor vessels may facilitate the differentiation and engraftment of endothelial cells into vessel walls. To understand the function of VEGFR-1 in hematopoietic cells, hematopoiesis was blocked by treatment of mice with the chemotherapeutic agent, 5-fluorouracil (5-FU). Elimination of hematopoietic cells with 5-FU activates embryonic programming of bone marrow stem cells to reconstitute hematopoiesis. This approach revealed that recovery of hematopoiesis is dependent on VEGFR-1 but not VEGFR-2.

In conclusion, VEGFR-2 activation triggers the release from bone marrow of endothelial progenitor cells that contribute to the expansion of the vasculature of some tumors. VEGFR-1 activation releases hematopoietic stem cells into the bloodstream, where they transit to the tumor vasculature and support tumor growth through effects on endothelial cell differentiation. VEGFR-1<sup>+</sup> hematopoietic stem cells and VEGFR-2<sup>+</sup> endothelial stem cells thus both contribute to tumor angiogenesis and growth. VEGF, through its action on VEGFR-1 and VEGFR-2, mobilizes stem cells. Blocking this mobilization is thus a potential strategy for reducing tumor angiogenesis. Finally, VEGFR-1 and VEGFR-2 on stem cells provide potential surrogate markers for assessing response to chemotherapy and evaluating tumor angiogenesis. Clinical trials may help to determine whether angiogenesis and tumor growth can be suppressed by blocking VEGFR-1 and VEGFR-2 in humans.

### Abnormalities of Tumor Vessel Structure and Function

Donald McDonald of the University of California, San Francisco, described the structural and functional abnormalities of blood vessels in tumors. These abnormalities are driven by changes in gene expression and environmental cues that alter the production of growth factors, receptors, transcription factors, and other molecules that influence angiogenesis and vascular stability in tumors. Although angiogenesis occurs in

some normal organs and is a feature in many chronic diseases, angiogenesis in tumors is unusual because the newly formed vessels have multiple abnormalities. The angiogenic switch in tumors activates the proliferation of blood vessels with features not found in other conditions.

Like normal blood vessels, tumor vessels have three components: endothelial cells, pericytes (mural cells), and basement membrane. The three components have multiple abnormalities that have been documented by genomics, proteomics, *in vivo* phage display, *in situ* hybridization, immunohistochemistry, and a variety of other approaches.

In normal blood vessels, tight junctions between adjacent endothelial cells create a barrier to extravasation of macromolecules. Endothelial cells naturally form a monolayer both *in vivo* and *in vitro*. Even in most pathologic states, including chronic inflammatory conditions accompanied by angiogenesis, the endothelial monolayer is well organized, even though focal leakage sites may be present. By comparison, endothelial cells in tumors range from reasonably normal to having multiple structural abnormalities and exaggerated sprouting, proliferation, and regression.<sup>13</sup> The endothelial cells of tumor vessels do not have a normal barrier function. The mean pore cutoff size ranges from ~200 to 2000 nm, which is more than 10-fold larger than that in normal vessels.<sup>14</sup> Although blood vessels in all tumors are leaky, the leakiness varies with tumor type and location. Electron microscopic studies have shown that defects in the endothelial monolayer explain the leakiness.<sup>13</sup>

Pericytes are present in the wall of capillaries and small venules, where they occupy the same position as smooth muscle cells of arterioles and larger vessels.<sup>15</sup> Pericytes are intimately associated with endothelial cells and together are surrounded by a basement membrane. Signaling between endothelial cells and pericytes is key to normal vessel structure and function. Platelet-derived growth factor (PDGF) secreted from endothelial cells drives PDGF receptor  $\beta$  signaling that is essential for pericyte formation and function.<sup>16,17</sup> Although the presence of pericytes in tumors has been debated over the years, they are clearly present in most tumors screened for multiple markers, such as  $\alpha$ -smooth muscle actin, desmin, NG2, and PDGFR- $\beta$ .<sup>15,18</sup> Pericytes on tumor vessels are both structurally abnormal and heterogeneous in their expression of these markers. Absence of  $\alpha$ -smooth muscle actin immunoreactivity, for example, is not indicative of absence of pericytes. Pericytes on tumor vessels have an unusual morphology and an abnormally loose association with endothelial cells, suggestive of vascular instability.

The vascular basement membrane is a dynamic, self-assembled layer of proteins, glycoproteins, and proteoglycans formed by endothelial cells and pericytes. Major constituents include type IV collagen, laminin, nidogen (entactin), fibronectin, and the heparan sulfate proteoglycan perlecan. Although basement membrane completely covers most tumor

vessels, it is loosely associated with the endothelial cells and pericytes and may have multiple layers. These abnormalities are particularly conspicuous in rapidly growing tumors. Redundant layers of basement membrane, which are loosely associated with endothelial cells and pericytes, reflect continuous vascular growth, regression, and remodeling.

Abnormalities of tumor vessels provide potential targets for selective delivery of drugs to tumors. Gene expression profiling has identified proteins that are selectively expressed in endothelial cells of tumors. The utility of such molecules as targets depends on amount of expression, uniformity of distribution within tumors, and accessibility to drugs in the bloodstream. The integrins  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ , and  $\alpha_5\beta_1$ , are overexpressed on tumor vessels.  $\alpha_5\beta_1$  integrin is an example of protein that is overexpressed on tumor vessels and is accessible from the circulation. This integrin is overexpressed in intestinal adenomas of adenomatous polyposis coli (APC) mice but is also expressed in smooth muscle of the normal intestinal wall and villi. The broad distribution of  $\alpha_5\beta_1$  integrin expression is readily seen by conventional immunohistochemistry. However, when an antibody to  $\alpha_5\beta_1$  integrin is injected intravenously, it labels the integrin on tumor vessels but not on normal smooth muscle or villi. This difference reflects the accessibility of  $\alpha_5\beta_1$  integrin. The integrin is accessible in tumor vessels but not in intestinal smooth muscle and villi because of the endothelial barrier.

The distribution and accessibility of  $\alpha_5\beta_1$  integrin has also been examined in spontaneous islet cell tumors in the pancreas of RIP-Tag transgenic mice.<sup>19</sup> Conventional immunohistochemistry has shown that  $\alpha_5\beta_1$  integrin is expressed in multiple locations in the normal pancreas, including pancreatic ducts, but there is little expression in islets. When an antibody to  $\alpha_5\beta_1$  integrin was injected into the bloodstream of normal mice, there was little staining in the pancreas, apart from faint labeling of islet blood vessels. However, when the antibody was injected into RIP-Tag mice, the tumor vasculature was strongly and uniformly labeled.<sup>20</sup> Scattered sites of leakage were also present. The amount of vascular labeling increased with the size of the tumors.

