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MEETING REPORT

Angiogenesis treatment, new concepts on the horizon

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Introduction

The bi-annual AACR special conference in Cancer Research focusing on anti-angiogenesis was held November 9–13 2005 in Waltham-Boston, Massachusetts. Boston was a well-chosen place to have an angiogenesis conference because it was here that Dr. Judah Folkman first hypothesized that tumor growth could be inhibited by interference of angiogenesis [1]. It was here that Dr. Harold Dvorak first identified VEGF [2], and it was also in Boston where Dr. Beverly Teicher first showed the promise of combination treatment of angiogenesis inhibitors and conventional treatment [3]. Three other distinguished investigators in the field chaired the meeting: Dr. Rakesh Jain, Dr. Lee Ellis and Dr. Luisa Iruela-Arispe.

Clinical trials and the importance of scheduling

Dr. Rakesh Jain opened by sharing the exciting news that over the past 3 years several clinical studies have demonstrated the benefits of the addition of anti-angiogenesis therapy, to be more precise anti-VEGF therapy, to chemotherapy in metastatic colorectal, lung and breast cancer clinical trials. Preclinical studies provided the foundation for these clinical trials, although the efficacy in preclinical studies far exceeded that observed in patients. Nonetheless comparing the results from a preclinical setting to a clinical setting, one can conclude that mouse studies can be a valuable indicator for the potential of these types of therapeutic strategies (Winkler et al. [4] versus Willet et al. [5], and Tong et al. [6] versus Willet et al. [5]). It must be remembered that the clinical studies thus far have been against late stage disease, a challenge that has proven more than difficult for almost every new experimental therapy tested.

Dr. Lee Ellis pointed out that it is critical to recognize that anti-angiogenesis treatment covers more than VEGF pathway inhibitors alone. Besides anti-VEGF compounds one can make the distinction between direct anti-angiogenesis compounds (i.e. affecting activated endothelial cells (EC) directly), vascular disrupting agents (VDA, e.g. combretastatin), and miscellaneous (e.g. IL-8, IL-2 and TNF-α). Yet, currently, this is not reflected in clinical trials where predominantly VEGF inhibitors are being tested. It begs the question whether the preclinical studies with non-VEGF directed therapies would also prove to be positive in humans. In the clinical trials thus far, the addition of bevacizumab (Avastin) to standard chemotherapy has in general improved survival advantages and response rate by 10–15%. However, it is important to also point out that not every individual combination study has demonstrated improved efficacy with the addition of bevacizumab. A Phase III trial in

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second/third line patients with metastatic breast cancer did not benefit from the addition of bevacizumab to capecitabine [7].

Dr. Christopher Willett brought the audience up to date on the current trials and accrual with bevacizumab and radiation therapy. Patients are being given a 2-week lead-in exposure to bevacizumab and then begin radiation therapy or fluorouracil chemotherapy [8]. Considering the increasing number of studies suggesting that the 'normalization window' may appear early on in treatment, and that after a few days there may be a decrease in tumor blood flow and oxygen [9, 10], the results of the first clinical combination studies will have to be carefully analyzed with proper perspective. Certainly, more basic and clinical studies elucidating optimal scheduling of combination treatments are warranted.

Dr. Donald McDonald reported on the effects of VEGF-R2 inhibitor treatment on non-mature and mature vasculature [11]. Already one day after start of treatment, non-mature tumor vessels are pruned: the endothelium retreats, but the extracellular matrix components stay behind. The so-called normalized blood vessels that remain are, however, far from normal. While their endothelium does contain intimate contact points with pericytes, normalization or pruning does not stop. A novel observation was that the anti-VEGF treatment also affected blood vessels in other tissues in the body. Depending on the location, blood vessels lost endothelial coverage and functionality (Fig. 1). In the pancreas, endothelial fenestrations were lost, and in renal glomerular vasculature proteinuria was induced due to loss of podocyte—endothelial based functionality. The important concept that in different organs and tumor capillaries variations in responsiveness to VEGF inhibition can occur, points to the existence of important vascular and endothelial heterogeneity throughout the body. Dissecting the molecular mechanism(s) underlying this heterogeneity will be an essential asset in the development of effective anti-VEGF treatment strategies for clinical application.

