VOLUME 31 · NUMBER 17 · JUNE 10 2013

JOURNAL OF CLINICAL ONCOLOGY

SPECIAL ARTICLE

Normalizing Tumor Microenvironment to Treat Cancer: Bench to Bedside to Biomarkers

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Published online ahead of print at www.jco.org on May 13, 2013.

Supported by grants from the National Cancer Institute (1980 to present), National Science Foundation (1976-1999), Department of Defense Breast Cancer Research Program (1996-2002; 2010 to present), American Cancer Society (1980-1992), National Foundation for Cancer Research (1998 to present), Goldhirsh Foundation (2002-2006), and Brain Tumor Society (2006-2008) and by grants from AstraZeneca, DuPont-Merck, Enzon, Genentech, Hybritech, ImClone, Medimmune, Miravant, Roche.

Presented as the American Society of Clinical Oncology (ASCO) Science of Oncology Award Lecture at the 50th Annual Meeting of ASCO, Chicago, IL, June 2-6, 2012.

Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

Author's disclosures of potential conflicts of interest and author contributions are found at the end of this article.

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0732-183X/13/3117w-2205w/$20.00

DOI: 10.1200/JCO.2012.46.3653

See video

ABSTRACT

For almost four decades, my work has focused on one challenge: improving the delivery and efficacy of anticancer therapeutics. Working on the hypothesis that the abnormal tumor microenvironment—characterized by hypoxia and high interstitial fluid pressure—fuels tumor progression and treatment resistance, we developed an array of sophisticated imaging technologies and animal models as well as mathematic models to unravel the complex biology of tumors. Using these tools, we demonstrated that the blood and lymphatic vasculature, fibroblasts, immune cells, and extracellular matrix associated with tumors are abnormal, which together create a hostile tumor microenvironment. We next hypothesized that agents that induce normalization of the microenvironment can improve treatment outcome. Indeed, we demonstrated that judicious use of antiangiogenic agents—originally designed to starve tumors—could transiently normalize tumor vasculature, alleviate hypoxia, increase delivery of drugs and antitumor immune cells, and improve the outcome of various therapies. Our trials of antiangiogenics in patients with newly diagnosed and recurrent glioblastoma supported this concept. They revealed that patients whose tumor blood perfusion increased in response to cediranib survived 6 to 9 months longer than those whose blood perfusion did not increase. The normalization hypothesis also opened doors to treating various nonmalignant diseases characterized by abnormal vasculature, such as neurofibromatosis type 2. More recently, we discovered that antifibrosis drugs capable of normalizing the tumor microenvironment can improve the delivery and efficacy of nano- and molecular medicines. Our current efforts are directed at identifying predictive biomarkers and more-effective strategies to normalize the tumor microenvironment for enhancing anticancer therapies.

J Clin Oncol 31:2205-2218. © 2013 by American Society of Clinical Oncology

INTRODUCTION

Since 1974, my colleagues and I have investigated solid tumors not just as a collection of malignant mutated cells but rather as aberrant organs composed of cancer cells and their stroma—also referred to as the tumor microenvironment. This microenvironment is composed of blood and lymphatic vessels and a variety of nonmalignant host cells—all embedded in an extracellular matrix (Fig 1A). Our work has shown that the tumor microenvironment is abnormal and that these abnormalities can fuel tumor progression and treatment resistance. Moreover, normalization of the microenvironment can improve treatment outcome in mice and patients with malignant and nonmalignant diseases.[2-5] Here I will discuss how we obtained these insights by imaging tumors in mice, and how we validated these concepts in patients. I will present our findings first in blood vessels, then lymphatic vessels, and finally the extracellular matrix.

TUMOR VASCULATURE IS ABNORMAL

Our initial work on the tumor microenvironment and drug delivery involved growing tumors in animals, excising them for various measurements, and then using mathematic models to gain insight into the inner workings of tumors.[6,7] Although insightful, this approach did not capture dynamic changes at a cellular or subcellular resolution. To overcome this, we developed transparent windows and sophisticated, high-resolution optical imaging techniques that allowed us to visualize events in tumors in real time.[8-10] Coupled with molecular probes, image analysis, and mathematic models, this approach has provided unprecedented insights into molecular, cellular, anatomic, and functional changes during tumor progression and in response to treatment (Data Supplement Fig S1).[10,11]

Unlike normal vessels, which are orderly, tumor vessels are tortuous, saccular, and chaotic in their organization (Fig 1B; Data Supplement Videos S1 [www.jco.org/site/v/3653/S1.mov] and

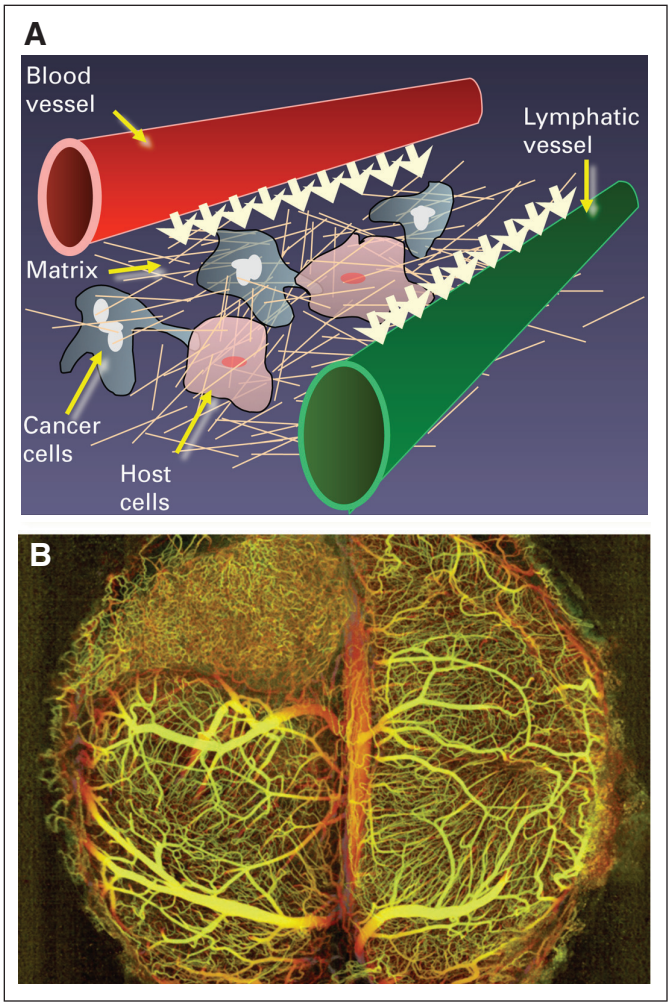

Fig 1. (A) The microenvironment is composed of blood and lymphatic vessels and a variety of nonmalignant host cells, all embedded in an extracellular matrix. The host cells include fibroblasts and a variety of resident and trafficking immune cells. (B) Vasculature of a brain tumor (upper left quadrant) and the surrounding brain of a mouse. Color overlay denotes scale of the depth of the vessel, with yellow vessels closest to the viewer, and red vessels deepest. Reproduced from.[1]

S2 [www.jco.org/site/v/3653/S2.mov]).[1,12] The structure of the vessel wall is also abnormal, with large gaps between endothelial cells, detached pericytes, and abnormally thick or thin basement membranes.[13-16] Consequently, tumor vessels are leaky in some places and not in others, with overall leakiness dependent on the host organ.[17-20] Moreover, these vessels change with tumor growth and treatment (Data Supplement Video S3 [www.jco.org/site/v/3653/S3.mov]).