The question of whether labeling in tumors by  $\alpha_5\beta_1$  integrin antibody in the bloodstream was a consequence of vascular leakiness was addressed by comparing the distribution of the integrin antibody with that of extravasated immunoglobulin (IgG). Unlike the uniform vascular labeling by the integrin antibody, IgG was restricted to scattered, patchy sites of extravasation.<sup>20</sup> Antibody to fibrinogen/fibrin had a similar patchy distribution.<sup>20</sup> This patchy distribution indicated that tumor vessels are not uniformly leaky and that antibodies do not extravasate and gain access to tumor cells homogeneously within tumors. Thus, the labeling of tumor vessels by  $\alpha_5\beta_1$  integrin antibody is not explained by leakage alone. Instead, binding sites for  $\alpha_5\beta_1$  integrin antibody were uniformly distributed and immediately accessible on the tumor vasculature,

consistent with expression on the luminal surface of endothelial cells.

In summary, tumor vessels have a spectrum of cellular and molecular abnormalities. Endothelial cells in tumors are structurally abnormal, express distinctive proteins, and have a defective barrier function. The abnormal proteins can serve as molecular targets. The leakiness governs the access of macromolecular therapeutics to extravascular compartments and contributes to the abnormal tumor microenvironment. Pericytes on tumor vessels have an abnormally loose association with endothelial cells and may express abnormal markers, indicative of defective endothelial cell-pericyte interactions. The vascular basement membrane reflects the abnormalities of endothelial cells and pericytes and the dynamic nature of tumor vasculature. These features can be used as readouts for monitoring the response of tumors to angiogenesis inhibitors.

### Evaluating Antiangiogenic Therapies in Mouse Models of Multistage Carcinogenesis

Douglas Hanahan, of University of California, San Francisco, discussed antiangiogenic therapies in mouse tumor models. Although subcutaneous tumors in mice do not recapitulate the full breadth of what is seen in tumors that arise naturally, genetically engineered mouse models of organ-specific cancer provide an opportunity to study angiogenesis inhibitors in tumors that develop from normal cells in normal organs. The RIP-Tag transgenic mouse is a particularly useful model of organ-specific multistage carcinogenesis. In these mice the SV40 early region is expressed in insulin-producing  $\beta$  cells of pancreatic islets.<sup>19</sup> This expression elicits an inevitable development of islet cell carcinomas. Before the development of large tumors by ~14 weeks of age, the mice exhibit a series of discrete premalignant stages, beginning with the hyperplastic/dysplastic phase at about 3–5 weeks. A subset of dysplastic islets switch on angiogenesis, and a subset of those go on to form varying grades of islet tumors and carcinomas. The mice thus provide a model for dissecting pathways wherein hyperplastic and dysplastic islets progress to angiogenic islets, which in turn progress to tumors and eventually invasive cancers.

Since Dr Hanahan introduced the concept of the angiogenic switch in the late 1980s, he has sought to understand why it occurs and how it is regulated. Because angiogenesis can be induced either by activation of angiogenesis inducers or by inactivation of angiogenesis inhibitors, angiogenesis activators can be targets, and endogenous inhibitors can be therapeutic agents.

VEGF is a prototypic example of an angiogenesis activator. VEGF is expressed in RIP-Tag tumors. Indeed, VEGF and its receptors VEGFR-1 and VEGFR-2 are expressed constitutively in normal islets as well as in tumors.<sup>21</sup> Expression of VEGF in normal islets would seem to be inconsistent with a role in the angiogenic switch. However, experiments have pro-

vided a plausible explanation. The importance of VEGF in tumor development in RIP-Tag mice was demonstrated by knocking out the VEGF gene using Cre-loxP-mediated site-specific recombination.<sup>22,23</sup> Absence of VEGF dramatically impaired the angiogenic switch in RIP-Tag mice and reduced the number, size, and growth of tumors. Tumors that developed were poorly vascularized and coopted vessels in the exocrine pancreas, which implies that VEGF is critically important for angiogenic switching during initial tumor growth in this model.

If VEGF and its receptors are expressed both before and after activation of the angiogenic switch, how can this growth factor be so important to tumor growth? A clue came from studies with an antibody that recognizes VEGF bound to VEGFR-2. Normal islets had little immunoreactivity with this antibody, but tumors had strong staining, indicating VEGF bound to VEGFR-2.<sup>24</sup> This finding suggests that, even if total VEGF does not change, the proportions of matrix-bound VEGF and mobilized VEGF increase during tumor growth from islets. The possible involvement of matrix metalloproteinases (MMP) in freeing VEGF from extracellular matrix was tested in MMP9 (gelatinase B) knockout mice crossed with RIP-Tag mice.<sup>24</sup> Absence of MMP9 impaired tumor growth in these mice, suggesting that the enzyme participates in mobilization of VEGF from extracellular matrix. Although the biochemistry of the sequestration and mobilization are not understood, it appears that MMP9 is an initial activator of angiogenesis by mobilizing sequestered VEGF. Surprisingly, the source of MMP9 in RIP-Tag tumors was innate immune cells, not tumor cells. Because MMP9 from infiltrating immune cells is involved in other cancer models as well, these cells appear to be angiogenesis-related cells and potential drug targets.

In reference to experimental therapeutics, Dr Hanahan described trials that examined the role of VEGF signaling and MMP9 activation in tumor angiogenesis in RIP-Tag mice. Gabriel Bergers, while a postdoc in his group (she is currently an Assistant Professor of Neurosurgery at UCSF), developed 3 types of trials.<sup>24</sup> Prevention trials addressed whether a drug could block the angiogenic switch in early-stage tumors. Intervention trials determined whether a drug could stop explosive tumor growth at an intermediate stage. Regression trials assessed whether a drug could cause tumor regression and prolong life in late-stage disease. In these experiments, SU5416, an inhibitor from Sugen, Inc. that targets VEGFR-2, impaired angiogenic switching in small tumors but did not regress late-stage disease.<sup>24</sup> This result was similar to the effect of knocking out VEGF. The MMP inhibitor BB94 reduced the frequency of angiogenic switching by about half, slowed tumor growth, and gave a modest prolongation of life but did not cause tumor regression.<sup>24</sup> MMP inhibitors were less potent than the VEGF inhibitor.

The poor response to SU5416 or BB94 in established disease in RIP-Tag mice is consistent with the results with

SU5416, Avastin, and MMP inhibitors in clinical trials against late-stage disease, suggesting that, as single agents, these drugs would be most effective against early-stage disease. Is there any hope of using angiogenesis inhibitors to treat late-stage cancer? This was examined by testing combinations of angiogenesis inhibitors and chemotherapeutics. Robert Kerbel and Judah Folkman converged on the really exciting concept that if chemotherapeutic drugs are delivered at lower doses without pause, myelosuppression is avoided, but endothelial cells are killed, and angiogenesis is suppressed.<sup>25,26</sup> This has become known as a metronomic regimen.<sup>27,28</sup> In a RIP-Tag tumor regression trial, SU5416 or BB94 was combined with vinblastine or cyclophosphamide (Cytoxan) in a metronomic regimen. SU5416 plus cyclophosphamide reduced tumor burden but did not improve survival. SU5416 plus vinblastine was even less effective. BB94 alone did not regress tumors but, when combined with vinblastine, stabilized tumor growth. However, BB94 combined with cyclophosphamide caused the greatest tumor regression and survival advantage, suggesting that metronomic chemotherapy with this drug combination has potential in late-stage disease.

