OUTLINE

- Normal Vasculature
  - Review of angiogenesis, the process
  - Properties of normal vasculature

- Tumor Vasculature
  - Properties of abnormal vasculature in tumors
    - Unique molecular profile
    - Overall irregularity
    - Abnormal pericyte coverage
    - Increased interstitial pressure

- Anti-angiogenic therapies, initial goals and actual effects

- Vascular normalization, an alternative hypothesis
- Well organized
- Vessel walls are solid and relatively impermeable
- Complete pericyte coverage - mature vessels
- Blood vessels evenly distributed

NORMAL VASCULATURE

Liver

Lung

Muscle

Nerves

Barrowed from Dr. William Li's 2010 TED Talk
DISEASE

Excessive

- Cancer
- Blinding Diseases
- Arthritis
- Alzheimer’s Disease
- Obesity
- Multiple sclerosis

Insufficient

- Chronic wounds
- Coronary Heart Disease
- Peripheral Arterial Disease
- Stroke
- Erectile dysfunction
- Solid tumors account for 85% of cancer mortality
- Tumors cannot grow without a blood supply
  - Once tumors reach 0.5mm³, they cannot continue to grow without a blood supply
  - Most of these tumors will die......
- When cancer cells mutate, they begin to produce pro-angiogenic factors which tip the balance and recruit new blood vessels to augment their growth
TUMOR VASCULATURE

- Tumor vessels have a unique molecular profile
- Leaky, diameter is irregular and their walls are thin
- Irregular pericyte coverage
- Tumor have increased interstitial fluid pressure resulting in impaired flow

TUMOR ENDOTHELIAL MARKERS (TEMs)

- Growth Factor Receptors
  - High levels of VEGFR1 and VEGFR2
  - Irregular expression of EPH receptors and their cell-surface ligands, ephrins
- Integrins
  - αvβ5 and αvβ3 integrins are upregulated in endothelial cells undergoing angiogenesis, and their level of expression in tumor vasculature correlates with the grade of malignancy of neuroblastoma
  - α5β1 is selectively expressed in angiogenic vasculature
- Extracellular Matrix components (ECM)
  - Collagen
- Matrix Metalloproteinases (MMPs)
  - MMP2
**IRREGULARITY OF TUMOR VASCULATURE**

- Lacking a defined endothelial barrier
- Tortuous
- Thin walls
- Hyperpermeable

“Leaky vessels” result in large collections of extravascular erythrocytes—blood lakes
- Phenomenon sometimes described as ‘vasculogenic mimicry’

Irregularly shaped
Loosely associated with endothelial cells
IRREGULAR PERICYTE COVERAGE

- Cell bodies not located on the vessel wall
- Some pericyte processes contact endothelial cells (arrows)

**INCREASED INTERSTITIAL FLUID PRESSURE**

- Defined as pressure exerted by the free interstitial fluid; if the pressure is negative this tends to suck fluid out of the vascular system and into the tissue space; if the pressure is greater than the intravascular pressure fluid tends to move out of the tissue space
  - Because tumor vessels tend to be “leaky”, fluid can leak from the vessels into the interstitium resulting in high IFP.

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**Graph:**

- Coordinates: Depth (mm) vs. Interstitial Pressure (mmHg)
- Data points represent measurements.
- Theoretical curve fit with parameters:
  - \( P_e = 10.2 \text{ mmHg} \)
  - \( \alpha^2 = 1200 \)

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INCREASED INTERSTITIAL FLUID PRESSURE