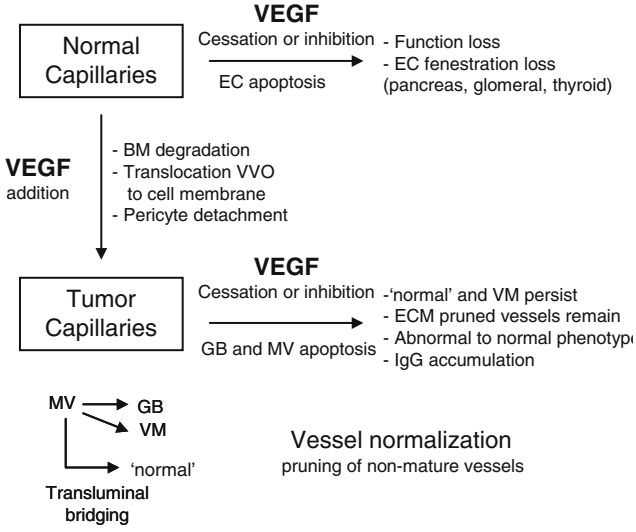

Fig. 1 Effect of anti-VEGF therapy on tumor and normal organ capillaries. MV, mother vessel; GB, glomeruloid bodies, Vm, vascular malformations; VVO, vesiculo-vacuolar organelle

Current status of surrogate markers for anti-angiogenesis therapy effects

Currently, effectiveness of an angiostatic compound is mostly defined by either response rate or survival. Although these are the crucial endpoints, efficacy of anti-angiogenesis treatment might be better monitored and calibrated with the use of surrogate markers, preferentially obtained by non-invasive methods. Successful early development of angiostatic and anti-vascular agents rests upon identification of reliable and potent biomarkers of clinical activity so that critical quantities, such as lowest active and maximum tolerated dose (MTD), can be established. By default, one surrogate marker of angiostatic activity is toxicity. Intriguingly, two patients with dose-limiting toxicities to bevacizumab, fluorouracil chemotherapy and radiation showed the best responses [8]. However, due to lack of severe toxicity indications for many anti-angiogenesis agents as monotherapies, dosing of angiogenesis inhibitors is extremely difficult. Chemotherapy dosing and scheduling is defined by its MTD, however this is not applicable for angiogenesis inhibitors, which are largely cytostatic rather than cytotoxic. The optimal biologic or therapeutic dose (OBD) has to be determined by some sorts of surrogate markers, especially in early clinical trials, as stated by Dr. Robert Kerbel in his presentation, on the merits and methodology of combining chemotherapy and agents targeting angiogenesis [12]. The current list of potential surrogate markers for treatment efficacy includes interstitial pressure, blood flow, perfusion, permeability and pO_2 levels, as well as identification of bone-marrow derived cell contribution to the tumor neovasculature or pericyte and basement membrane changes in the tumor. Non-invasive monitoring methods include functional imaging strategies such as dynamic contrast enhanced magnetic resonance imaging (DCE-MRI) and computed tomography (CT) based perfusion defect analysis and many minimally-invasive techniques to assess physiological status. Biopsies can be taken for histological analysis of changes in tumor parenchyma and

stroma, and less invasive blood analysis may lend information on circulating endothelial cells (CEC). At present, no consensus has been reached on how to define cells such as CEC that originate from the bone marrow in response to solid tumor growth. Some groups have used only a single pan-endothelial marker CD146, as Dr. Dan Duda presented [8], while others include staining of VEGF-R2 [12]. Interestingly, Shaked et al. showed that the vascular targeting agent combretastatin can actually cause a rapid and transient increase in viable CEP levels, in response to tissue damage which appear to contribute to the regrowth of the tumor. Combination treatment with agents to prevent bone marrow mobilization (i.e. anti-VEGF-R2 antibodies) were able to reduce tumor growth, which is a novel application of therapy to prevent 're-vascularization' of the tumor after treatment.

