Tumor Angiogenesis


Donald McDonald

University of California, San Francisco, CA

The formation of normal blood vessels is very complex. The appropriate factors are needed in the appropriate amounts and at the right time. That is one reason why blood vessels formed during pathological neovascularization are abnormal. These abnormalities provide targets for specific treatments. It is important to define the abnormalities of tumor blood vessels and determine the effects of angiogenesis inhibitors that exploit these abnormalities.

The elements of tumor blood vessels are endothelial cells, pericytes, and extracellular matrix in the form of basement membrane that envelopes endothelial cells and pericytes. Endothelial cells in tumors have abnormal gene expression. Abnormalities in intercellular junctions lead to vessel leakiness. The vessels are also sprouting and active in ways that normal endothelial cells are not. Importantly, a dependency on growth factors provides the rationale for treatment with growth factor antagonists. Pericytes are abnormal in tumor vessels; they have an unusual association with endothelial cells. The vascular basement membrane is also abnormal in tumor vessels, having multiple layers and exposed cryptic sites.

Normal blood vessels in all organs have a hierarchical organization, with arterioles branching down to capillaries, which in turn are connected to venules that become progressively larger and eventually connect to veins. This hierarchy is missing in tumor vessels. Blood vessels of different sizes are interconnected in a seemingly random fashion; none has characteristics of arterioles, capillaries, or venules. The endothelium of tumor vessels is so thin in some regions that erythrocytes are visible through the wall. Filopodia are abundant on the external surface of endothelial cells. Filopodia are normally most common at the tips of growing vessels, but in tumors they occur not only at the tip but also scattered over the vessel surface in a disorganized pattern. Tumor blood vessels have an abnormal pattern of protein expression. Injection of antibody to \( \alpha_5\beta_1 \) shows the expression of this integrin on tumor vessels, but not normal vessels. Expression of the integrin on the luminal surface as well as abluminal surface of tumor vessels indicates loss of endothelial cell polarity.

Pericytes on normal vessels form a cable overlying endothelial cells and are very intimately associated, particularly at junctions. This is not the case in tumor vessels, where pericytes have a loose association.

What about the effect of angiogenesis inhibitors? The effect on tumor vessels of a variety of VEGF antagonists was examined to determine the effects of this class of agent on the normal microvasculature of adult mice. The inhibitors examined included: (1) adenoviral vectors that express soluble VEGFR1 or VEGFR2 (Calvin Kuo, Stanford), (2) VEGF Trap (Regeneron), (3) DC101 antibody directed against VEGFR2 (ImClone), (4) a tyrosine kinase inhibitor that blocks VEGFR1, VEGFR2, and VEGFR3 and at a 10-fold higher concentration blocks PDGF receptors (Pfizer). Not only are many tumor vessels dependent on VEGF for survival, but the normal microvasculature also has some distinct properties with respect to VEGF.
dependency in adult mice. Each microvascular bed in the body has a different degree of VEGF-
dependency, ranging from none (independent) to fairly dependent. An example of the latter is
the thyroid gland in which about half the capillaries in adult mice are VEGF-dependent. This is
a dose- and time-dependent phenomenon. After treatment with a VEGF antagonist for about 3
weeks, about half the capillaries in the thyroid have regressed. It appears that capillaries that are
fenestrated are particularly sensitive to VEGF inhibitors in the adult.