<table>
<thead>
<tr>
<th>Types of Tissue</th>
<th>Number of Patients</th>
<th>Mean Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal breast</td>
<td>8</td>
<td>0.0</td>
</tr>
<tr>
<td>Normal skin</td>
<td>5</td>
<td>0.4</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>1</td>
<td>38.0</td>
</tr>
<tr>
<td>Cervical carcinoma</td>
<td>26</td>
<td>22.8</td>
</tr>
<tr>
<td>Colorectal liver metastases</td>
<td>8</td>
<td>21.0</td>
</tr>
<tr>
<td>Head and neck carcinoma</td>
<td>27</td>
<td>19.0</td>
</tr>
<tr>
<td>Breast carcinoma</td>
<td>8</td>
<td>15.0</td>
</tr>
<tr>
<td>Metastatic carcinoma</td>
<td>12</td>
<td>14.3</td>
</tr>
<tr>
<td>Lung carcinoma</td>
<td>26</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Renal cell carcinoma \(n=1\)
Ovarian carcinoma \(n=3\)
Cervical carcinoma \(n=26\)
Colorectal carcinoma liver metastases \(n=8\)
Metastatic melanoma \(n=14\)
Head and neck carcinoma \(n=27\)
Breast carcinoma \(n=81\)
Rectal carcinoma \(n=13\)
Lung carcinoma \(n=26\)
Sarcoma \(n=6\)
Brain tumors \(n=17\)
Lymphoma \(n=7\)
Normal skin \(n=5\)
Normal breast \(n=8\)

Barrowed from Genentech website

INCREASED INTERSTITIAL FLUID PRESSURE

Vascular hyperpermeability

Inability to maintain gradients between vascular and interstitial pressures

Contributes to interstitial hypertension

Impairs the flow of fluid and marcromolecules
1971 – Judah Folkman hypothesized that inhibition of angiogenesis would be an effective strategy to treat human cancer.


However, in initial clinical trials, a response rate of only 3.3% was observed among chemotherapy-pretreated colorectal cancer patients receiving monotherapy.
2004 – Genetech published direct evidence that the VEGF-specific antibody bevacizumab has antivascular effects in human rectal cancer.

Figure 1  Effect of treatment on tumors in patients who completed entire combined treatment regimen, and surgery. (a) Endoscopic and pathological evaluation of rectal tumors. Surgical specimens showed grade II tumor regression in patients 1–5 and grade III in patient 6, by Mandard criteria (see Supplementary Note). Endoscopic image (instead of surgical specimen) was taken for patient 6, 3.5 weeks before surgery. BV, bevacizumab. (b) Representative functional CT images of blood perfusion before treatment (day 0), after bevacizumab (day 12) and after completion of treatment (day 104) in patient 5. (c) Tumor FDG uptake before treatment (pretreatment), 12 d after bevacizumab treatment and 6–7 weeks after completion of all neoadjuvant therapy (presurgery). Sagittal projections of FDG-PET scans for patient 1 are shown. Tumor is outlined in box, posterior to bladder.
Certain anti-angiogenic agents can also transiently normalize the abnormal structure and function of tumor vasculature to make it more efficient for oxygen and drug delivery.
VASCULATURE NORMALIZATION

1. Increased homogeneity of functional vascular density and more orderly arrangement of vessels could reduce heterogeneity in blood flow in different regions with a tumor

2. Improved connections between adjacent endothelial cells, an increased proportion of PVC-covered vessels and a tighter association between PVCs and ECs would reduce vascular permeability, resulting in drop in intratumoral IFP
Two-photon microscopic images of tumor vessels reveal anti-VEGFR2 antibody prunes and reduces tumor vessel size but does not cause complete vessel regression.
VASCULAR NORMALIZATION – PERICYTE COVERAGE INCREASES

- Pericyte coverage increases after anti-angiogenic treatment

Winkler et al., Cancer Cell 6: 553-563 (2004)

DC101 decreased the interstitial fluid pressure in 54A (top panel) and U87 (bottom panel) tumors implanted in s.c. nude mice.
VASCULAR NORMALIZATION

Table 2  Studies reporting the impact of anti-angiogenic/vascular normalization strategies upon delivery of therapeutic compounds/systemically administered molecules into tumors