Clinical experience indicates that a primary tumor may respond to certain therapies, whereas its metastases might not.[21] To understand the role of different host microenvironments in tumor biology or response to treatment, we examined tumors in various organs of mice, such as the brain, mammary fat pad, liver, pancreas, and skin.[19,22-27] For instance, when we inoculated the same breast cancer cells in three different sites, the resulting vasculature was abnormal yet vastly different in each site (Fig 2A).[1]

We also examined blood vessels in spontaneously arising tumors in various organs, such as skin, breast, pancreas, liver, and colon.[30] Figure 2B shows the vasculature of a spontaneously arising colon cancer in a genetically engineered mouse model.[28] Note the highly organized blood vessels in normal colon. However, similar to transplanted tumors, the vessels in the spontaneous colon cancer are abnormal, and this abnormality increases as tumors progress. More crucially, these blood vessels are as structurally abnormal as those in colon carcinomas in patients (Fig 2C).[29]

ABNORMAL VASCULATURE LEADS TO HOSTILE TUMOR MICROENVIRONMENT

The abnormal vascular structure leads to spatially and temporally heterogeneous blood perfusion in tumors.[31] In any given vessel, blood flow can change with time. Compounding this heterogeneity, blood flow can be quite brisk in one region of the tumor and static in another region, and the flow in each region can also change with time.[22,23,32-34] There are two major causes of this flow heterogeneity: one, physical forces, known as solid stresses, generated during tumor growth can compress vessels, resulting in reduced or no flow in the pinched vessels and the ones downstream[35,36]; and two, excessive vessel leakiness, leading to plasma escape and hemoconcentration, can itself cause flow stasis, even when that vessel has an open lumen.[37,38]

Independent of the cause, this heterogeneity in perfusion has multiple adverse consequences. It limits the access of blood-borne drugs and effector immune cells to poorly perfused regions of tumors[39] and leads to hypoxia and low extracellular pH (Data Supplement Fig S2).[40,41]

Hypoxia is known to aid tumor progression and metastasis by inducing genetic instability, angiogenesis, immunosuppression, inflammation, the cancer stem-cell phenotype, epithelial-mesenchymal transition, resistance to cell death by apoptosis and autophagy, and altered metabolism.[42-47] Hypoxia also confers resistance because various treatments, such as radiation, certain chemotherapies, photodynamic therapy, and even immunotherapies, require oxygen to be effective.[43,44,48,49]

Because of their excessive leakiness, tumor vessels are unable to maintain pressure gradients across their walls, causing interstitial fluid pressure (IFP) to rise close to microvascular pressure levels.[50,51] Hence, unlike normal skin or breast, where IFP is close to zero, tumors in mice and patients exhibit elevated IFP (Data Supplement Fig S3).[52-60] Moreover, IFP is uniformly elevated inside the tumor and drops precipitously in the tumor margin to normal values.[61-63] As a result, the transport of drugs occurs primarily by diffusion within tumors, and the interstitial fluid oozes out from the tumor margin into the surrounding tissues, where the pressure is low. This fluid not only causes peritumor edema but also carries growth factors and cancer cells with it, further fueling tumor progression.[64,65] Finally, as stated earlier, excessive leakiness and vascular compression cause flow stasis in tumor vessels, resulting in hypoxia, which further upregulates vascular endothelial growth factor (VEGF) and other angiogenic molecules, thus establishing a vicious cycle (Data Supplement Fig S4).

VASCULAR NORMALIZATION HYPOTHESIS: BENCH TO BEDSIDE

Realizing the adverse consequences of hypoxia and interstitial hypertension, we began to look for ways to remedy these microenvironmental abnormalities. There are only two ways to increase oxygen levels in

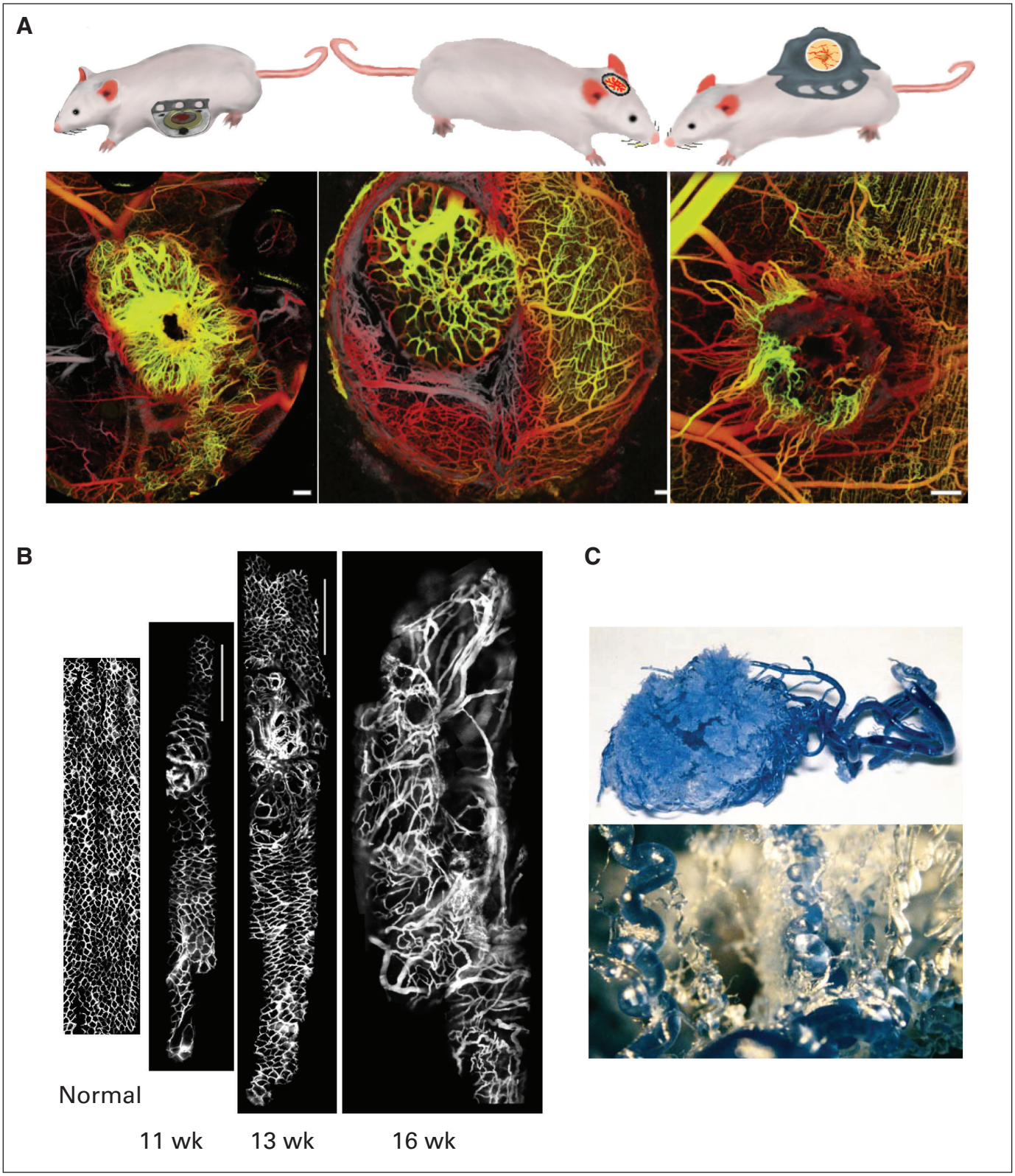

Fig 2. (A) Transparent windows were implanted in the mammary gland, cranium, and dorsal skin of mice. Vasculature of breast tumors grown in these different sites is abnormal yet significantly different. Reproduced from.[1] (B) The vasculature of the normal colon and that of a spontaneously arising colon cancer in a genetically engineered mouse model. Note that the blood vessels in a normal colon are highly organized. However, similar to transplanted tumors, the vessels in the spontaneous colon cancer are abnormal, and this abnormality increases as the tumor progresses over weeks 11 to 16. Reproduced from.[28] (C) A polymer cast of the vasculature of a 1-lb human colon cancer. Note that this vasculature is as abnormal as that in the murine tumors. Reproduced from.[29]

tumors: one, increase the supply of oxygen via blood vessels; and two, decrease the consumption of oxygen by cells. Initially, we attempted to improve the blood supply of tumors using vasoactive agents.[31,66-69] However, the improvements in blood perfusion were short lived because the blood vessels of the tumor remained abnormal. Hence, we looked for translatable approaches to normalize tumor vessels.[2-4,13]