About 20% of the capillaries in the trachea of adult mice are VEGF-dependent. In the
immediate postnatal period, 100% of tracheal capillaries are VEGF-dependent, but the
proportion gradually drops to 20% by 16 weeks of age. The tracheal microcirculation of mice
lends itself to detailed analysis of the effects of VEGF antagonists because the vasculature is
highly organized, distributed as a monolayer, and readily viewed in whole mount preparations.
Injection of a fluorescent lectin into living mice shows which vessels are patent, because blood
flow is required to carry the lectin to the vessels it labels. Under baseline conditions, all tracheal
blood vessels are painted with the lectin as shown by the tight correlation between lectin staining
and postmortem staining with CD31, which stains all endothelial cells. Two days after the onset
of treatment with a VEGF antagonist, there is mismatch between lectin and CD31 staining,
indicating that some vessels are not perfused. By 10 days, some capillaries are neither perfused
with the lectin nor stained with CD31. Apoptotic endothelial cells, identified by activated
caspase 3 immunoreactivity are present in regressing capillaries. The sequence seems to be,
first, loss of vascular lumen, followed by apoptosis of endothelial cells, and then disappearance
of endothelial cells, without loss of pericytes or the vascular basement membrane.

In pancreatic islet tumors in RIP-Tag2 transgenic mice, all vessels have both lectin and
CD31 staining under baseline conditions, although there is some variability because of
heterogeneous blood flow. Twenty-four hours after onset of treatment with a VEGF antagonist,
many tumor vessels are not perfused, but CD31 staining is little changed. By 2 days after
initiation of treatment, there is a significant reduction in both lectin staining and CD31 staining,
with greater reduction in perfusion. At 7 days, there is a match again, with about 70% reduction
in both, indicating that all the remaining vessels are perfused. These findings show that the
pruning occurs very quickly, first by vessel closure and then by apoptosis and removal of
endothelial cells. Once flow is lost, the stimulus for vessel maintenance is lost and the vessels
regress.

In the trachea, at baseline there is complete colocalization of endothelial cells and
basement membrane, but after 10 days of treatment, about 20% of basement membrane sleeves
have no endothelial cells. The same thing happens in tumors, but a much larger proportion of
basement membrane sleeves are left behind when vessels regress. Once treatment is stopped,
explosive vascular regrowth occurs. The regrowth appears to take advantage of existing
basement membrane sleeves, which act like railroad tracks. As long as basement membrane
sleeves persist and the VEGF drive for regrowth remains, the entire microcirculation of tumors
can be reconstructed within a few days.

PDGF is an essential growth and survival factor for pericytes. In tumor vessels, many
endothelial cells are sensitive to VEGF inhibition, but most pericytes are not. In RIP-Tag2
tumors treated with a VEGF antagonist for 7 days, the microcirculation is pruned. Staining with
α-smooth muscle actin (αSMA), which stains pericytes, shows that pericytes are not just located
on the surviving tumor vessels but are also associated with strands of basement membrane that
lack endothelial cells. Basement membrane and pericytes appear to be key elements for vascular
regrowth.
The effects of several PDGF antagonists have been examined: (1) an anti-PDGF aptamer (Archemix), (2) adenoviral vectors expressing soluble PDGF receptors (Calvin Kuo, Stanford), (3) Glivec, a kinase inhibitor that blocks PDGF receptors, abl and c-kit. Systemic delivery of the anti-PDGF aptamer to mice with implanted Lewis lung carcinomas resulted in rapid decrease in number of pericytes with the tumors. A 50% reduction in pericytes on tumor vessels after 24 hours progressed to about 80% reduction after 4 weeks. About 4 days after loss of pericytes begins, endothelial cells start to die. By 4 weeks, there was equal reduction of pericytes and endothelial cells. It appears that many endothelial cells are dependent upon pericytes in this particular tumor. One reason is that the pericytes are a source of VEGF. As the tumor cells themselves do not make a lot of VEGF, loss of pericytes eliminates a major source of VEGF.

Three other markers for pericytes, NG2, desmin, and PDGFRβ, have been examined in addition to αSMA. In Lewis lung carcinomas treated with anti-PDGF-B aptamer, all of the markers show about the same 80% reduction over 4 weeks of treatment. Disappearance of pericytes, not just a change in phenotype, was confirmed by electron microscopy. Because pericyte phenotype can change after treatment with VEGF antagonists, loss of a single marker, such as αSMA, may reflect downregulation of that molecule rather than loss of pericytes. In addition, pericytes that remain may undergo normalization as reflected by closer association with endothelial cells. A similar phenomenon is seen after treatment with VEGF antagonists, where many tumor vessels are eliminated and the remaining ones appear more normal, both by overall geometry and by close association with endothelial cells.