Promising results were also obtained with SU6668, another receptor tyrosine kinase inhibitor from Sugen, which inhibits PDGF receptors with 200-fold higher affinity than VEGF and FGF receptors. SU5416, which primarily targets VEGFR-2, had a dramatic effect in the prevention trial, but SU6668 caused only a modest reduction, implying that SU5416 is much better in early-stage disease.<sup>29</sup> However, in the regression trial, SU5416 was relatively ineffective, but SU6668 stopped tumor growth during the 4-week treatment period. SU6668 also reduced vascular density in the tumors, disrupted the association of endothelial cells and pericytes, and increased the size of surviving vessels.<sup>29</sup> To understand these effects of SU6668, the location of PDGF receptors was determined. In RIP-Tag tumors, PDGF receptors are not expressed on tumor cells, but 3 of the 4 PDGF ligands are made by endothelial cells, and PDGFR- $\beta$  and perhaps PDGFR- $\alpha$  are expressed in pericytes. Although it was not appreciated until recently, pericytes are abundant on RIP-Tag tumor vessels.<sup>15</sup> Pericyte-endothelial interactions are dictated in part by paracrine signaling networks, one component of which involves PDGF ligands talking to PDGF receptors.<sup>16,17</sup> The ligands are made by endothelial cells, and the receptors are expressed on pericytes. SU6668 apparently interrupts endothelial-pericyte signaling, interferes with pericyte survival, and favors regression of tumor vessels.

The most promising results were obtained with the combination of SU5416 and SU6668.<sup>29</sup> In a prevention trial, no enlarged islets or tumors were found in 10-week-old RIP-Tag mice receiving combination therapy. Moreover, in the regression trial, SU5416 and SU6668 produced marked tumor regression. The tumor vasculature was even more abnormal than with either agent alone. After 4 weeks of treatment, there was

a dramatic reduction in tumor burden and conspicuous changes in gross morphology of the tumors. Gleevec (STI-571), which inhibits PDGF receptors as well as Bcr-Abl and c-Kit, has poor pharmacokinetics in mice and little effect as a stand-alone agent in RIP-Tag tumors. However, in combination with SU5416, Gleevec complements the effects of SU5416. In a regression trial, SU5416 plus Gleevec produced tumor regression comparable to that caused by SU5416 plus SU6668.<sup>29</sup> These results suggest a new strategy for antiangiogenic therapy by targeting both endothelial cells and pericytes in tumors. The findings also raise the possibility of combining VEGF receptor inhibitors with PDGF receptor inhibitors to increase efficacy against human cancers beyond that attainable with single agents.

To summarize, two receptor tyrosine kinase inhibitors are showing efficacy in the RIP-Tag mouse model of multi-stage carcinogenesis. The VEGF receptor inhibitor SU5416 blocks progression of early-stage disease, and SU6668, which inhibits PDGF receptors among other kinases, produces regression of late-stage disease. When combined, the two inhibitors have more than additive effects. SU5416 combined with Gleevec, which targets Bcr-Abl and c-Kit and also blocks PDGF receptors, mimics the effects of SU5416 plus SU6668. Because Gleevec is an approved drug, clinical trials of Gleevec in combination with anti-VEGF agents should be considered. These effects are explained by the growth factor dependence of tumor endothelial cells and pericytes. However, normal endothelial cells and pericytes may be affected if doses exceed the therapeutic window. Although there is a question of the predictive value of the RIP-Tag model in assessing responses to angiogenesis inhibitors in humans, if it is predictive, targeting the tumor vasculature may provide real benefit in human cancer. This benefit is dependent on the clinical trial design taking into account the stage at which the drugs are likely to be most efficacious. When used alone, inhibitors of VEGF or MMPs may be most effective in early-stage disease. Combinations of complementary agents such as SU5416 and SU6668 or SU5416 and Gleevec are more likely to achieve objective responses in late-stage disease.

## SESSION 2: ANGIOGENESIS UNDER INVESTIGATION: EXPERIMENTAL MODELS AND ASSAYS

### Need for Better Assays of Angiogenesis

Therapeutic targeting of angiogenesis is a novel approach to cancer therapy that has achieved “proof of principle” in animal models of tumor progression<sup>30–33</sup> and has demonstrated success in early clinical trials with cancer patients.<sup>34,35</sup> The recent discovery of specific molecular targets that modulate endothelial cell responses has given further impetus for the development and therapeutic application of therapy targeted to angiogenic blood vessels.<sup>32,33</sup> However, one of the major

problems in angiogenesis research, at both the basic and applied levels, remains the development of suitable methods for assessing and quantifying the angiogenic response in vivo.<sup>36,37</sup>

Several key steps in the process of endothelial sprouting have been identified.<sup>31,38</sup> The process begins with vasodilatation and an increase in vascular permeability, initiated by vascular endothelial growth factor (VEGF) produced in response to hypoxia or other factors. Increased vascular permeability results in extravasation of plasma proteins that become organized into a provisional matrix that, along with the endothelial basement membrane, forms a physical barrier that must be crossed by migrating endothelial cells. Changes in the adhesion of endothelial cells to the extracellular matrix, induction of protease activities and cell migration, as well as endothelial cell proliferation also are involved. Finally, endothelial sprouts undergo lumen formation, anastomosis, and initiation of blood flow.

Myriad in vitro, in vivo, and ex vivo assays have been developed and used by researchers to understand angiogenic mechanisms and to evaluate the efficacy of novel angiogenesis inhibitors. These assays have contributed to the characterization of the cellular events of angiogenesis<sup>36,37</sup> (see Table 1 for summary). However, in vitro angiogenesis assays, based on endothelial cell cultures or tissue explants, focus on an isolated endothelial cell function (eg, endothelial cell proliferation, migration, or invasion) and do not examine the coordination of cell functions required for a successful angiogenic response.<sup>36,37,39,40</sup> In contrast, in vivo angiogenesis assays examine the entire spectrum of molecular and cellular processes, with the endpoint being formation of new, functional blood vessels. However, many in vivo angiogenesis assays are expensive, technically difficult to perform, require substantial amounts of test compound, and rely on selective morphometric analysis (eg, vessel counts, vascular morphology) for quanti-

**TABLE 1. Current Angiogenesis Assays**

In vivo Assays	In vitro Assays
1. Microcirculatory preparations	1. Tube formation
2. Biocompatible polymer implant	2. Boyden chamber
3. Excision of vascular tissue	3. Cell proliferation
Advantages	
1. Recapitulates full spectrum	1. Ability to control variables
2. Temporal and spatial visual	2. Lower cost, technical ease
Disadvantages	
1. Expensive	1. Lack standardization
2. Quantitation morphology	2. Quantitation morphology
3. Technically demanding	3. Extrapolation to in vivo?
4. Not suitable for screening	
Modified from Jain et al <sup>36</sup> and Auerbach et al <sup>37</sup>	

fication. Because of these limitations, current drug development strategies for identifying and testing novel angiogenic agents or inhibitors rely principally on in vitro systems. Although in vitro angiogenesis assays are useful for identifying potential molecular targets or to augment, alter, or block endothelial cell responses and preliminary screening of novel pharmacologic agents, their results may not correlate with in vivo angiogenesis measurements<sup>36,37,39,40</sup> because of the complex cellular mechanisms involved in blood vessel formation in vivo.