In physiologic angiogenesis, the effects of proangiogenic molecules, such as VEGF, are exquisitely counterbalanced by endogenous antiangiogenic molecules, such as sVEGFR1 and thrombospondins (Fig 3A, panel 1).[13,71] During tumor angiogenesis, because of genetic and epigenetic factors, this balance is tipped in favor of new vessel formation. However, the resulting vessels are highly abnormal both structurally and functionally (Fig 3A, panel 2). We posited that by mopping up some of the excess VEGF using bevacizumab or by blocking VEGF signaling, we could restore this balance. This would prune some abnormal vessels and remodel the remaining vessels, resulting in a normalized vasculature (Fig 3A, panel 3). In turn, this would reduce tumor hypoxia and IFP, resulting in enhanced efficacy of various therapies. If the antiangiogenic agent is too potent or the dose is too high, the balance can tip in the other direction and cause excessive vessel pruning. This may cause necrosis and delay tumor growth (Fig 3A, panel 4, top). However, increased hypoxia can decrease the efficacy of various therapies and even increase metastasis. Alternatively, tumors might begin to make abnormal vessels again by activating other proangiogenic pathways (Fig 3A, panel 4, bottom). Hence, as discussed later in the Biomarkers of Response and Resistance to Antiangiogenic Therapy section, considerable effort is now directed toward blocking these escape pathways.

The normalization hypothesis offered a potential resolution of an outstanding paradox: Bevacizumab increased survival only when used with chemotherapy or immunotherapy for metastatic colorectal, lung, and kidney cancers (Data Supplement Table S1).[72-75] But the

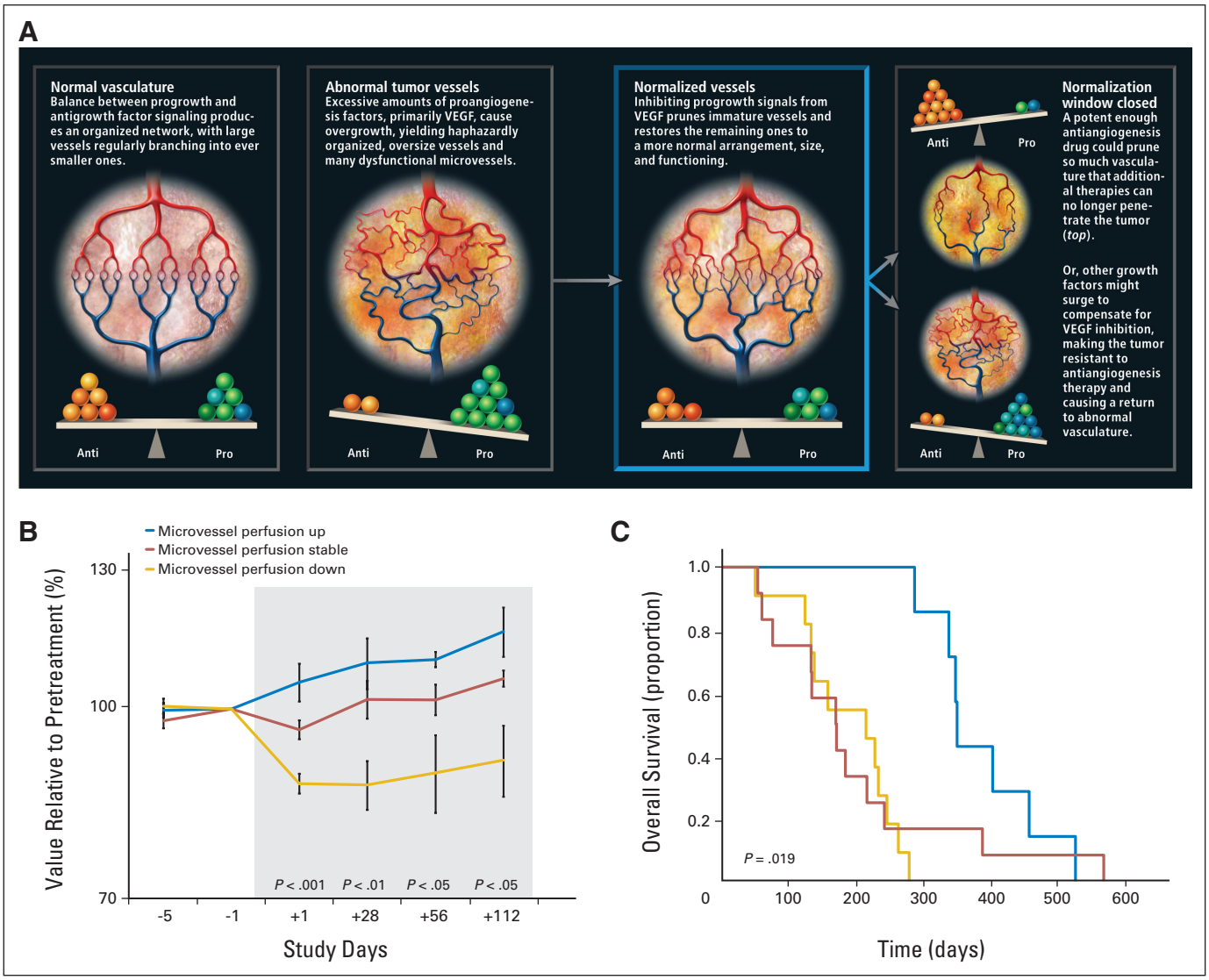

Fig 3. (A) Normalization hypothesis. Originally published by Scientific American, Inc. Illustration © 2013 Kasnot Illustration, Inc. All rights reserved.[2-4] (B) Blood perfusion in recurrent glioblastomas during treatment with cediranib, a pan–vascular endothelial growth factor receptor tyrosine kinase inhibitor. Note that the perfusion goes up in some patients, remains stable in others, and goes down in the rest. Data adapted.[70] (C) Kaplan-Meier survival curves for patients with recurrent glioblastoma treated with cediranib. Note that the patients whose tumor perfusion increased survived longer than the rest. Data adapted.[70]

original goal of anti-VEGF therapy was to destroy the blood vessels of tumors,[76,77] and chemotherapy or immune therapy requires functional blood vessels to deliver these therapeutics. So how could vessel destruction help these therapies (Data Supplement Fig S5)? Our thought was that if the vessels began to function better in response to anti-VEGF therapy, they would enhance both the delivery and effectiveness of concurrent therapies.[2-4,13] However, this concept was new and naturally required validation.

We first tested this idea in a number of animal models, using both direct and indirect angiogenesis inhibitors. Indeed, these agents normalized blood vessels (Data Supplement Video S3 [www.jco.org/site/v/3653/S3.mov]).[1-4,13,78-84] These findings raised the next question: Is normalization an artifact of animal models? So, in collaboration with my colleague, Dr Christopher Willett, we tested this concept in patients with rectal carcinoma receiving bevacizumab. The clinical data confirmed our preclinical finding that blocking VEGF could normalize tumor vessels and lower IFP.[57,59] But this raised a new set of questions, such as: When does normalization begin? When does it end? Is the outcome superior when the therapy is administered during the time window of normalization?

