Leukemia (2007) 21, 1606–1609

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EDITORIAL

The history of angiogenesis inhibitors

Leukemia (2007) **21,** 1606–1609; doi:10.1038/sj.leu.2404756

In 1971, J Folkman published, in the *New England Journal of Medicine*, a hypothesis that tumor growth is angiogenesis-dependent. The hypothesis predicted that tumors would be unable to grow beyond a microscopic size of 1–2 mm^3 without continuous recruitment of new capillary blood vessels. Folkman[1] introduced the concept that tumors probably secreted diffusible molecules that could stimulate the growth of new blood vessels toward the tumor and that the resulting tumor neovascularization could conceivably be prevented or interrupted by drugs called angiogenesis inhibitors.

Beginning in the 1980s, the biopharmaceutical industry began exploiting the field of antiangiogenesis for creating new therapeutic compounds for modulating new blood vessel growth in angiogenesis-dependent diseases.

The antiproliferative activity of interferons (IFN) against human tumors was first demonstrated in the 1960s with partially purified IFN-α by Strander[2] at the Karolinska Institute. A mixture of IFN inhibited the migration of capillary endothelial cells *in vitro*[3] and lymphocyte-induced angiogenesis *in vivo,*[4] as well as tumor angiogenesis.[5] The first angiogenesis inhibitor IFN-α administered at low doses was reported in 1980.[3] Since 1988, IFN-α has been used successfully to cause complete and durable regression of life-threatening pulmonary hemangiomatosis, hemangiomas of the brain, airway and liver in infants, recurrent high-grade giant cell tumors refractory to conventional therapy and angioblastomas.[6–9] These tumors all express high levels of fibroblast growth factor-2 (FGF-2) as their major angiogenic mediator.

Cartilage has been studied as a potential source of angiogenesis inhibitor because of its avascularity. In fact, cartilage is a relatively tumor-resistant tissue and the tumor associated with cartilage, chondrosarcoma, is the last vascularized of all solid tumors. In 1980, Langer *et al.*[10] partially purified extracts of cartilage, which inhibited tumor-induced neovascularization when delivered regionally (via controlled release polymer) and when delivered systemically (via infusion). Ten years later, Langer and co-workers[11] purified an angiogenesis inhibitor from bovine scapular cartilage and obtained amino-terminal sequence.

In 1982, Taylor and Folkman characterized protamine, a sperm-derived cationic protein, able to inhibit neovascularization in the chick embryo chorioallantoic membrane (CAM) assay, and tumor growth and metastases when administered systemically, although its efficacy was limited to its toxicity at high doses.[12]

In 1982, Denekamp[13] hypothesized that the local disruption of the tumor vasculature would result in the death of many thousands of tumor cells, and that only a few endothelial cells within the vessels need to be killed to completely occlude the vessels. This strategy relies on the ability of vascular disrupting agents (VDA) to distinguish the endothelial cells of tumor capillaries from normal ones based on their phenotype, increased proliferative potential and permeability, and dependence on tubulin cytoskeleton. VDA work best in the poorly perfused hypoxic central tumor areas, leaving a viable rim of well-perfused cancer tissue at the periphery, which rapidly regrows.[14] Combination of VDA and chemo and/or radiation therapy, which targets cancer cells at the tumor periphery, has produced promising responses in preclinical models.

When heparin and cortisone were added together in the CAM assay to study angiogenesis activity in fractions being purified from tumor extracts, tumor angiogenesis was inhibited.[15] When this combination of heparin and steroid was suspended in a methylcellulose disk and implanted on the CAM, growing capillaries regressed leaving in their place, 48 h later, an avascular zone up to 4 mm in diameter. The anticoagulant function of heparin is not necessary for its angiogenic activity with steroids. Angiostatic steroids administered with heparin fragments that lack anticoagulant activity inhibit angiogenesis.