### Ex Vivo Aortic Ring Assay

Dr William Figg described a modified aortic ring assay, originally developed by Roberto Nicosia,<sup>41</sup> in which aortas from male rats were removed, cut into rings, and cultured in Matrigel and endothelial growth medium. Five days of incubation yielded substantial microvessel outgrowth, which were shown to be proliferating endothelial cells by BrdU and Factor VIII staining.<sup>42</sup> Quantification of outgrowth can be easily done using image analysis programs. This ex vivo assay represents various aspects of angiogenesis from endothelial cell proliferation, migration, and tube formation to invasion through extracellular matrices. A similar assay using mouse aortic rings was also mentioned. One of the advantages of using murine aorta is that knockout or transgenic models can be used to study the molecular pharmacology of agents whose targets are deleted or altered. Dr Nicosia alluded to the dramatic variations in aortic microvessel outgrowth dependent on the age and genetic background of the animals, indicating the importance of internal controls in these assays. In collaboration with Dr Steven Libutti, Dr Figg developed a humanized form of the assay using human saphenous vein to allow the assessment of targeted therapies specific for human receptors. Complementing the ex vivo models, Dr Figg's laboratory also routinely uses the human umbilical vein endothelial cell (HUVEC) proliferation and tube formation assays to screen novel antiangiogenic agents. HUVECs are derived from large vessels, the characteristics of which may not always reflect microvessel endothelial cells. Tube formation is considered to be a fairly specific assay for angiogenesis in that it measures the ability of endothelial cells to form three-dimensional tubular structures in extracellular matrix. One concern, however, is that cultured cells of nonendothelial origin such as fibroblasts may also form tubes in Matrigel. Recent observations that some tumor cells are also capable of forming tubes and lining vascular channels only complicate matters further.<sup>43</sup> The use of multiple assays is essential in evaluating the efficacy of new angiogenesis inhibitors.

### Directed In Vivo Angiogenesis Assay

Dr William G. Stetler-Stevenson described the development of a novel in vivo assay of angiogenesis. This assay, referred to as the DIVAA system for directed in vivo angiogen-

esis assay, was designed to be technically simple, reproducible, and directly quantifiable independent of morphometric parameters.<sup>44</sup> The design also recognizes that the development of the angiogenic response to tumors in vivo occurs vectorially; that is, blood vessels grow toward the primary or metastatic tumor. In addition to recapitulating the directed response to tumors, the DIVAA system has the potential to allow investigators to examine changes in endothelial responses (gene expression) that may occur during the development and progression of vascular sprouts.

The DIVAA system uses a piece of surgical silicon tubing that is sealed at one end. Before implantation in the experimental animal (mouse), this tubing, measuring approximately 1 cm in length by 0.15 cm diameter, is filled with a small volume (18  $\mu$ L) of extracellular matrix suspension containing an angiogenic factor (either bFGF or VEGF). These "angioreactors" can be filled with provisional-type extracellular matrix such as a fibrin clot, basement membrane matrix such as Matrigel, or interstitial-type matrix such as type I collagen. These components are then allowed to gel at 37°C before implantation. The contents of the angioreactors can be modified as experimental design dictates. Various combinations of angiogenic factors, angiogenic factor concentrations, differing extracellular matrix composition, as well as various angiogenesis inhibitors or combinations of inhibitors can be tested.

Quantification of the angiogenic response is limited to the vessel growth within the angioreactors and is achieved by 1 of 2 methods. The invasion of endothelial cell-lined structures into the extracellular matrix of the angioreactors is demonstrated by direct staining of endothelial cells using FITC-labeled *Griffonia* lectin, which is an endothelial cell-selective reagent. Alternatively, angiogenic responses are quantified by digesting the angioreactor matrix in dispase solution, recovering the cellular contents by centrifugation, and staining for endothelial cells with FITC-labeled *Griffonia* lectin. The second method of quantification uses an intravenous injection of FITC-labeled dextran ( $M_R > 250$  kDa) before surgical recovery of the angioreactors. Comparison of these methods demonstrates that even in VEGF-induced angiogenic responses, vascular leakage does not significantly contribute to the FITC-dextran signal.

Dr Stetler-Stevenson described the characterization of the DIVAA system, including intra- and interassay variation (coefficient of variation less than 10%) as well as the kinetics of the angiogenic response (optimally 9 days following implantation). For example, the DIVAA system allows accurate dose-response analysis of the angiogenesis inhibitor TNP-470 in vivo. The results of this experiment demonstrated for the first time that the  $EC_{50}$  for TNP-470 in vivo is 88 pM, which is in excellent agreement with the  $IC_{50}$  value reported for the in vitro inhibition of endothelial cell growth. Dr Stetler-Stevenson also demonstrated the utility of the DIVAA system for characterization of novel angiogenesis inhibitors, such as

thrombospondin peptides, as well as the application of DIVAA in genetic models, such as the MMP2-knockout mouse.

### Modifying Angiogenesis Assays to Meet Specific Needs

Dr Robert Auerbach briefly discussed several *in vivo* assay systems as well as their advantages and pitfalls. In the chick chorioallantoic membrane (CAM) assay, a window is made in the egg shell to expose the CAM of the chick embryo on which tissue or organ grafts are placed. The grafts are scored for growth, vascularization, or for the number of bifurcation points in a defined surrounding area after a period of incubation. Although this assay is cheap and easy to perform, the fact that chick cells are used limits its utility.

The mouse corneal angiogenesis assay involves the implantation of test tumors or tissues, which elicit the ingrowth of new vessels from the peripheral limbal vasculature, into a pocket in the avascular cornea. Intravenous injection of fluoro-chrome-labeled high-molecular-weight dextran allows the extent of angiogenesis to be visualized and quantified by fluorescence microscopy and image analysis programs. Furthermore, the efficacy of oral and intravenous antiangiogenic agents can be evaluated. The appeal of this model stems from its ability to follow the angiogenic process and the absence of an existing background vasculature in the cornea. However, the surgical technique is demanding and time consuming. The induction of inflammatory responses during surgery may itself result in angiogenesis, masking the effects of the actual angiogenic stimuli under investigation.

In the Matrigel plug assay, Matrigel containing tumor cells or test agents is injected subcutaneously into mice. The solidified plug is recovered after 7–21 days when blood vessels have entered, examined histologically, and quantified. Similar to the mouse corneal assay described above, fluoro-chrome-labeled dextran can be injected to visualize the vessels. This assay is less challenging surgically than the mouse corneal model.

Several important points were highlighted in this section of the workshop. First, endothelial cells are not all alike. Differences exist between large vessel-derived and microvascular endothelial cells and between endothelial cells obtained from different organ sites. Second, cell cultures are different from organ cultures. HUVECs are likely to behave differently in response to stimuli or inhibitors compared with rat aortic rings. Species differences need to be taken into account. Different adaptations of techniques dependent on the objective of each experiment are also critical. Third, although *in vitro* methods are useful, expeditious, and readily quantifiable, they seldom reflect tumor–host interactions. *In vivo* tests are therefore absolutely essential for validation. One of the most commonly used *in vivo* models is human tumor xenografts implanted in immunodeficient mice. In this case the vasculature and vascular targets in these xenografts are of mouse origin. In fact, most

of the candidate drugs for molecular targeted antiangiogenic therapies currently being tested in the clinic are optimized for mouse targets, which is a limitation when extrapolating results to patients. Last but not least, standardization for using specific assay systems is necessary to allow comparison and validation of test results among laboratories.