To address these questions, we returned to the bench—this time using mice with human glioblastoma (GBM) xenografts in cranial windows—and found that normalization began by day 1 and lasted 5 to 6 days, alleviating hypoxia during this time window. More crucially, the outcome of radiotherapy was superior when administered during the normalization window. We also discovered the underlying molecular mechanisms: Activation of Ang1/Tie2 signaling contributed to recruitment of pericytes, and activation of matrix metalloproteinases (MMPs) contributed to thinning of the vascular basement membrane.[84]

Moreover, in collaboration with my colleagues, Drs Tracy Batchelor and Gregory Sorensen, we confirmed vascular normalization in patients with recurrent GBM treated with the oral pan-VEGFR kinase inhibitor cediranib. As a result, brain edema resolved, and white matter tracts were restored (Data Supplement Video S4 [www.jco.org/site/v/3653/S4.mov]). Similar to our mouse model, normalization began by day 1, but the normalization window in patients lasted for at least 1 month—long enough for almost a full course of radiation therapy (Data Supplement Fig S6).[85]

All this clinical work showed that anti-VEGF therapies could normalize tumor vessels. But a crucial question arose: How does tumor vascular normalization affect patient survival? In fact, patients whose blood vessels normalized the most had the highest progression-free and overall survival (OS) rates (Data Supplement Fig S7).[86] But the most compelling support for the role of vascular normalization came when we looked at the blood perfusion changes in tumors. Anti-VEGF therapy actually increased tumor perfusion in some patients for nearly 4 months. Moreover, the patients whose tumor perfusion increased survived 6 months longer than those whose blood perfusion did not (Figs 3B and 3C).[34,70]

In a subsequent trial, we saw the same pattern in patients with newly diagnosed GBM treated with cediranib and chemoradiation. In 20 patients, tumor blood perfusion increased for 1 month, and in 10, it decreased.[87] Our hypothesis is that increased blood perfusion alleviates tumor hypoxia. Oxygen is a well-known radiation sensitizer and should improve tumor response to radiation therapy. Oxygen can also increase antitumor immunity. So we would expect better survival in the patients with increased perfusion. Indeed, the patients whose tumor blood perfusion increased survived approximately 9 months longer than those whose did not. These two trials provide compelling clinical evidence that antiangiogenic agents, which were originally developed to starve tumors, can increase tumor blood perfusion in some patients and that those patients survive longer. Moreover, these observations suggest that a noninvasive imaging test—perfusion magnetic resonance imaging—may enable clinicians to identify early on patients most likely to benefit from antiangiogenic therapy.

BIOMARKERS OF RESPONSE AND RESISTANCE TO ANTIANGIOGENIC THERAPY

To date, 10 antiangiogenic drugs have been approved for treatment of 12 different malignancies. However, similar to many targeted therapies, the OS benefit from antiangiogenic therapies remains modest (Data Supplement Table S1). Unfortunately, unlike many targeted therapies, there are no validated biomarkers for antiangiogenic agents.[88] If we could find biomarkers to identify patients more likely to benefit from these drugs, the survival benefit in patients receiving anti-VEGF drugs may be comparable to that from other targeted therapies (Data Supplement Table S2). To answer these critical questions, we need to better understand the intrinsic and evasive resistance mechanisms.[16,89,90] Treated tumors could release additional proangiogenic molecules or recruit tumor vessels via mechanisms less dependent on VEGF (Data Supplement Fig S8). Tumor endothelial cells could develop cytogenetic abnormalities, and cancer-like stem cells may differentiate into endothelial cells—processes that may reduce sensitivity to anti-VEGF drugs. In addition, recruitment of myeloid cells, activation of cancer-associated fibroblasts, or coverage of tumor vessels by pericytes may also render tumor vessels insensitive to VEGF blockade. In collaboration with Drs Dan Duda, Lei Xu, and Yves Boucher and our clinical colleagues, we are examining these questions in more than 20 multidisciplinary trials by measuring tissue, circulating and imaging biomarkers at various time points during and after treatment, and correlating these with the various outcome measures (Data Supplement Table S3). The following two examples illustrate emerging insights from these trials.

Circulating sVEGFR1 As Potential Biomarker of Intrinsic Resistance

In 2009, we found that patients with rectal carcinoma who had elevated levels of sVEGFR1 before treatment were less likely to benefit from bevacizumab combined with chemoradiotherapy.[91] Now, we see the same association in patients with newly diagnosed GBM, triple-negative breast cancer, hepatocellular carcinoma (HCC), and metastatic colorectal carcinoma (Data Supplement Table S4).[87,92-94] Our hypothesis is that sVEGFR1 functions as an endogenous VEGF trap. Thus, adding external anti-VEGF agent is not likely to have significant biologic effects in patients with high sVEGFR1 levels. Indeed, high levels of sVEGFR1 were also associated with fewer adverse effects in patients with rectal or breast cancer or HCC.[93-95] Additionally, a retrospective analysis has shown that a genetic variation in the *VEGFR1* gene correlates with increased VEGFR1 expression and poor outcome of bevacizumab treatment in patients with metastatic renal cell carcinoma or pancreatic ductal adenocarcinoma.[96] Of course, these findings need to be tested prospectively.

SDF1α/CXCR4 As Potential Evasive Pathway

We found that circulating levels of the chemokine SDF1α increase in patients who evade various anti-VEGF therapies: rectal carcinoma with bevacizumab, GBM with cediranib, HCC with sunitinib, and sarcoma with sorafenib (Data Supplement Table S5).[60,93,97-99] Interestingly, the sources of SDF1α are different in each of these diseases, as is the role of the SDF1α/CXCR4 pathway.[100] For example, in GBM, this pathway seems to facilitate invasion of cancer cells and co-option of host vessels by invading cancer cells.[101] On the basis of this finding, our collaborator, Dr Patrick Wen, has started a clinical trial with AMD3100 (an anti-CXCR4 drug) plus bevacizumab in patients with recurrent GBM (ClinicalTrials.gov identifier: NCT01339039).

EMERGING INSIGHTS INTO VASCULAR NORMALIZATION

Since 2001, when I formally proposed the normalization hypothesis, more than 100 studies have demonstrated normalization in a variety of tumors using both direct and indirect antiangiogenesis agents, including metronomic chemotherapy.[5,102,103] Several new insights have emerged from these studies.

First, the dose of the anti-VEGF drug matters. As originally hypothesized, vascular normalization is dependent on the dose of anti-VEGF drug (Fig 4A).[2] High doses of anti-VEGF agents could cause rapid vessel pruning and might not improve the outcome of concurrent therapies. High doses may even increase invasion and metastasis, as seen in a number of preclinical studies. In contrast, lower doses might improve perfusion and outcome. In fact, in two independent studies, we found this to be the case with breast cancer in mice.[104,105] These data suggest that the 15 mg/kg dose of bevacizumab might have been too high and responsible for the lack of OS benefit in phase III breast cancer trials with bevacizumab.[106-108] In fact, a recent trial using a dose of 15 mg/kg resulted in decreased perfusion and uptake of docetaxel in patients with non–small-cell lung cancer.[109]

Second, the size of therapeutic agents matters. We have previously shown that tumor vessels have large holes (pores) in their walls, and anti-VEGF treatments lower the size of these holes.[19,110] An outstanding question is whether this decrease in pore size would outweigh the benefits of vascular normalization. Indeed, in a breast cancer model in mice, we found that VEGFR2 blockade improved the treatment benefit from 10-nm nab-paclitaxel (Abraxane; Abraxis Oncology, Los Angeles, CA) but not that from 100-nm liposomal doxorubicin (Doxil; Janssen Pharmaceuticals, Beerse, Belgium; Data Supplement Fig S9).[105]

Third, vascular normalization can improve the outcome of immunotherapy. More than a half dozen studies have demonstrated that vascular normalization can improve the delivery of immune cells into the tumor and/or convert the immunosuppressive microenvironment of tumors into an immunostimulatory one, presumably by alleviating hypoxia (Fig 4B).[104,111-116] With the recent approval of a number of immunotherapeutics for cancer and many more in clinical trials, judicious use of vascular normalizing drugs offers new hope of improving the modest survival benefit of immunotherapies.[117]

Fourth, vascular normalization can decrease intravasation of cancer cells. We had hypothesized that the normalized tumor vasculature may also inhibit the shedding of cancer cells into the circulation—a prerequisite for metastasis.[3] In an elegant study, Carmeliet et al[118] have now demonstrated this in mice.