Imaging of angiogenesis and anti-angiogenic drug efficacy

While tumor tissue or blood samples can provide some useful data, non-invasive imaging of the tumor yields direct and immediate information about global changes in tumor vasculature, without the need for invasive procedures. Several imaging modalities have been used to image tumor vasculature, of which DCE-MRI is the most established. Although this technique produces data that correlate well with a number of histological, biochemical and clinical outcome parameters, there are some caveats with the imaging of angiogenesis, according to Dr. Gordon Jayson. A high degree of heterogeneity is seen between patients, especially in Phase I clinical trials, and even between tumor deposits within the same patient [13]. The consequence of the heterogeneity is that dose response relationships are blurred so that only threshold effects are seen. This translates into the situation that as cohorts of patients are treated with higher doses, a dose can usually be identified below which the patients do not seem to derive any benefit. Additional technical constraints include respiratory artifact and the heartbeat, both of which reduce resolution in the chest thus confining most current imaging studies to the abdomen and pelvis.

Cytogenetic abnormalities of endothelial cells

Although it has been postulated for many years that tumor endothelial cells are genetically stable [14], new evidence suggests that tumor-associated endothelial cells in solid tumors are cytogenetically abnormal. These results were presented by Dr. Kyoko Hida who did some fascinating work with Dr. Michael Klagsbrun [15]. Mouse endothelial cells from two different human tumor xenografts (melanoma and liposarcoma) were compared to EC of skin and adipose tissue. Tumor associated endothelial cells expressed typical EC markers, such as CD31 and tumor endothelial cell markers (TEM), but had relatively large, heterogeneous nuclei and were cytogenetically abnormal. Aneuploidy and abnormal multiple centrosomes were identified in freshly isolated tumor endothelial cells by fluorescence in situ hybridization (FISH) analysis. These cytogenetic alterations were neither clonal, nor was there any evidence of human tumor derived chromosomal material in the mouse EC. All these observations were made exclusively in tumor derived EC and not in EC isolated from normal skin or adipose tissue. These results raise several questions to be elucidated in the future, such as, (1) exactly how genetically stable are endothelial cells associated with various disease states? (2) Can tumors, more specifically the tumor EC, become resistant to anti-angiogenic treatment in part because of chromosomal abnormalities? and (3) What if the tumor microenvironment or the carcinogenic process causes or influences the EC to acquire cytogenetic abnormalities?

Abnormal cellular content of the tumor vasculature

Tumor blood vessels have multiple molecular and cellular abnormalities on all levels of the vessel wall, including EC, pericytes, and vascular basement membrane. This was nicely demonstrated in presentations by Dr. Donald McDonald and Dr. Luisa Iruela-Arispe. Besides the cytogenetic abnormalities of EC as noted above, tumor EC are known to express abnormal proteins, undergo sprouting and proliferation and have a defective barrier function. In addition, tumor vessel associated pericytes are abnormally low in relation to EC. Lastly, the basement membrane has redundant loose layers that reflect the dynamic nature of tumor vasculature [11]. Therefore, these abnormalities and the associated extracellular matrix components are promising additional therapeutic angiogenesis targets [16]. Dr. Zena Werb's talk focused on the interaction of leukocytes within the tumor microenvironment [17]. She proposed that developing tumors undergo an inflammatory switch. Leukocytes at the tumor stroma interface are very

motile using amoeboid-like migration patterns, and stayed in close proximity to the tumor cells and the neo-vasculature. T-cells preferentially migrated along blood vessels and interacted with the blood vessels as well as with each other. Leukocytes within the tumor (i.e., those that have extravasated into the interstitial space) are much more stationary, while those at the tumor margin were very active in general. A fascinating additional observation made by their group was that the degree of motility of leukocytes correlated closely to the oxygen tension in the blood stream. This has interesting implications for the role of hypoxia on immune response to tumor tissue. Taken together, all these abnormalities on the molecular and cellular level of tumor parenchyma, stroma and immunological cell types contribute to the dramatic changes in tissue physiology that occur as tumor growth drives the angiogenic process.