### SESSION 3: TRANSLATION TO THE CLINIC AND CLINICAL TRIAL DESIGN

#### Challenges for the Clinical Development of Angiogenesis Inhibitors

Dr Roy Herbst discussed some of the issues involved in clinical trial design, general concepts, and use of surrogate markers in trials of angiogenesis inhibitors in cancer.<sup>45–47</sup>

In phase 1 trials, where identifying a maximum tolerated dose is the goal, assays for biologic dose are needed. In phase 2 trials the same problem exists. As with cytostatic drugs, when the dose of angiogenesis inhibitor is increased, a delay in tumor growth may be observed. This change may be meaningful in early-stage disease but may have less impact in advanced tumors that are still growing. Even if the tumor grows less than it would without treatment, it is difficult to obtain meaningful information from individual patients without controls. What is needed is a way to determine optimal or maximal biologic dose. Many newer agents go from phase 1 to phase 3 and skip phase 2 because the dose is based on pharmacokinetics rather than on target validation. Evidence of drug activity is difficult to obtain without phase 2 data, and phase 3 trials are large and expensive.

Antiangiogenic agents may be useful in maintenance therapy. After a maximal response is obtained with cytostatic drugs, patients could be randomized into groups receiving angiogenesis inhibitor or placebo. There are pros and cons to this. The pro is the use of angiogenesis inhibitors in the setting of minimal disease. There is some time to give the drug before the patient progresses. Metastases may be prevented with relatively nontoxic agents, and many of these agents are oral. The con is that the effects in phase 2 are often unknown. These are large trials. The biologically active dose is not always known. The long-term side effects are not always known, and a large number of patients are required. So although all would agree that earlier disease is the place to study these agents, a signal is needed before then to know that the right dose/schedule has been identified before going forward.

Another approach that may be used, not only with antiangiogenic agents but also with many biologic therapies, is to add an antiangiogenic agent to standard chemotherapy. This is a design based on preclinical data that show synergy. Where there is minimal added toxicity, treatment is given to maximal response, and then the antiangiogenic drug is used as a maintenance therapy to get back to a minimal or stable disease setting. The pros are that synergy has been shown in numerous



model systems for increased response without increased toxicity. Often full-dose chemotherapy can be administered, and there is the ability to use maintenance, which currently does not exist. It would be nice if there were something that was safe that could be used as maintenance therapy. That is the goal of most of the biologic regimens. It seems like a good idea, but many of these approaches are still in phase 1 or phase 2. It is necessary to look at pharmacokinetics. The M. D. Anderson team did this with the EGFR inhibitors, and there have been two negative randomized trials of adding EGFR agents to chemotherapy.

Dr Herbst stressed the need for serum/plasma markers and for the validation of the target enzyme or site. He also emphasized the need to obtain tissue from patients. Many have suggested using time to tumor progression as a clinical indicator. With that as background, the trial with human recombinant endostatin provided an example. Three trials of endostatin were initiated 3 years ago. The phase 1 trial was designed as a trial to look for the optimal biologically effective dose. Tissue biopsy was mandated in the trial, so patients had a biopsy to get into the study and another at 8 weeks. The patients were selected on the basis of having tissue that was easily amenable to biopsy. Serum markers of activity and noninvasive radiology, mainly PET, were used.

Many are familiar with endostatin and the compelling data from animal studies with the induction of tumor dormancy. Endostatin is a 20-kDa protein whose effects are endothelial cell specific, and there has been an absence of drug resistance. Endothelial cell apoptosis was examined as one of the mechanisms by which this drug was working. At M. D. Anderson, this trial was a team effort to ask a phase 1 question to determine dose, safety, pharmacokinetics, and whether apoptosis was induced in samples; the trial looked at pre- and posttreatment biopsies of endothelial cells, used noninvasive imaging with PET, and looked for some hint of antitumor activity. The patients had to have measurable disease that was amenable to the required biopsies at time 0 and at 8 weeks. Pharmacokinetics was done during the first week and the fourth week. The imaging studies were done every 4 weeks.<sup>47,48</sup>

Doses were based on the animal pharmacology. It was predicted that activity would be seen around the 30 mg/m<sup>2</sup> level. The dose went up to 600 mg/m<sup>2</sup>; 26 patients were entered, and 25 were studied. Most patients had melanoma or sarcoma, and the rest had other solid tumors. There was no serious toxicity. There was a pharmacokinetic target. The AUC target from Entremed was based on some of the preclinical work and was reached at the 300 mg/m<sup>2</sup> dose and above. The data are being reanalyzed looking at a C<sub>MIN</sub> as the target rather than a total AUC.

The result was typical of phase 1 trials. Here are some patient examples. One patient stayed on study for a year and a half. A couple of patients stayed on and showed some hint of

activity, but most of the patients stayed on for a median of 69 days, and the median time to progression was 49 days. VEGF serum levels were determined during the time the patients were on study, but no trends were seen. VCAM and E selectin were measured, but no trends were observed. Serum markers were not useful for following the agent.<sup>47,49,50</sup>

PET scanning, using both fluorodeoxyglucose PET for metabolism as well as H<sub>2</sub>15<sub>15</sub>O PET for blood flow, showed interesting results. All patients were studied with PET. The data analysis was done independently, and there was a trend ( $P = 0.027$ ) toward a measurable response by imaging. There was decreasing blood flow with increasing dose of endostatin. There was a 20% decrease in blood flow; however, notwithstanding the decrease in blood flow, many tumors grew larger. This is the reason for including surrogate assays in trials. A laser scanning cytometer was used to look at the tissue biopsies. Snap frozen tissues were processed, and the slides were stained for the biomarkers; staining was confirmed using a fluorescent microscope, and then the slides were scanned very much like a cell sorter or a FACS analysis to look for the different labels. There was very little endothelial cell apoptosis pretreatment and some endothelial cell apoptosis afterward with an area of tumor decay just adjacent. The apoptotic endothelial cells, before and after treatment, were counted, and the apoptotic tumor cells before and after were counted as well. In both cases the number went up. However, there was no a correlation with dose.<sup>51</sup>

This was a successful phase 1 study, a very safe drug meeting its pharmacokinetic requirements and some hints that it had an effect biologically in a few patients. Similar activity was seen at Dana Farber.<sup>52</sup>

Combinations with radiation and chemotherapy and other biologic agents are currently being studied in the laboratory.<sup>53</sup> One can think of angiogenesis inhibitors as either indirect or direct. The indirect inhibitors might be the EGFR agents. An inhibitor such as endostatin is a more direct inhibitor working on the endothelial cell. There may be a rationale for combinations and using these 2 molecules together. Avastin and OSI774 are Genentech drugs, both indirect inhibitors. Six advanced patients have been treated, and 2 of them have responded and are tolerating the combination very well.<sup>54</sup>

A paradigm for developing combinations of angiogenesis inhibitors, perhaps with chemotherapy, is important. The recent experience with the EGFR inhibitors suggests that chemotherapy can be given before or after, and using these drugs sequentially may be advantageous. There is a lot of hope and many prospects for these drugs in the clinic, and it behooves us now to use some of these new trial designs and to use surrogate markers to better design trials for the future.