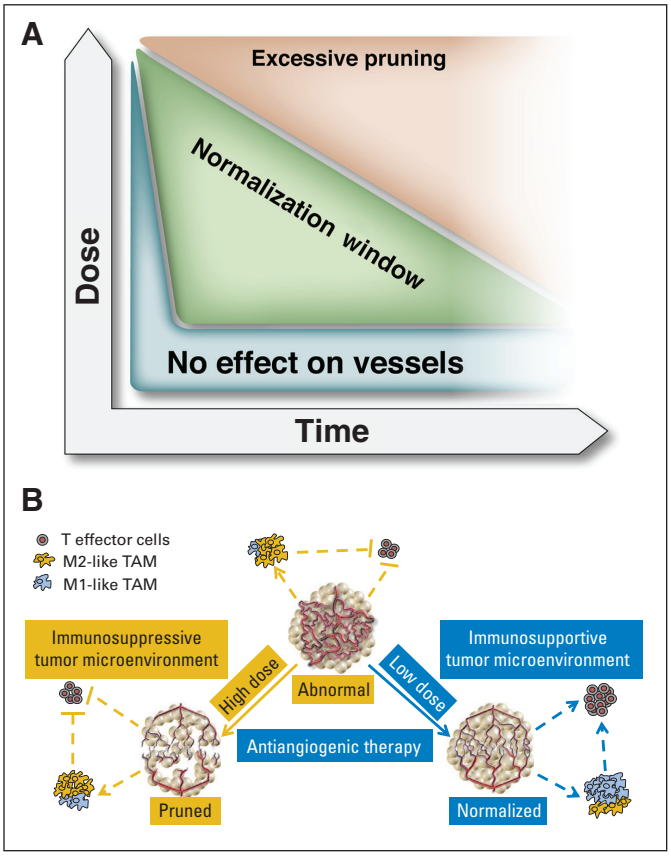

Fig 4. (A) Normalization window is dose dependent; the higher the dose, the shorter the window. Higher doses can also lead to adverse effects in normal tissues. Data adapted.[3] (B) Vascular normalization can convert the immunosuppressive microenvironment of a tumor to an immunostimultaory microenvironment and improve the outcome of various immunotherapies by increasing flow and oxygenation. Data adapted.[104] TAM, tumor-associated macrophage.

Fifth, vascular normalization is also a useful strategy for treating a number of nonmalignant diseases. Pathologies characterized by abnormal vessels afflict more than a half billion people worldwide.[4,16] These include wet age-related macular degeneration and diabetic macular edema—leading causes of blindness. Anti-VEGF agents have been shown to normalize leaky vessels and control these ocular pathologies.[119] Neurofibromatosis type 2 is another such disease. The blood vessels of these benign tumors are also abnormal and contribute to hearing loss in affected patients. My collaborators, Drs Scott Plotkin and Emmanuelle di Tomaso, demonstrated in a trial involving 10 patients with neurofibromatosis type 2 that normalizing the vessels with bevacizumab contributed to improved hearing in 60% of participants.[120] Other emerging applications of vascular normalization include controlling plaque rupture and mitigating neurovascular complications stemming from radiation therapy.[121,122]

NORMALIZATION OF LYMPHATIC VESSELS

So far, I have discussed our attempts to fix the abnormal vasculature using antiangiogenic agents. As mentioned earlier, all components of

the tumor microenvironment are abnormal. So even if we normalize blood vessels, the tumor microenvironment will remain far from normal. For example, by normalizing tumor vessels, VEGF blockade lowers tumor IFP in mice and patients, but never to values seen in normal tissues (Data Supplement Fig S10).[57,60,83,93] Our hypothesis is that the lymphatics within tumors are also dysfunctional and remain so despite vascular normalization with these treatments.[123]

Lymphatics play a critical role not only in maintaining fluid homeostasis in tissues but also in immune response and lymphatic metastasis.[124] To this end, we began investigating lymphatic function in mice approximately 20 years ago using intravital fluorescence lymphangiography (Data Supplement Video S5 [www.jco.org/site/v/3653/S5.mov]) and more recently using a new imaging technique that does not require injection of a fluorescent tracer (Figs 5A and 5B).[1,128-131] These two distinct approaches have revealed that lymphatic vessels in the tumor margin are hyperplastic and functional, and they are adequate for transporting cancer cells from the tumor to nearby lymph nodes (Data Supplement Videos S6 [www.jco.org/site/v/3653/S6.mov] and S7 [www.jco.org/site/v/3653/S7.mov]).[58,125,126] However, functional lymphatics are absent within tumors in mice (Figs 5A and 5B) and humans.[58,126,132] Our hypothesis is that forces generated during tumor progression mechanically compress fragile blood and lymphatic vessels associated with tumors, even from the early stages of tumorigenesis.[30,36,133] In fact, if we deplete different components of tumors (cancer cells, fibroblasts, collagen, and/or hyaluronan), both blood and lymphatic vessels open up (Data Supplement Fig S11).[35,36,134] Other investigators have also shown increased blood perfusion with stroma depletion (reviewed by Hidalgo and Von Hoff[135]). Blood begins to flow in previously compressed blood vessels, but the lymph flow is not resumed in the decompressed lymphatic vessels.

The pressure gradients generated by the heart drive blood flow in vessels. In contrast, lymphatic contractions, along with the valves in the lymphatics, are responsible for normal physiologic lymph flow. So we examined the lymphatic valves in the tumor margin and the contraction of the draining lymphatics. Indeed, the leaflets of the valves did not fully close, presumably because of hyperplasia in the draining lymphatics in the tumor margin, and the contractions were abnormal (Figs 5C and 5D; Data Supplement Video S8 [www.jco.org/site/v/3653/S8.mov]).[127,136] Our current efforts are directed toward restoring normal function in these draining lymphatics to restore fluid homeostasis and immune response in tumors.

In 2008, my colleague, Dr Dai Fukumura, showed that restoring nitric oxide (NO) gradients around tumor blood vessels could normalize their function.[137-139] If the same principle holds for lymphatics, restoring NO gradients around the tumor draining lymphatics should also restore their function (Data Supplement Fig S12). Indeed, we can restore lymphatic contraction in a model of cutaneous inflammation by restoring NO gradients (Data Supplement Video S9 [www.jco.org/site/v/3653/S9.mov]).[127] Previously, we demonstrated that blocking endothelial NO synthase could decrease lymphatic hyperplasia and prevent lymphatic metastasis (Data Supplement Figs S13 and S14).[140] Currently, in collaboration with Drs Timothy Padera, Dai Fukumura, Lance Munn, and Lei Xu, we are building on these findings to slow tumor progression and lymphatic metastasis and further improve treatment outcome by manipulating the function of tumor-associated lymphatics.