Langer and co-workers[15,16] produced a series of heparin fragment, which were tested for their angiostatic activity in the CAM assay. A hexasaccharide fragment with a molecular weight of approximately 1600 was found to be the most potent inhibitor of angiogenesis in the presence of a corticosteroid. Tetrahydrocortisol, a natural metabolite of cortisone, is one of the most potent naturally occurring angiostatic steroids. Synthetic angiostatic steroids exhibit greater antiangiogenic activity than most of the natural steroids. The mechanism of action of angiostatic steroids is not understood completely. However, in the presence of steroid–heparin combinations, the basement membranes of growing capillaries undergo rapid dissolution.[17]

Fumagillin was found by Ingber *et al.*[18] in the Folkman lab to inhibit endothelial cell proliferation without causing endothelial cell apoptosis, when a tissue culture plate of endothelial cells became contaminated with a fungus *Aspergillus fumigatus fresenius*. Scientists at Takeda Chemical Industries (Osaka, Japan) made a synthetic analogue of fumagillin called TNP-470, which inhibits endothelial proliferation *in vitro* at a concentration 3 logs lower than the concentration necessary to inhibit fibroblasts and tumor cells. TNP-470 showed significant inhibition of tumors in clinical trials, including durable complete regression.[19] The clinical utility of TNP-470, however, was limited by neurotoxicity. This side effect was overcome when Satchi-Fainaro *et al.*[20] in the Folkman lab conjugated TNP-470 to N-(2-hydroxypropyl)methacrylamide to form caplostatin. Caplostatin can be administered over a dose range more than 10-fold that of the original TNP-470 without any toxicity. In addition to its antiangiogenic activity, caplostatin is the most potent known inhibitor of vascular permeability.[20]

In 1990, Maione *et al.*[21] demonstrated that recombinant human platelet factor-4 (PF 4) inhibited endothelial cell migration and proliferation *in vitro* and angiogenesis *in vivo*. Other studies have demonstrated that PF 4 inhibited tumor growth in mice.[22,23]

Thrombospondin-1 (TSP-1) was the first protein to be recognized as a naturally occurring inhibitor of angiogenesis.[24] TSP-1, a heparin-binding protein that is stored in the extracellular matrix, was able to inhibit proliferation of endothelial cells from different tissues[25] and appeared to destabilize contacts between endothelial cells.[26] Tumors grew significantly faster in TSP-1 null mice than in wild-type mice.[27]

O'Reilly *et al*.[28] joined Folkman lab as a postdoctoral fellow in July 1991 and began to test the hypothesis that a primary tumor might suppress growth of its distant metastases by releasing an angiogenesis inhibitor into the circulation by screening a variety of transplantable murine tumors for their ability to suppress metastases. A subclone of Lewis lung carcinoma was isolated, which could not suppress metastasis. When the metastasis-suppressing primary tumor was present in the dorsal subcutaneous position, microscopic lung metastases remained dormant at a diameter of less than 200 μm surrounding a pre-existing microvessel, but revealed no new vessels. Within 5 days after surgical removal of the primary tumor, lung metastases became highly angiogenic and grew rapidly, killing their host by 15 days. This striking evidence demonstrated that the primary tumor could suppress angiogenesis in its secondary metastases by a circulating inhibitor.

O'Reilly *et al*.[28] succeeded in purifying this inhibitor from the serum and urine of tumor-bearing animals and identified a 38-kDa internal fragment identical in amino-acid sequence to the first four kringle structures of plasminogen, and it was named angiostatin. Angiostatin first revealed that an antiangiogenic peptide could be enzymatically released from a parent protein that lacked this inhibitory activity. Angiostatin inhibited growth of primary tumors by up to 98%[29] and was able to induce regression of large tumors (1–2% of body weight) and maintain them at a microscopic dormant size.

In 1950s, thalidomide was developed as a sedative that showed non-toxicity in preclinical animal models. In 1962, the association between limb defects in babies born to mothers who used thalidomide during pregnancy was established.[30] D'Amato *et al*.[31] in the Folkman lab suggested that thalidomide's mechanism of teratogenicity was related to inhibition of angiogenesis in the developing fetal limb bud. Thalidomide has been shown to have pleiotropic effects including antiangiogenic (downregulation of tumor necrosis factor-α, FGF-2 and vascular endothelial growth factor (VEGF)) immunomodulatory, neurologic and anti-inflammatory effects.[32] Thalidomide was approved in Australia for the treatment of advanced multiple myeloma in 2003 and now is used as a first-line therapy. Many patients have been kept on the drug for 3–5 years without evidence of drug resistance.[32]