Morphological traits and gene expression of tumor vasculature

Tumor blood vessels are also highly abnormal on an organizational, structural and functional level. VEGF, besides inducing angiogenesis and being a survival factor, also disrupts normal vascular barrier functions of EC. Dr. Harold Dvorak showed that VEGF-A_{164} introduced by injection of adeno-viral vectors into nude mice induced vascular hyperpermeability, edema, and fibrin deposition and generated several distinct types of tumor vessel surrogates: so-called mother vessels (MV), glomeruloid bodies (GB), vascular malformations (VM), and capillaries [18]. MV, large thin-walled hyperpermeable and pericyte-poor sinusoids, were the first new vessel types to form, beginning to develop within 18 h from pre-existing venules by a process of basement membrane degradation, pericyte detachment and transfer of internal vesiculo-vacuolar organelle (VVO) membranes to the cell surface. Subsequently, MV evolved into GB, VM or into normal appearing capillaries, the latter by a process of transluminal bridging (Fig. 1). Fascinatingly, upon cessation of VEGF-A exposure MV and GB underwent apoptosis, whereas VM did not and persisted indefinitely, apparently because they are VEGF independent. Interestingly, PLGF (platelet derived growth factor) only induced one type of tumor vessel to grow, namely VM, without the induction of significant vascular hyper-permeability or edema. Once these VM were formed they also persisted indefinitely and were PLGF independent.

Dr. David Cheresh pointed out in his talk that VEGF is also able to uncouple endothelial cell-cell junctions, causing the characteristic tumor vessels that are dilated, torturous and leaky [19]. This disruption may potentiate tumor cell extravasation leading to widespread dissemination of tumor cells from the primary site and possibly increased metastatic growth. Apparently, the degree of abnormality in tumor growth and physiology is tightly linked to the levels of growth factors, such as VEGF and PLGF that are present.

Dr. Gabriele Bergers discussed in great detail the challenges experienced in studying pericyte behavior during neovascular activity [20]. Whereas pericyte coverage of neovasculature has been a subject of debate for quite some time now, the use of multiple marker proteins may now solve the main problem of cell detection in different tissues. Although not being pericyte-specific, and representing dynamic, tissue and cell differentiation stage-specific markers, the combination of desmin, NG2, PDGF-R2 and α-SMA can differentiate the pericyte from other cell types. The kinetics and dynamics of pericyte marker protein expression could be clearly demonstrated when endothelial cells are co-cultured in Matrigel with pericyte progenitor cells. Maturation stages observed by flow cytometry dynamically evolved from PDGF-R2 to NG2 to α-SMC positive. The importance of the presence of pericytes on endothelial cell behavior and survival in *in vitro* systems, could be visualized in Matrigel tube formation assays: while endothelial derived tubes fell apart after 3 days of culture, co-culture of endothelium with pericytes resulted in viable tubes even at 14 days after start of incubation.

Dr. Beverly Teicher from Genzyme Corp. reported on their strategy to use SAGE and long-SAGE libraries to screen lung carcinomas for new targets on the tumor vasculature. Extensive studies in their laboratories demonstrated that overall, endothelium in capillaries in normal tissue and in tumor tissue express similar genes. Only a small subpopulation of genes is differentially expressed in the tumor vasculature. Interestingly, of the new targets identified, not the regularly used HUVEC or HMVEC, but the human endothelial progenitor cells (EPC) demonstrated expression of the genes under study. Based on this result, their strategy of studying angiogenic markers in culture systems now includes human EPC cultured in Matrigel systems. In combination with pericytes derived from neonatal brain tissue that are commercially available, this experimental set-up can now be employed for more detailed studies on target gene functionality in vitro.