NORMALIZING THE EXTRACELLULAR MATRIX

Even if we were to normalize the function of blood and lymphatic vessels, the extracellular matrix could still impede drug penetration and efficacy, especially in highly desmoplastic tumors (eg, pancreatic ductal adenocarcinomas).[135,141] We suspected nearly 30 years ago that the extracellular matrix could be a barrier when we observed a lack of penetration of a large molecule (150,000 MW dextran) in some regions of tumors grown in rabbits (Data Supplement Fig S15).[8,9] We saw the same when we intravenously administered 90-nm liposomes—approximately the size of liposomal doxorubicin, an approved nanomedicine—in tumor-bearing mice (Fig 6A).[20] These particles leaked out of some tumor vessels but did not move far from the vessel wall. Even 150-nm particles directly injected into a tumor do not move too far from the injection site (Fig 6B).[142]

The answer to this riddle came from two developments in our laboratory. First, Dr Paolo Netti made an unexpected discovery in 2000: Diffusion of macromolecules is low in tumors that are stiff and collagenous.[144] Second, Dr Edward Brown employed second harmonic generation microscopy to image collagen in vivo.[145] By imaging collagen and 150-nm viral particles simultaneously in vivo, we discovered that fibrillar collagen restricts the movement of these particles (Fig 6B). And when we coinjected bacterial collagenase with these particles, the volume of distribution tripled (Data Supplement Fig S16).[142]

Because collagen is a structural molecule, we cannot administer bacterial collagenase systemically. So we searched for antifibrotic agents that could deplete collagen. Initially, we tested relaxin—a hormone released during pregnancy and known to reorganize collagen matrices.[145] Indeed, 2 weeks of treatment with relaxin reorganized the collagen matrix in a desmoplastic tumor and increased the penetration of large molecules (Data Supplement Fig S17).[145,146] Although we did not see any increase in metastasis in our tumor models, others reported that relaxin could increase metastasis from prostate cancer in mice.[147]

We next tried to permeabilize the collagen matrix by employing enzymes known to degrade collagen I: MMP1 and MMP8.[148] The latter is also known to reduce metastasis. Indeed, ectopic expression of MMP8 in tumors increased the volume of distribution and efficacy of gene therapy (Data Supplement Fig S18). Faced with the challenge to translate this finding, we began a search for US Food and Drug Administration–approved antifibrotic agents.

After considering a number of options, we realized that the widely prescribed angiotensin II receptor blocker (ARB) losartan—known to reduce collagen production by blocking transforming growth factor beta activation—could be a potential candidate. Indeed, losartan treatment for 2 weeks led to a dramatic decrease in collagen as well as an increase in penetration and accumulation of 100-nm particles in collagen-rich tumors (Figs 6C to 6E).[143] And this, in turn, increased the efficacy of gene therapy and liposomal doxorubicin in desmoplastic tumors (Fig 6F).

It is worth noting that a retrospective analysis has shown that patients with pancreatic ductal adenocarcinoma receiving ARBs or angiotensin-converting enzyme inhibitors survive approximately 6 months longer than those who do not (Data Supplement Table S6).[149] Similar retrospective analyses have shown increased survival in patients

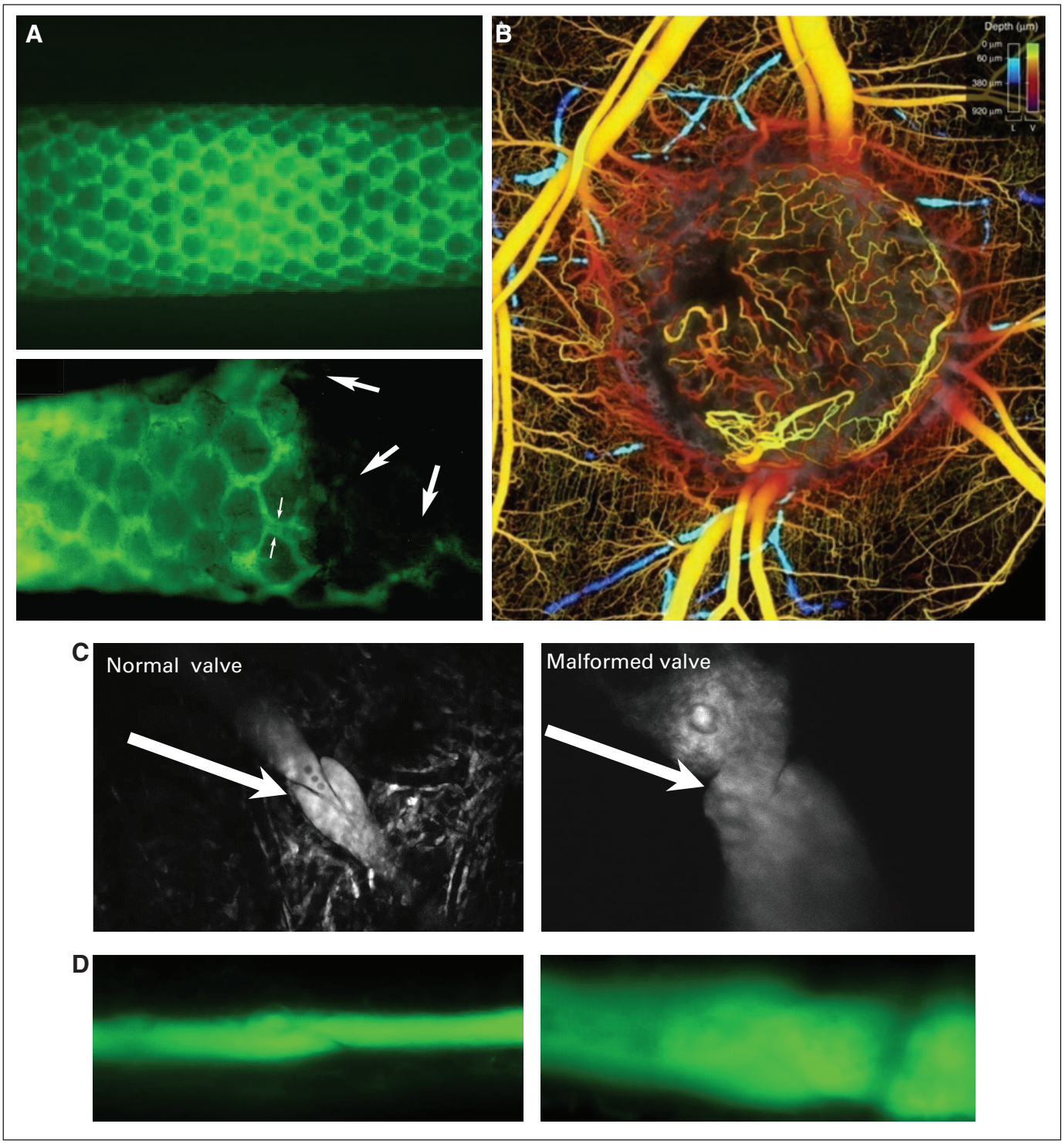

Fig 5. (A, B) Functional lymphatic vessels are not detected within tumors with two different techniques. (A) Top panel shows functional lymphatic vessels (green) in a mouse tail. Bottom panel shows the functional lymphatic vessels are absent within a sarcoma growing a mouse tail, but they have a larger diameter in the tumor margin (arrows). Reproduced from.[125] (B) Functional lymphatic vessels (blue) are present around a breast tumor grown orthotopically in a mouse, but they are absent within the tumor mass. Yellow-red indicates blood vessels. Reproduced from.[1] (C) Unlike normal valves (left), the valves in the lymphatic vessels draining a tumor are malformed (right). Reproduced from.[126] (D) Unlike normal contraction and valve (left), both are abnormal in a lymphatic vessel draining a tumor. Data adapted (unpublished data; Dr Shan Liao).[127]

with lung and renal cancers treated with ARBs or angiotensin-converting enzyme inhibitors.[150,151] Finally, a prospective phase I study recently showed that candesartan—another ARB—is safe in patients with pancreatic ductal adenocarcinoma; it is being followed by a phase II trial.[152] In parallel, in collaboration with Drs Lei Xu and Yves Boucher, we have shown that blocking transforming