<table>
<thead>
<tr>
<th>Systemically Administered Molecule</th>
<th>Normalization Strategy</th>
<th>Tumor Model(s)</th>
<th>Effect on Delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional cytotoxics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Irinotecan</td>
<td>A4.6.1</td>
<td>Colon carcinoma</td>
<td>↑ [293]</td>
</tr>
<tr>
<td>Topotecan, etoposide</td>
<td>Bevacizumab</td>
<td>Neuroblastoma</td>
<td>↑ [71]</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>Sunitinib</td>
<td>Glioma</td>
<td>↑ [317, 318]*</td>
</tr>
<tr>
<td>Cyclophosphamide, cisplatin</td>
<td>TNP-470</td>
<td>Lung carcinoma</td>
<td>↑ [274]</td>
</tr>
<tr>
<td>Temozolomide</td>
<td>TNP-470</td>
<td>Glioma</td>
<td>↓ [192]</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Thalidomide</td>
<td>Liver carcinoma</td>
<td>↑ [255]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>PDGF-D overexpression</td>
<td>Breast carcinoma</td>
<td>↑ [190]</td>
</tr>
<tr>
<td>Topotecan</td>
<td>IFN-β overexpression</td>
<td>Neuroblastoma</td>
<td>↑ [70]</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>Anti-TGF-β antibody or overexpression sTβrII</td>
<td>Breast carcinoma</td>
<td>↑ [Liu et al., unpublished data]</td>
</tr>
</tbody>
</table>

Nanoparticles

| Liposomal doxorubicin              | Anti-TGF-β antibody or overexpression sTβrII | Breast carcinoma | ↑ [Liu et al., unpublished data] |

Antibodies

| Nonspecific IgG, anti-E-cadherin Ab | Axitinib           | Lung carcinoma, pancreatic tumor | ↑ (per vessel) [212] |

Viral particles

| Oncolytic virus                   | Cilengitide         | GBM                             | ↑ [176]             |

Other molecules

| BSA                               | DC101               | Breast carcinoma, colon carcinoma | ↑ [279]             |
| FDG                               | Bevacizumab         | Rectal carcinoma                 | ↑ (per vessel) [294, 297]* |
VASCULAR NORMALIZATION – IMPROVED DRUG PENETRATION

- Vascular normalization improves the penetration of molecules into tumors

Tong et al., Cancer Research 64, 3731-3736 (2004)

Liu et al., Clin Cancer Res 17: 3638-3648, (2011)
A combination of radiation and antiangiogenic therapies is only synergistic during a "normalization window" when tumor hypoxia is greatly diminished.

**A**

Day -9 -6 -3 0 3 6 9
Radiation RT1 RT2 RT3 RT4 RT5 + DC101

**B**

Tumor growth delay (days)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>DC101</th>
<th>RT1</th>
<th>RT2</th>
<th>RT3</th>
<th>RT4</th>
<th>RT5</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

*P < 0.05, compared with RT; **P < 0.05, compared with EAE.

**C**

Normalization Time Window

- Vascular density
- Diameter
- Pericyte Coverage
- Basement Membrane Thickness
- Radiation Response

Day 0 Day 8

Winkler et al., Cancer Cell 6: 553-563 (2004)

- A combination of radiation and antiangiogenic therapies is only synergistic during a "normalization window"
VASCULAR NORMALIZATION – TIMING IS CRITICAL

Of these studies, the majority have employed agents targeting the VEGF pathway.

1. Anti-VEGF agents

One strategy widely explored in preclinical studies utilizes specific blockers of VEGF, which prevent its binding to its receptors VEGFR1, VEGFR2, and neuropilin (NRP)-1 and -2. These include anti-human VEGF antibodies (e.g., A4.6.1, the murine anti-human VEGF IgG which is the precursor of bevacizumab), bevacizumab (a humanized anti-human VEGF IgG), anti-murine VEGF-A antibodies, and the “VEGF-Trap” aflibercept (a fusion protein binding and sequestering all isoforms of VEGF and placental growth factor, PlGF).