### Advanced Imaging as a Surrogate Marker

Dr King Li discussed the need for surrogate markers for imaging. A perfect surrogate marker would allow determina-

tion of which patients to treat, what agent to use, response to therapy, and which combination therapy to give. Those data allow for the design of a very good clinical trial. No available clinical imaging test today can provide that set of data. Future trials need to integrate development of new imaging tests in tandem with therapeutics to get those data while doing the clinical trial.

There are many types of agents under development. The imaging tests performed are global tests including studies of blood flow, glucose uptake, permeability, and other variables. It has been proven that much of the time imaging changes do not correspond to increasing survival or decrease in the tumor growth rate. To establish imaging tests, they must be incorporated at the beginning of the clinical trial. There is a need to combine the development of the imaging test with the therapeutic to have a surrogate marker that is built in with the therapeutic agent. To develop the imaging test requires engineering the biodistribution of the test agent.

Using a single ligand or antibody to simulate the interaction between the leukocytes and the endothelium will fail. A particle that has a biodistribution similar to leukocytes and the ability to have multivalency and polyvalency are important. To target different markers, it is necessary to develop a biocompatible particle that has intravascular confinement and different ligands and protein conjugates on the surface. A patient is selected with the marker, and then it is necessary to know exactly how the marker and its density change in a spatial manner by using imaging. After the drug is delivered, the same imaging can be used to look at response. Therefore, the goal is not imaging alone but imaging and therapy together as a combined regimen.

Many different methods of making polymer liposome particles have been tested. UV light is used to lock the particular configuration. Different types of lipids and different functional groups can be cross-linked by use of UV light. The novelty is the use of the polydiacetylene cross-link. Different metals can be loaded for imaging tests such as PET scanning, MRI, and CT. Finally, the same particle can be used to deliver a drug.

The antibody LM609 against  $\alpha_v\beta_3$  integrin was used to prove that particles in vivo could be targeted to activated endothelial cells. The V2 rabbit carcinoma was implanted subcutaneously into the thigh. A particle labeled with LM609 and gadolinium was injected, and the in vivo distribution determined. The same tumor line implanted in 2 different sites gave different distributions of the biomarker. The enhancement was in the tumor periphery and very little in the tumor center. Using biopsy as a surrogate marker requires knowledge of where to biopsy using imaging as a guide. Select the enhanced area, pick out tissue using the image, and then, from the tissue, do the standard test. Stain for integrins and then stain for the particle. Be certain that the particle localizes to the vessels that have the receptors and that the vessels that do not stain with the integrin

do not show the particle. It is a very specific localization technique.

The particle can be labeled with indium and used for SPECT imaging. In the tumor it is only localized in the activated vessel, so there is a huge concentration effect. Only the tumor vessels that express  $\alpha_v\beta_3$  integrin bind the particle. A significant target-to-background ratio can be achieved.

The accumulation of the targeted particles can be tracked over time, and the same particle can be used for imaging or therapeutics. The metal can also be yttrium. Yttrium is used in radioimmunotherapy, and 1 dose of 6  $\mu\text{Ci}/\text{kg}$  can control tumor growth in V2 carcinoma in a rabbit. If the particle is used with M609 and yttrium, the growth curve is much slower than controls. Without the yttrium, just using the antibody on the particle, there is also suppression of the tumor growth rate, but the antibody alone gives less reduction. There is a synergy between the particle and the antibody, resulting in a significant increase in tumor response between the antibody alone and the particle with the antibody. Of course, with a payload that is therapeutic, there is more effect. With a platform, the payload and the targeting ligand can be changed.

But the question is: how can this be translated into the clinic? A reporter gene, viral tyrosine kinase, which will track a PET-labeled analogue of gancyclovir, was cloned into a plasmid carried by the particle. Then if  $\alpha_v\beta_3$  integrin is present in vivo, it could be visualized by using the reporter gene to track PET emission and the efficiency of in vivo gene transfer before the therapeutic agent is administered. After the therapeutic agent is given,  $\alpha_v\beta_3$  integrin up-regulation can be monitored over time.

This paradigm is just an example of looking forward and not using nonspecific, generalized tests to assay specific phenomenon. In a platform technology, varying the targeting is possible. The contrast agent can be varied depending on the sensitivity and the receptor density using different types of tests. PET provides more sensitivity with lower spatial resolution. MRI provides more spatial resolution with lower sensitivity. Diagnostic agents are not useful if they are not coupled with the development of therapeutics.

There is no clinically available imaging test that is useful for selecting the patient who will respond to the therapy, selecting the right dose for that patient, monitoring the effect, and finally choosing the combination therapy. Imaging can provide assessment of the overall vascularity. These concepts are not being applied in the imaging tests that are currently available, and without a dedicated paradigm shift and development of new imaging paradigms, it will not be possible to use imaging as a surrogate marker.

### Circulating Endothelial Cells as Surrogate Markers

Dr John Heymach discussed circulating endothelial cells as surrogate markers of antiangiogenic activity.<sup>55,56</sup> For about

30 years it has been known that endothelial cells can be detected in the circulation and may be useful as surrogate markers of vascular injury.

Initially these cells, detected with P1H12/CD146 antibody, were assumed to be endothelial cells sloughed from vessel walls. Bone marrow-derived circulating endothelial precursors (CEP) can contribute to pathologic angiogenesis, particularly in tumors. CEC is a general term for circulating cells bearing endothelial markers. In the circulation, mature endothelial cells have a higher apoptotic fraction, about 50%, than hematopoietic cells.

Tumor products such as VEGF that induce CEP mobilization and tumor neovascularization can be inhibited by therapy, so there can potentially be a decrease in CEP, but there could also be an increase in tumor endothelial apoptosis so, overall, there may be an increase in the shedding and an increase in the apoptotic fraction of EC. Pre- and posttherapy, the total number of circulating EC is constant, but the percentages that are proangiogenic precursors and apoptotic endothelial cells change. The tools for distinguishing CEP from vessel wall EC are limited. CEP are proliferative; vessel wall-derived endothelium by contrast have a very limited proliferative capacity, so the formation of colonies in culture is a sign of CEP. The stimuli for CEP are proangiogenic signals such as VEGF, whereas endothelial cells of vessel walls are mobilized by vascular injury or things that damage vessels. Both CEP and CEC bear KDR receptor and most other endothelial antigens. It appears that P1H12 is not expressed in CEP but is strongly expressed in vessel wall endothelium, and when a number of different markers are tested, P1H12 has proven to be the most specific marker. AC133 is a marker of CEP. In humans these two markers help distinguish precursors from mature endothelial cells. Endothelial cells from different tumors may differ, so there may not be one marker that is perfect for endothelial cells or tumor-derived endothelial cells.