Endostatin, a 20 kDa protein with an N-terminal amino-acid sequence identical to the C terminus of collagen XVIII, provided the first evidence that a basement membrane collagen contained an angiogenesis inhibitory peptide.[33] O'Reilly in the Folkman lab found endostatin in the blood and urine of mice bearing tumors, which suppressed angiogenesis in remote metastases. In tumor-bearing animals, continuous dosing of endostatin by an intraperitoneal mini-osmotic pump inhibited tumor growth 10-fold more effectively than the same dose administered once per day as a bolus dose.[34]

When endostatin is overexpressed in the vascular endothelium of mice, tumors grow 300% more slowly in mice expressing only 1.6-fold more endostatin than wild-type mice.[35] Recombinant endostatin was at first produced in *Escherichia coli*. Preparations of inclusion bodies that were endostatin-free and of low solubility were capable of regressing a variety of established murine tumors when administered subcutaneously.[36] When soluble recombinant endostatin was produced in yeast, active endostatin was produced by numerous laboratories and a wide range of inhibited tumors was reported.[37]

More than 750 reports on endostatin reveal significant inhibition of more than 20 different rat and human tumors (in mice) by administration of the recombinant endostatin protein. Endostatin counteracts virtually all the angiogenic genes upregulated by either VEGF or FGF-2, and also downregulates endothelial cell Jun B, HIF-1α, neuropilin and the epidermal growth factor receptor (EGFR).[38]

Browder *et al*.[39] in the Folkman lab was the first to demonstrate this novel concept: by optimizing the dosing schedule of conventional cytotoxic chemotherapy to achieve more sustained apoptosis of endothelial cells in the vascular bed of a tumor, it is possible to achieve more effective control of tumor growth in mice, even if the tumor cells are drug resistant. Browder *et al*.[39] reported that conventional chemotherapy such as cyclophosphamide administered by the traditional schedule of maximum-tolerated doses interspersed with off-therapy intervals of 3 weeks to permit recovery of bone marrow led to a drug resistance in all tumors when therapy was started in Lewis lung carcinomas at tumor volumes of 100–650 mm^3.

Conventional chemotherapy is administered at maximum-tolerated doses followed by off-therapy intervals of 2–3 weeks to allow the bone marrow and gastrointestinal tract to recover. In contrast, antiangiogenic chemotherapy is administered more frequently at lower doses, without long interruptions in therapy, and with little or no toxicity. In contrast, when cyclophosphamide was administered at more frequent intervals and at lower doses, it acted as an angiogenesis inhibitor. Proliferating endothelial cells in the tumor vascular bed underwent a wave of apoptosis about 4 days before the tumor cell apoptosis began. All tumors completely regressed and animals remained tumor-free for their normal lifespan (up to 657 days).

In an editorial, Hanahan *et al*.[40] coined the term 'metronomic chemotherapy' to indicate the new schedule itself. During antiangiogenic chemotherapy, endothelial cell apoptosis and capillary dropout precede the death of tumor cells that surround each capillary.[39] Cyclophosphamide, 5-fluorouracil, 6-mercaptopurine ribose phosphate and doxil (the pegylated liposomal formulation of doxorubicin) inhibit angiogenesis when administered on an antiangiogenic dose schedule.

Kerbel and co-workers[41] showed that continuous administration of cyclophosphamide in the drinking water inhibited tumor growth in mice by 95% and significantly increased circulating levels of TSP-1. The low-dose 'metronomic chemotherapy' was ineffective in TSP-1 null mice, indicating that the low-dose oral chemotherapy was in part dependent on its capacity to induce an increase in circulating TSP-1.

Pediatric oncologists use a metronomic-like modality of chemotherapies called 'maintenance chemotherapy' to treat various pediatric malignancies such as acute lymphoblastic leukemia, neuroblastoma or Wilm's tumor.[42]

A combination of cytotoxic drugs (taxanes, cisplatin or 5-fluorouracil) with angiogenesis inhibitors (TNP-470, endostatin, SU11248) produced at least additive, but in certain cases synergistic, antitumoral effects.[43]

Combinatorial therapies with antiangiogenic agents are not limited to those including cytotoxic chemotherapy. Several preclinical and clinical trials are exploring the combination of various angiogenesis inhibitors with other targeted therapies, such as EGFR or Ger2 inhibitors (cetuximab, erlotinib, trastuzumab), PDGFR/bcr-abl inhibitors (imatinib), proteasome inhibitors (bortezomib) and other antiangiogenic agents, such as inhibitors of integrins.