Our laboratory provided the first insights into the mechanisms of action of anti-VEGF therapy in tumors in 1996 (313). We implanted three different human tumors [colorectal cancer, glioblastoma multiforme (GBM), and melanoma] into severe combined immunodeficient (SCID) mice. Tumors were xenografted under surgically fashioned transparent windows, allowing serial microscopic and dynamic imaging of the tumor vessels' structure and function in real time (95). Specifically, GBM was implanted orthotopically under a transparent cranial window, and the other tumors were implanted subcutaneously and examined using a dorsal skinfold window system. Mice were subjected to A.4.6.1 therapy, neutralizing human VEGF released by tumor cells. After a single bolus of A4.6.1, a reduction in vascular diameter and tortuosity was observed, accompanied by dramatic drop in vascular permeability to albumin. Together, these findings provided early evidence that neutralization of tumor cell-derived VEGF could reverse some of the abnormalities that are hallmarks of the tumor microvasculature. We also examined the temporal kinetics of these changes. After a single dose of intravenous A4.6.1, vascular permeability dropped within 6 h but was increased to baseline again by 5 days, implying reversibility of the normalization phenotype with discontinuing therapy. In mice subjected to continuous therapy, features of vascular normalization were eventually lost and replaced by pronounced vascular regression, presumably caused by excessive neutralization of VEGF. These data provided the first evidence of the transient nature of the pharmacologically induced normalization phenotype and hence the existence of the normalization window.

Table 3

<table>
<thead>
<tr>
<th>Tumor Model</th>
<th>Mechanism of (Ab)normalization</th>
<th>Vessel Phenotype</th>
<th>Effect on Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pancreatic carcinoma</td>
<td>Anti-PlGF Ab</td>
<td>Pruning of immature vessels</td>
<td>Normalizing vessels reduces metastasis</td>
</tr>
<tr>
<td>Pancreatic carcinoma, lung carcinoma</td>
<td>PHD2 haploinsufficiency</td>
<td>Tighter EC junctions, permeability</td>
<td>Normalizing vessels reduces metastasis</td>
</tr>
<tr>
<td>Pancreatic/H922 -cell tumor</td>
<td>Murine pericyte deficiency (PDGFB ret/ret mice)</td>
<td>Pericyte deficiency, hyperpermeability</td>
<td>Normalizing vessels reduces metastasis</td>
</tr>
</tbody>
</table>

EC, endothelial cell; PDGFB, platelet-derived growth factor B; PHD, prolyl hydroxylase domain protein; PlGF, placental growth factor. Reference numbers are given in parentheses.

FIGURE 5

Preclinical and clinical studies suggest the presence of a vascular normalization “window” in response to pharmacological anti-angiogenic therapies. The vascular normalization hypothesis posits that a well-designed strategy should passively prune away immature, dysfunctional vessels and actively fortify those remaining, while incurring minimal damage to normal tissue vasculature, thus improving delivery of systemically administered cytotoxic compounds. Excessive or prolonged dosing of anti-angiogenic therapy can lead to heavy pruning of tumor vessels, but judicious dosing may restore the vasculature towards a more normal phenotype (during the normalization window, green). Vascular normalization will occur only in regions of the tumor where the imbalance of pro- and antiangiogenic signaling has been corrected. [From Jain (143), with permission.]

noma was chosen given the proven benefits of bevacizumab plus chemotherapy in this tumor type in the metastatic setting (135, 246) and the relative ease of access to tumor tissue via flexible sigmoidoscopy. The trial was designed to evaluate the effects of bevacizumab monotherapy on 1) vascular physiology: tumor perfusion, tumor blood volume, permeability-surface area (P-S) product and IFP; and 2) vascular structure: microvessel density and PVC coverage. To this end, patients underwent extensive investigation at the time of enrollment [sigmoidoscopic biopsy, IFP measurements, dynamic computed tomography (CT) scanning to determine tumor blood flow, and positron emission tomography (PET) scanning to determine tumor uptake of the radioactive tracer 18-Fluorodeoxyglucose (18-FDG)]. Patients then received a single dose of bevacizumab (5 mg/kg body wt for most patients) and 12 days later repeated the same investigations. After this initial cycle, standard concurrent chemoradiation was given in combination with ongoing fortnightly bevacizumab. The initial report from this study described features observed in the first 6 patients, but total accrual continued to a total of 32 patients (296, 297).