Vascular identity of the premetastatic niche

Over the years many theories have tried to describe the mechanism by which tumor cells are able to stimulate tumor growth at metastatic sites; seed-and-soil theory and clonal theory are some of the most established. Dr. David Lyden proposed an intriguing new theory by which certain tumor types maintain a similar metastatic pattern to certain tissues in the body [21]. They have identified a subpopulation of hematopoietic progenitor cells (HPC), which are VEGF-R1$^+$, that mobilize to the peripheral circulation along with bone marrow derived VEGF-R2$^+$ EPC, contributing to the neovascularization in primary tumors. These HPC home to tumor specific premetastatic sites and form cellular clusters that are apparently permissive to angiogenesis prior to the arrival of tumor cells. Critical for the mobilization of HPC are Id (inhibition of differentiation) genes and removal or blocking of these HPC prevents metastases. Furthermore, yet to be identified tumor-specific growth factors provide a permissive niche for α4ß1 integrin$^+$ HPC and tumor cells, by upregulating fibronectin in resident fibroblasts. A final convincing study performed by their group used conditioned media from distinct tumor types. After injection of this media into the mice, unique patterns of fibronectin expression in specific organs occurred and furthermore, metastatic growth of tumors could be 'directed' to certain organs depending on the conditioned medium that was injected. This appears to be a significant advance in our understanding of why certain tumors spread to specific organs, and the identification of the growth factor and cytokine expression patterns that guide the location of premetastatic niches could be revolutionary for therapeutics against metastasis. In general, targeting VEGF-R1$^+$ and α4ß1$^+$ cells may be a step forward in preventing the early events involved in tumor spread.

Challenges for the future, and the future is now!

The identification of neovascularization as an essential process in the growth of both primary and metastatic tumors and subsequent unraveling of the molecular pathways underlying the cell biological reactions involved, have re-shaped our thinking about cancer therapy. Due to relentless efforts of many great scientists gathered at this excellent meeting, new (patho) physiological concepts generated in the last decades, have given rise to the development of a large variety of new drugs to interfere with angiogenesis. Currently, bevacizumab, thalidomide and the recent approval of endostatin in China (renamed Endostar [22]) represent the forefront of the field. Moreover, all large pharmaceutical industries possess one or more angiostatic compound in certain stages of development, representing a multi-billion dollar market foreseen for the future. The stakes are clearly high for the pharmaceutical industry, but more importantly the stakes are high for those 50% of patients diagnosed with cancer today who cannot be successfully treated with currently available therapies. Some years of experience with anti-angiogenesis drugs in clinical studies have revealed a number of challenges imperative to be addressed in future translational research. Combination of (non-invasive) neovascular imaging and predictive biomarkers of cell biological effects of the drugs under study will be essential for proper interpretation of pharmacological effectiveness in relation to clinical efficacy (see also Table 1). In parallel, we need analytical tools to dissect the molecular basis for undesired vascular effects in non-target blood vessels from desired effects in the tumor vasculature. For this a paradigm shift from in vitro to in vivo study of endothelial pharmacology needs to be strongly advocated and initiated [23, 24]. Ultimately, effective treatment strategies will only be accomplished by personalized

Table 1 Status quo of tumor associated angiogenesis research and possible futures

Anti-Angiogenesis topic	Preclinical proof	Clinical evidence	Routine use	Future studies and uses
Tumor growth reduction by combination treatment with VEGF inhibition	√	√	±	VEGF-independent angiogenesis inhibition
Vessel normalization by VEGF inhibitors	√	√	±	VEGF-independent vessel normalization
Angiogenesis imaging	√	√		Standard procedure and prognosis
Surrogate markers (pO_2, perfusion, CEC, CEP)	√	±		Standard procedure and prognosis
Abnormal cellular and morphological vessels	√	√		Novel targets and re-designed treatment strategy
Abnormal vessel associated pericytes and ECM	√	√		Novel targets
Vascular mimicry	√	√		Novel treatment strategies
EC anergy	√			Novel immune enhancers
Cytogenetic abnormal EC	√			Cocktail of angiogenesis inhibitors
Premetastatic niche	√			Prophylactic, site directed treatment

disease identification. Miniaturizations of newly developed analytical tools (e.g. genomics, proteomics and quantitative real-time PCR) are instrumental for this purpose, to bridge the gap in knowledge on the true molecular and cell biological nature of success and failure of agents such as anti-angiogenesis drugs. And as we move forward with a collective goal to implement these newest ideas, it is wise to retain a healthy perspective on where the field has come to date. All that is necessary is to think back to the days of standard chemotherapy, surgery and radiation therapy, and realize that we have the ability and the responsibility to create the best opportunities to implement treatments of low toxicity and simplicity with a real chance to revolutionize treatment of cancer and other disease states.

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