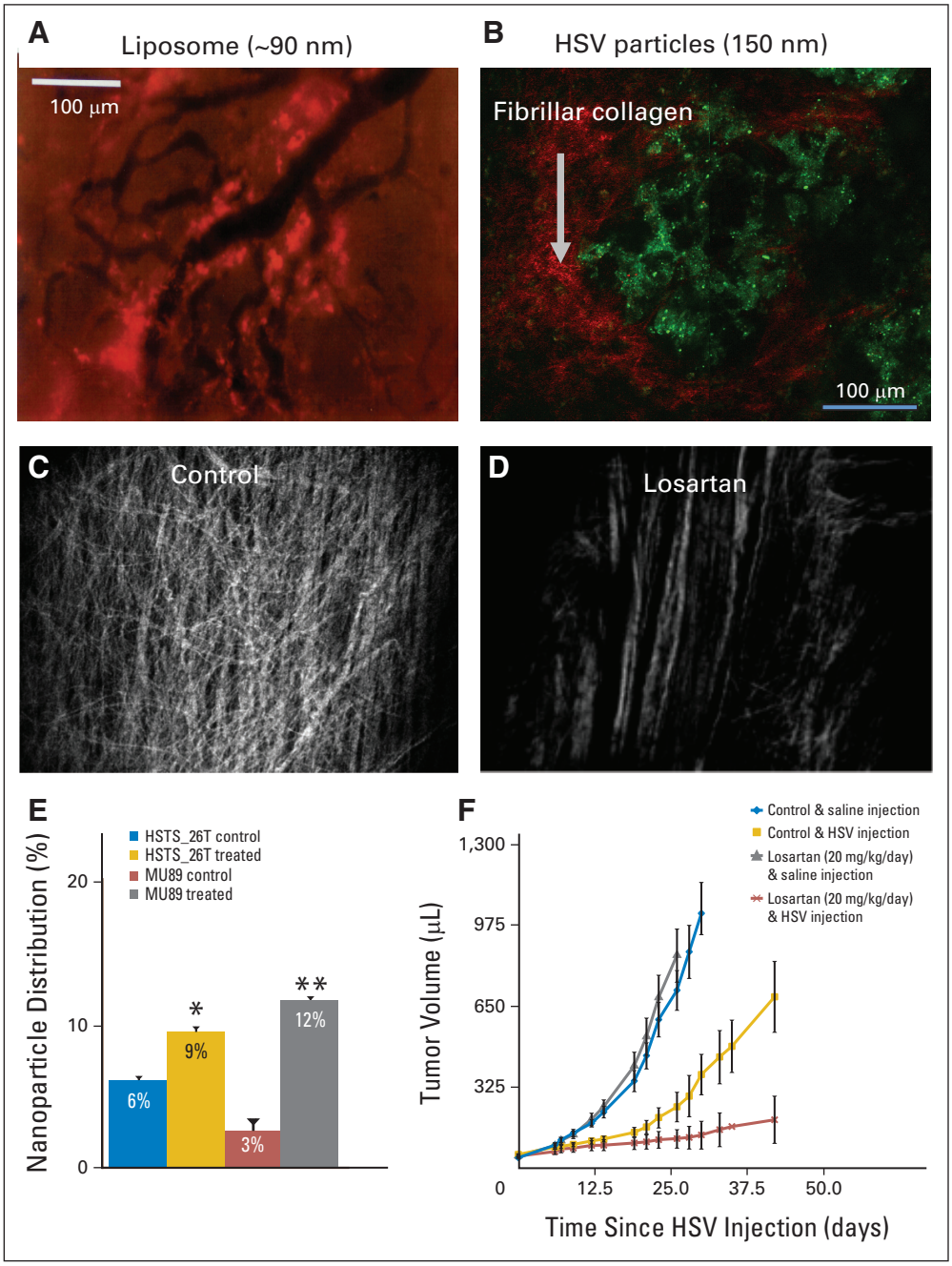

Fig 6. (A) Intravenously administered 90-nm liposomes—approximately the size of liposomal doxorubicin—were injected into tumor-bearing mice. These particles leaked out of some tumor vessels but did not move far from the vessel wall. Reproduced from.[20] (B) Similarly, 150-nm viral particles injected into a tumor do not move far from the injection site. Reproduced from.[142] (C, D) Losartan treatment for 2 weeks led to a dramatic decrease in collagen as well as (E) an increase in penetration and accumulation of 100-nm particles in collagen-rich tumors. (F) This, in turn, increased the efficacy of gene therapy and liposomal doxorubicin in desmoplastic tumors. Data adapted.[143] HSV, herpes simplex virus.

growth factor beta signaling can normalize both blood vessels and collagen matrix in breast cancer models in mice and improve the treatment outcome of liposomal doxorubicin (Data Supplement Figs S19 and S20).[153,154]

In addition to signaling and obstructing the movement of drugs, the extracellular matrix—in concert with cancer cells and fibroblasts—also contributes to the elevated mechanic forces in tumors.[36] These forces are large enough to compress the fragile blood and lymphatic vessels within tumors. These forces can also compress the surrounding normal tissues and contribute to morbidity and mortality. In collaboration with Dr Lance Munn, we have shown that these compressive forces can also directly increase cancer cell invasion.[155] Thus, the tumors seem to use mechanical forces to aid progression and treatment resistance. We must take this into account as we develop and test new stroma-depleting therapeutics in the clinic (Data Supplement Tables S1 and S2).

SUMMARY AND PERSPECTIVE

In summary, our work has demonstrated that blood and lymphatic vessels as well as the extracellular matrix of tumors are abnormal, and these abnormalities create a hostile microenvironment. Other stromal cells, such as activated fibroblasts, macrophages, and other immune cells, are also part of the abnormal tumor microenvironment.[135,156-160] This microenvironment fuels tumor progression, metastasis, and immunosuppression and induces a stem-cell phenotype—all of which

contribute to treatment resistance. Normalization of blood vessels and matrix can alleviate some of these problems, not only in mice but also in patients. Crucially, in two brain tumor trials, patients whose tumor blood perfusion increased survived longer than those whose tumor perfusion did not increase.

These exciting discoveries notwithstanding, great challenges remain ahead of us. Perhaps the biggest unmet need is improving antimetastasis therapies. Here, the disparity between preclinical and clinical research in antiangiogenesis remains profound. Most preclinical studies have been performed on primary tumors; only a handful have occurred in the metastatic or adjuvant setting that recapitulates the clinical situation. Even in these studies, the dose of antiangiogenic agent used is high compared with that in the clinical setting. Thus, it is not surprising that the resulting hypoxia may enrich for cancer stem cells or increase metastasis. In our own limited adjuvant studies with two different VEGFR tyrosine kinase inhibitors at lower doses, we have not detected an increase in metastasis—an observation consistent with clinical trials (Data Supplement Fig S14).[161,162] In addition, with improved systemic therapy, there is an alarming increase in incidence of brain metastasis, regarded as the last frontier in the war against cancer.[21,163] Our recent work using a VEGFR2-blocking antibody combined with trastuzumab and lapatinib has shown a dramatic effect in an experimental model of brain metastasis of human epidermal growth factor receptor 2–positive breast cancer (Data Supplement Fig S21).[164] This finding needs to be tested in the clinic.