Although tumors did not shrink, vascular structure and function changed significantly as early as day 12 after bevacizumab monotherapy (294). Macroscopically, tumors reverted from hyperemic, hemorrhagic lesions to pale masses, consistent with reduced vessel density and permeability. Indeed, dynamic CT scanning revealed an almost 40% reduction in tumor blood flow and immunohistochemistry confirmed a drop to approximately half in microvascular density after bevacizumab. Moreover, a dramatic reduction in tumor IFP (50%) was observed after bevacizumab monotherapy, consistent with a reduction in vascular permeability and reversion to a more normal vascular phenotype. Histological data also showed an increase in the proportion of vessels covered by SMA-positive PVCs after bevacizumab, in keeping with increased vessel maturity and stability (294). Moreover, despite the reduction in tumor blood flow and permeability, tumor uptake of 18-FDG was not reduced by bevacizumab monotherapy, further supporting the notion that the remaining tumor vessels had an improved function after treatment. Taken together, the results provided the first convincing evidence of normalization in human tumors.

Additional mechanistic insight was gained from a study we conducted in collaboration with Batchelor, Sorensen, and colleagues in 31 patients with recurrent GBM, an aggressive primary brain tumor (14, 15, 263) (FIGURE 13). In that study, patients whose GBMs had progressed despite conventional treatment (whole brain radiotherapy and concurrent cytotoxic chemotherapy) were treated with cediranib, the same agent used in the previously described preclinical study of mice bearing orthotopic GBMs (160). This important study was designed to answer several outstanding questions pertaining to vascular normalization in GBM patients:

1) What is the timing of the onset and end of the vascular normalization window

2) How can normalization be reversed or prevented?

3) What is the long-term impact of normalization on patient survival?

Table 5  Clinical studies demonstrating vascular normalization in humans

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Anti-Angiogenic Therapy</th>
<th>Changes in Vessel Structure</th>
<th>Changes in Vessel Function</th>
<th>Clinical Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal carcinoma</td>
<td>Bevacizumab</td>
<td>↓ Density, ↑ PVC coverage</td>
<td>↓ tumor blood flow, ↓ IFP,</td>
<td>Tumors became pale (294), improved delivery of FDG per vessel</td>
</tr>
<tr>
<td>(n = 32)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glioblastoma</td>
<td>Cediranib</td>
<td>↓ Vessel size</td>
<td>↓ permeability</td>
<td>↓ Tumor-associated edema, reduced patient need for corticosteroids (14, 15)</td>
</tr>
<tr>
<td>(n = 31)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-grade glioma</td>
<td>Bevacizumab</td>
<td>↓ Vascular arcades and</td>
<td></td>
<td>(89)</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td>glomeruloid vessels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate carcinoma</td>
<td>Androgen ablation</td>
<td>Pruning of immature vessels,</td>
<td></td>
<td>(22)</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td>↑ PVC coverage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HER2+ breast cancer</td>
<td>Lapatinib</td>
<td>↓ Vessel tortuosity</td>
<td></td>
<td>(43)</td>
</tr>
<tr>
<td>brain metastases</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 22)</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

FDG, fluorodeoxyglucose; HER2, human epidermal growth factor receptor-2; IFP, interstitial fluid pressure. Reference numbers are given in parentheses.
Radiologic evidence of vascular normalization in human glioblastoma patient treated with anti-VEGF therapy

- Anti-angiogenic cocktail with radiation/chemotherapy
- Continue efforts to identify novel targets and multiple cells within the tumors
REFERENCES