Dr Heymach looked at the effect of tumors on CEC or different tumor types on CEC in mice before treatment. First, HSVF, a pancreatic tumor line that expresses high levels of VEGF, was used. The number of CEC was dramatically up-regulated compared with control non-tumor-bearing mice. With Lewis lung carcinoma, it was surprising to find that the number of CEC was about half of control non-tumor-bearing mice. On further reflection, this was reminiscent of the observation that Lewis lung carcinoma suppresses distant metastases. This may reflect the balance of pro- and antiangiogenic stimulators released at a distance by the tumor.

Dr Heymach looked at the effect of endostatin on CEC in mice with no tumor, or mice with the HSVF tumor that is untreated or treated. Endostatin completely abrogated the tumor-induced rise in CEC. Type 2 Lac Z mice that express Lac Z in the endothelium specifically were examined, and blue cells were counted. Once again adeno-VEGF significantly increased the number of blue cells, and endostatin abrogated that

increase. VEGF was delivered with myoblast-expressing VEGF, and treatment with a small-molecule VEGF receptor inhibitor completely abrogated the VEGF-induced increase. It was shown that antiangiogenic therapy can prevent a VEGF-induced rise in CEC and CEP based on colony formation.

Lewis lung carcinoma of an established size was used, and then the animals were treated with a small-molecule VEGF receptor antagonist or taxotere or the combination. The goal was to find a marker that, after 3 days, predicted the response that would eventually occur. There was about 90% growth inhibition with combination therapy. Treatment with the VEGF receptor inhibitor or taxotere increased the number of CEC. With the combination, there was an increase in CEC, and the apoptotic fraction was about 80%, which was an increase. CEC measurement is noninvasive. CEC may measure the net angiogenic output of the tumor, again by looking at the baseline level of CEC. It measures the biologic response to therapy. Even if CEC does correlate with tumor endothelial apoptosis, it may be unrelated to the clinical efficacy.

Samples from the phase 1 and phase 2 trials of endostatin at the Dana Farber Institute were examined.<sup>52</sup> The phase 1 work was initiated using a cytospin analysis and staining with a P1H12 antibody. In phase 2, flow cytometry using markers for both CEP and mature EC were used. One observation was that patients have different levels at baseline. In 2 out of 3 patients who were on therapy for more than 6 months, there was a decrease after being on therapy for a long period. A 2-color flow cytometry using the markers and standard flow cytometry techniques was applied. Less than 0.02% CEC as a percentage of peripheral blood mononuclear cells could be detected reliably.

Given these changes in CEC, there was a need to further characterize this endothelial population and to tell whether they were apoptotic or nonapoptotic precursors. Two panels were established for each patient: CD31, P1H12, CD34 to exclude hematopoietic cells, and CD133 for CEP. Mature endothelial cells would be CD31 positive, P1H12 positive, CD45 negative, and AC133 negative; and CEP would be AC133 positive. There was also a panel incorporating 7AD and annexin V to look at apoptotic populations. The CD45-positive cells were isolated. In regard to P1H12 versus CD31 in normal blood, there are no positives; therefore, there were fewer than 0.01% positive CEC. When spiked *in vivo*, there is a rise in the CD31-positive, P1H12-positive population, and when AC133 versus CD31 was examined, there was a rise in CD31-positive cells but not in AC133-positive cells. In the animal experiment, there was an over 2.8-fold rise in CEC, but many were apoptotic. There was greater than 75% apoptosis, which is a significant rise from baseline.

Using this assay, differences could be observed if they occurred. One key issue in measuring circulating endothelial cells is the method. Flow cytometry is the most widely used because the instruments are readily available and quantitative

results can be obtained. Cytospin and immunopurification are other methods, and a combination with these increases assay sensitivity. Another key issue is reproducibility, not only within a laboratory but also between labs. In an effort to quantify this and to control quality, samples from different labs can be compared. There is a need for methods whereby cells can be fixed, frozen, and stored and then analyzed in batches. Being able to ship samples to a central location would facilitate multicenter trials. One possibility is the use of activated caspase 3 antibody for monitoring apoptosis.

In conclusion, antiangiogenic therapy can inhibit VEGF-induced increases in circulating endothelial cells and circulating endothelial cell precursors in murine models. Different tumor types may stimulate or suppress these cells in murine tumor models, so all cancer patients may not have elevated levels. In tumor-bearing mice, antiangiogenic therapy can cause an increase in CEC and apoptotic fraction and in tumor vessel wall-derived endothelial cells. Our limited data suggest that there is not a clear correlation between tumor burden and CEC. Some patients with advanced malignancies do not have elevated CEC levels, and some with modest disease have high levels.

### Isolated Limb Perfusion in Patients with Large Sarcomas or Metastatic Melanomas

Dr Alexander Eggermont addressed the importance of antivasular therapies through his discussions of the successful track record of isolated limb perfusion (ILP) with melphalan as the treatment of choice for multiple (small) melanoma metastases. The application of tumor necrosis factor- $\alpha$  (TNF) in ILP has made it a successful modality to treat locally advanced extremity soft tissue sarcomas and other large tumors and salvage limbs. Moreover, the efficacy of TNF plus melphalan ILP was shown against various other limb-threatening tumors such as skin cancers and drug-resistant osteosarcomas. In patients with melanoma in transit metastases, overall response rate with TNF-based ILP has been reported to vary from 90% to 100% with complete response rates varying from 64% to 91%.

TNF was approved by the EMEA in Europe for the treatment of advanced limb-threatening grade II–III extremity sarcomas on the basis of the results from multicenter trials involving 270 ILPs (TNF + Melphalan) in 246 patients: 1 ILP (22 patients) or 2 ILPs (24 patients). Very advanced tumors included recurrences in 45%, multiple tumors in 22%, metastases in 15%; size was over 10 cm in 46%, Grade III in 66%, previous radiation therapy in 13%, and chemotherapy in 15%. Resection of the tumor remnant was usually (75%) done 2–4 months after ILP. Independent review agreed in 80% of the cases (196 patients) that only ILP offered a chance for limb sparing. Results demonstrated major responses in 76%, rendering tumors resectable in most cases. Clinical responses were CR (18%, pCR 28%), PR(47%), NC (17%), PD (6%), missing (1%). At median follow-up, there was over 3 years of

limb salvage in 71% of the 196 patients considered justified for ILP by independent reviews. There was little toxicity with no toxic deaths. Matched-pair analysis with cases from the Scandinavian STS database demonstrated that TNF-ILP had no negative effect on survival ( $P = 0.96$ ).