In summary, endothelial cells, pericytes, and vascular basement membrane are all abnormal in tumor vessels. Treatment with a VEGF inhibitor results in rapid pruning of susceptible blood vessels, leaving a population of VEGF-resistant blood vessels behind. Blood vessel pruning also leaves behind the pericytes and basement membrane of the pruned vessels. When the VEGF antagonist is stopped, tumor vessels rapidly regrow along the remaining sleeves of basement membrane and pericytes. A second round of VEGF inhibition can take out the regrown tumor vessels, indicating that the new vessels are VEGF-dependent. PDGF antagonists can reduce the population of pericytes and secondarily the population of endothelial cells in susceptible tumors. Still unanswered is the question of whether the entire vasculature of some tumors can be eliminated by a combination of VEGF and PDGF antagonists.

Questions

1. Is Lewis Lung carcinoma a special target for pericytes?
   It is unclear whether Lewis lung carcinoma is a special case, but it clearly differs from RIP-Tag2 tumors. Like beta cells in normal pancreatic islets, tumor cells in RIP-Tag2 tumors are VEGF factories. PDGF inhibitors by themselves have very little effect on the microvasculature of RIP-Tag2 tumors. Some pericytes are removed, but most endothelial cells survive. Apparently tumor cell production of VEGF in these tumors is sufficient to support the survival of the endothelial cells and hence the microvasculature. An important, unanswered question is, “Do tumor vessels become more vulnerable to VEGF inhibitors after their pericytes are lost?”

2. Does loss of pericytes eventually result in vessel occlusion?
   That is not yet known.
3. Have you looked at early time points to determine why VEGF antagonists result in occlusion of tumor vessels?

Vascular occlusion is not simply the consequence of endothelial cell swelling. Loss of vessel patency may result from loss of a signal that keeps the lumen open. It is important to identify the molecular basis of what keeps the lumen open and what changes to result in closure, but it is not known at this time.

4. Given the importance of integrin binding in regulating growth factor signaling, in terms of specificity and extent of the signal and the upregulation of $\alpha_5\beta_1$, do you see any functional role for $\alpha_5\beta_1$ in VEGF antagonist-induced vascular regression?

It is a reasonable possibility, but we don’t have a function blocking antibody for mouse $\alpha_5\beta_1$ to be able to answer that question.

5. Do you see upregulation of other integrins on the cell surface?

There is upregulation of $\alpha_v\beta_3$ on endothelial cells of many tumors, but it is much more spotty than $\alpha_5\beta_1$ in RIP-Tag2 tumors.

6. The same sequence of events occurs in regression of hyaloid vessels during development. The vessels narrow, become occluded, and then there is synchronous apoptosis and loss of endothelial cells. Then macrophages come in and remove the basement membrane. Do you know the status of macrophages in the tumors you have studied?

Macrophages are present in the tumors we have studied, but their association with blood vessels has not been systematically examined. This needs to be done. We have followed tumors treated with VEGF antagonists for as long as 4 weeks. Basement membrane strands are still present at that point, so indicative of slow clearance of the basement membrane sleeves. I suspect that the process of vessel regression in tumors is similar to that occurring elsewhere. It will be important to identify the mechanism of vessel closure after inhibition of VEGF signaling. Fibrin accumulates within the lumen of some regressing vessels before closure, but it is unknown whether this is a primary or secondary event. One possibility is that phospholipids (phosphatidylserine) in the luminal plasma membrane of regressing endothelial cells flip in polarity, making the luminal surface thrombogenic.

7. Do pericytes of normal vessels depend on PDGF for survival?

This does not seem to be the case under baseline conditions. Subtle changes may occur, but few pericytes are lost in the normal organs we have examined.