Although our work has focused on vascular normalization, we are cognizant of other potential mechanisms of benefit from antiangiogenesis alone or when combined with chemotherapy.[165,166] These include killing both endothelial and cancer cells by antiangiogenics (eg, PlGF/Nrp1 antibodies in medulloblastoma or ramucirumab in gastric and liver cancers).[167-169] Antiangiogenic agents may also sensitize endothelial cells to cytotoxic drugs and impair the recruitment of bone marrow–derived cells that can differentiate to endothelial cells or release proangiogenic molecules. Finally, cytotoxic agents may kill stromal cells.[77,170] Although all these mechanisms have been previously examined in preclinical models, their roles in progression-free survival and OS need to be carefully investigated in patients.[166] Specific changes in biomarkers may inform these mechanisms.[88]

In terms of biomarkers, our work suggests that patients who have elevated pretreatment levels of plasma sVEGFR1 are not likely to benefit from anti-VEGF therapies, and increased levels of SDF1α seem to correlate with escape from anti-VEGF therapies. Other evasive pathways emerging from preclinical and clinical studies include Ang2 and cMET.[16,89,90,97,171-173] These biomarkers and pathways need to be tested prospectively.[88] With the discovery of biomarkers to identify appropriate patients, the survival advantage from antiangiogenic drugs is likely to be comparable to that from other targeted drugs (Data Supplement Tables S1 and S2).

AUTHOR'S DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) and/or an author's immediate family member(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.
Employment or Leadership Position: Rakesh K. Jain, XTuit Pharmaceuticals (C), H&Q Healthcare Investors and H&Q Life Sciences Investors (C) **Consultant or Advisory Role:** Rakesh K. Jain, Dyax (C), Enlight Biosciences (C), NOXXON Pharma (C), SynDevRx (C), Zyngenia (C) **Stock Ownership:** Rakesh K. Jain, Enlight Biosciences, SynDevRx, XTuit Pharmaceuticals **Honoraria:** Rakesh K. Jain, WebMD Global **Research Funding:** Rakesh K. Jain, Dyax, MedImmune, Roche
Expert Testimony: None
Other Remuneration: None

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GLOSSARY TERMS

Angiotensin II receptor blocker (ARB): Blocks the effect of angiotensin II, a chemical that narrows blood vessels, and thus helps widen blood vessels to allow blood to flow more easily, which lowers blood pressure. ARBs, at the present time, are generally prescribed when one cannot tolerate an angiotensin-converting enzyme (ACE) inhibitor to lower blood pressure.

Desmoplastic: Refers to growth of dense connective tissue or stroma by transformation of fibroblastic-type cells to a myofibroblastic phenotype that stain positive for smooth-muscle actin. Furthermore, an increase in total fibrillar collagens, fibronectins, proteoglycans, and tenascin C is distinctive of the desmoplastic stromal response in several forms of cancer.

Genetically engineered mouse: Mouse model in which the genetic makeup of the mouse has been modified by transgenic or gene-targeting technologies to affect expression of a gene of interest or to express a mutant.

Interstitial fluid pressure: The pressure exerted by the free interstitial fluid in a tissue, which is nearly zero mmHg in most normal tissues, but much higher in tumors because of leaky blood vessels. Interstitial fluid pressure cannot permanently collapse leak vessels.

Nanomedicine: Collectively refers to application of nanotechnology to medicine, including the use of nanomaterials for drug delivery to tumors and nanoelectronic biosensors. Delivery of effective amounts of drugs with current nanomedicine remains a challenge for this field. One nanometer is one millionth of a millimeter.

Solid stresses: The stresses exerted by the solid components of a tissue and accumulated within solid structural components (ie, cancer and stromal cells, collagen, and hyaluronan) during growth and progression. Solid stress is elevated in tumors because of growth and is independent of the high interstitial fluid pressure.

Stromal cells: Refers to the noncancer cells in tumors. The stroma is distinct from the parenchyma, which consists of the key functional elements of an organ.

sVEGFR1: Soluble form of VEGF receptor 1 (also known as sFlt-1), a truncated version of the cell membrane–spanning VEGFR1, can bind to circulating VEGF and PlGF.

Vascular normalization: The process whereby genetic or pharmacologic approaches result in pruning and/or remodeling of abnormal tumor vessels, which become closer to normal tissue vasculature in terms of structure and function.

Acknowledgment

I am enormously honored and pleased to receive the 2012 Science of Oncology Award. Every scientist's dream is that his or her findings will someday translate from bench to bedside. I have been very fortunate to see this happen in my career multiple times. Therefore, being recognized by the world's largest society of oncologists for contributions to basic and translational oncology is an enormous honor. I am profoundly grateful to the American Society of Clinical Oncology for this recognition.

In the words of the Nobel Laureate Paul Nurse, "Science is both intensely individual and completely collaborative." I have had the good fortune of collaborating with more than 200 graduate students and postdoctoral researchers from multiple disciplines who contributed to the research summarized in this article (Data Supplement Table S7). None of this would have been possible without the long-standing and very close collaborations with my faculty colleagues in the Edwin L. Steele Laboratory—Yves Boucher, PhD, Dan G. Duda, DMD, PhD, Dai Fukumura, MD, PhD, Peigen Huang, MD, Igor Garkavstev, MD, PhD, Lance L. Munn, PhD, Timothy P. Padera, PhD, and Lei Xu, MD, PhD (http://steele.mgh.harvard.edu)—and biostatistician Marek Ancukiewicz, PhD, and the outstanding technical support of the highly talented staff—notably Sylvie Roberge, Phyllis McNally, and Julia Kahn—over my entire career. I am most grateful to them as well as to more than 200 collaborators all over the world who have made my journey as a chemical engineer into the world of solid tumors both productive and compelling. The clinical insight I have presented here would not have been possible without my clinical collaborators—Christopher Willett, MD, Tracy Batchelor, MD, Gregory Sorensen, MD, and others—who led the multidisciplinary clinical studies summarized in this article or the patients with cancer who participated in these trials (Data Supplement Table S3). I have also had the enormous pleasure of collaborating with world leaders in the areas of imaging, angiogenesis, nanotechnology, molecular biology, genetics, pathology, and radiation, medical, neuro-, and surgical oncology—notably Moungi Bawendi, PhD, Brett Bouma, PhD, Peter Carmeliet, MD, PhD, Jeffrey Engelman, MD, PhD, Napoleone Ferrara, MD, Raju Kucherlapati, PhD, Brian Seed, PhD, and Andy Yun, PhD. I am grateful to my PhD mentors —James Wei, ScD, and the late Pietro M. Gullino, MD—for encouraging me to work at the interface of engineering and tumor pathophysiology; my colleagues—Shu Chien, MD, PhD, Harold Dvorak, MD, Marcos Intaglietta, PhD, and the late Judah Folkman, MD—for inspiring me to work on blood vessels; my former and current chairs—Herman D. Suit, MD, DPhil, and Jay S. Loeffler, MD—for their unyielding support; and Dan G. Duda, DMD, PhD, Keith Flaherty, MD, Dai Fukumura, MD, PhD, Stephen Isakoff, MD, PhD, and Lance L. Munn, PhD, for their help with this article. Finally, I am grateful to my family members—especially to my wife Jacqueline Samson, PhD, and my daughters Anjali and Natalie Jain—for their love, inspiration, patience, and support.

This article focused on the contributions of my laboratory, but obviously, there have been many significant contributions by others. I would like to apologize to those authors whose seminal work I could not cite because of the limit on the number of words and references.