Recently they have shown that the TNF-based ILP is safe not only under leakage-free conditions but also when significant leakage has occurred during the ILP, provided patients are well hydrated and adequate perioperative monitoring of the patient is done.<sup>57</sup> Moreover, they have demonstrated excellent results as well as safety in elderly patients over 75 years old.<sup>58</sup>

ILP models in their laboratory have provided a system to elucidate their insights into mechanisms of action and to develop new treatment modalities. These models have identified the prerequisites for the TNF-mediated toxic effects on the tumor vasculature,<sup>59</sup> and, of crucial importance, they have shown that addition of TNF to the perfusate results in a 3- to 6-fold increase in melphalan or doxorubicin uptake in tumors.<sup>60</sup> New (vasoactive) drugs and new mechanisms of action are being discovered.<sup>61,62</sup> Moreover, they have established that the degree of success correlates with the vascularity of the tumors<sup>63</sup> and that low-dose TNF can significantly enhance homing of long circulating liposomes to tumors and increase the uptake of cytostatic drugs, leading to tumor control in their models.<sup>64,65</sup> Their conclusion was that TNF is here to stay.<sup>66</sup> Furthermore, ILP provides an excellent system for the study of vascular targeted therapies.

### Novel Clinical Trials and Designs for Angiogenesis Inhibitors

Dr Michael Gordon made additional comments on trial designs for angiogenesis inhibitors. When investigators started to develop a strategy for testing angiogenesis inhibitors, the thinking was that angiogenesis is similar in all tumors. It is clear that there are molecular differences in endothelial cells in both normal and abnormal tissue, and that likely leads to differential sensitivity, and that as tumors progress there is evolution of angiogenic profiles that results in different sensitivity to treatment.<sup>67–69</sup>

Some investigators had the perspective that an antiangiogenic therapy that was effective in 1 disease could be applied to all diseases. There have been large-scale phase 1 trials and broad diagnoses. This may be one flaw in clinical development. The mistake was to regard antiangiogenic agents as another form of chemotherapy.

Certainly dose-limiting toxicity must be identified, and it has been occasionally. There has been a search for biologically active dose or optimal biologic dose, but unfortunately it is difficult to define this. It is important to recognize that there have been very few responses in phase 1. A few drugs have demonstrated dose-limiting toxicity, and the maximal deliverable dose has been a common phenomenon for antibodies and

small molecules, which leaves the question of whether, if we push the dose higher, more distinct biologic activity would be observed. As a result, phase 1 has been completed with a sense of safety of the drug and pharmacokinetics but a lack of antitumor activity to direct phase 2 into specific diseases or areas. Phase 2 trials have not been adequately powered and ended up running without a good “go/no go” signal.

A number of years ago, a concept for looking at new drugs was described, hypothesizing that drugs that cause disease stabilization or can be vascular toxic and might kill vascular endothelial cells could lead to tumor regression but that we did not know how long this would take. This is a complicating issue because there is no sense of how to characterize the drugs or to know the true potential benefit. Combining a chemotherapeutic with an antiangiogenic agent might provide synergy, targeting both the vascular compartment and tumor compartment and leading to better antitumor activity. For most drugs, rapid development was prioritized over rational development. Most studies have been done in colorectal cancer and non-small-cell lung cancer, not because those are great targets for antiangiogenic therapy but because there are many patients, and thus it is relatively easy to accrue a 900-patient study.

Most of us would agree with the classic paradigm for moving a drug to use in earlier-stage disease if it has some activity in extensive disease or more advanced disease. Getting into adjuvant trials is highly complex and requires a large number of patients; the cost is more than for randomized phase 3 trials. These commit large numbers of patients to studies that take years and years.

Dr Gordon discussed the VHL and VEGF story and the fact that most people who treat renal cell carcinoma believe that there is a link between VEGF, particularly in the clear cell carcinomas, and the development of kidney cancer. Renal cell carcinoma is a very angiogenic tumor and fits Dr Folkman's paradigm for dormancy with long periods of time between nephrectomy and relapse.

Many are familiar with data indicating that the higher dose of bevacizumab (10 mg/kg) produced a progression-free survival advantage compared with placebo that is statistically significant.<sup>34</sup> The crossover precluded assessment of overall survival, and there are a few caveats such as the definition of progression but there were few objective responses.

There are other rationales for directing antiangiogenic therapy. Melanoma and primary glioblastoma express large numbers of integrins on the cell surface. Among these are  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  integrin. This provides a rationale for antiintegrin therapy, which was thought of as specifically antiangiogenic in these diseases. This may not be specifically an antiangiogenic approach, but to get drugs on the market, there is a need to maximize efficacy in clinical trials. The rational combination of targeted therapies should be based on preclinical data.

The increasing availability of these new drugs provides the ability to think broadly, and many companies are developing platforms of antiangiogenic drugs. The potential to combine drugs early in development needs to be aggressively pursued. Some of the drugs such as the thrombospondin mimetics including Abbott's ABT510 and ABT726, broad-acting VEGF inhibitors that absorb or act not only against one isoform of VEGF but against multiple isoforms, and some of the broader-acting inhibitors that work like the EGFR inhibitors and combinations of these drugs. The expense and time expended will be for naught unless active combinations are identified and the surrogates are focused in active settings. There is nothing better than a study that shows a shrinking tumor. CT scans are ultimately going to be the best surrogate for showing an active drug, but the points that were made about targeting and looking at tumors that are responding and understanding why a particular tumor is responding are going to be critical.

To summarize, it has been 5 years, and there is a need to change our mind set relating to combining drugs and scientifically driving the development of these agents. To maximize the future, the focus must be on developing combinations to get the best antitumor activity. Understanding the relationship between the endothelial compartment via genomics or proteomics or CEC and assessing the changes in those CEC as a biomarker and the tumor are going to be critical. This may lead to the conclusion that among the broad phase 1 trials, there is a need to do some targeted trials.

## SUMMARY AND FINDINGS OF THE WORKSHOP

The outstanding presentations and discussions of the Workshop participants highlighted some important considerations for the future of angiogenesis research in cancer. With the growing body of information on the molecular mechanisms underlying the process of tumor angiogenesis, more targeted therapies are needed. Several targets were presented, and the challenge will now be the development of novel therapeutics to exploit them. Much of this work is ongoing and should provide the next generation of angiogenesis inhibitors for the clinic.

The importance of good assay systems was emphasized, and several new and improved preclinical models were discussed. Some of these lend themselves to high-throughput screening, which should be important in the evaluation of novel targeted therapies as they become available. There should be an emphasis on assays that provide data on the molecular activity of the test compound as well as a readout on its effects on vessel growth.

Finally, new surrogate endpoints are needed for monitoring novel angiogenesis inhibitors in clinical trials. The Workshop highlighted some exciting techniques in imaging such as dynamic MRI and advanced PET scanning. In addition, markers such as circulating endothelial cells are being validated in clinical trials and may allow for novel phase 1 trial

designs geared toward optimal biologic dose. Novel trial designs for this new class of agents should be strongly considered as we move beyond old paradigms used for cytotoxic agents that may not be applicable. Since this Workshop was held, some exciting data have emerged supporting the clinical activity of angiogenesis inhibitors applied to renal cell cancer and colon cancer.<sup>34,35</sup> These data from trials of an antibody (Avastin, Genentech, CA) that blocks VEGF has shown in prospective randomized trials that the use of an angiogenesis inhibitor can prolong time to progression in renal cancer and improve survival in stage IV colon cancer. These exciting results underscore the importance of the findings of this Workshop and should provide encouragement to the investigators in this field.

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