Over the past 15 years, considerable research has been conducted in this area, and several molecules have made it through the preclinical challenges to enter clinical development. A variety of small-molecule receptor tyrosine kinase (RTK) inhibitors targeting the VEGF receptors have been developed. The most advanced are SU11248/sutent and BAY43-9006/

sorafenib. SU11248 inhibits VEGFR-1, VEGFR-2, PDGFR, c-kit and Flt-3.[44] BAY43-9006 was initially identified as a raf kinase inhibitor and subsequently shown to inhibit several RTK including VEGFRs. Phase III trials have demonstrated the efficacy of SU11248 and BAY43-9006 in the treatment of patients with renal cancer.[45] An additional VEGF RTK inhibitor in late-stage clinical trials is PKT 787.[46] Other anti-VEGF agents including VEGF-Trap (Regeneron, Tarrytown, NY, USA), a soluble receptor targeting VEGF-A, VEGF-B and placental growth factor, an antisense oligonucleotide VEGF-AS (Vasgene Therapeutics Inc., Los Angeles, CA, USA) targeting VEGF-A, VEGF-C and VEGF-D, are at various stages of clinical development.[45]

Inhibitors of VEGF signaling not only stop angiogenesis but also cause regression of some tumor vessels,[47] causing robust and rapid changes in all components of the vessel wall of tumor, consisting in loss of endothelial fenestrations, regression of tumor vessels and appearance of basement membrane ghosts.[48]

Moreover, targeting VEGF/VEGFR system transforms some tumor capillaries into a more normal phenotype.[45] Finally, VEGF inhibition might have direct cytotoxic effects on tumor cells that aberrantly express VEGF receptors and depend to some extent on VEGF for their survival.

Avastin (bevacizumab) is a humanized anti-VEGF monoclonal antibody. As approximately 60% of human tumors express VEGF-A, Avastin can be very effective against tumors that produce VEGF. Avastin was the first angiogenesis inhibitor approved by the Food and Drug Administration (FDA) for the treatment of colorectal cancer in February 2004,[49] administered in combination with bolus IFL (irinotecan, 5-fluorouracil and leucovorin). This followed for a phase III study showing a survival benefit.[49] Median survival was increased from 15.6 months in the bolus-IFL plus placebo arm of the trial to 20.3 months in the bolus-IFL plus bevacizumab arm. Similar increases were seen in progression-free survival, response rate and duration of response.

Bevacizumab was associated with gastrointestinal perforations and wound-healing complications in about 2% of patients. In addition, the incidence of arterial thromboembolic complications was increased about twofold relative to chemotherapy alone, with patients 65 years or older with a history of arterial thromboembolic events being at higher risk.

In December 2004, the FDA approved pegaptanib sodium (macugen), an aptomer that blocks the 165-amino-acid isoform of VEGF-A, for the treatment of the wet (neovascular) form of age-related macular degeneration, the most common of severe, irreversible vision loss in the elderly.[50] Macugen is injected into the eye once a month under local anesthesia in the ophthalmologist's office. In phase III clinical trials, it arrested progress of the disease in 90–95% of patients. In patients who were legally blind, sight was restored in 40%. Data from clinical trials have shown improved outcomes with the use of bevacizumab as a single agent in metastatic renal cell carcinoma (benefit in progression-free survival but not in overall survival).[51] In subsequent phase III trials, bevacizumab in combination with standard chemotherapy improved overall survival in lung cancer patients and progression-free survival in breast cancer patients.[45] Administration of bevacizumab in combination with paclitaxel and carboplatin to patients with non-small cell lung cancer resulted in increased response rate and time to progression relative to chemotherapy alone in a randomized phase II trial.[52]

In 2002, it was estimated that over 10 000 patients with cancer worldwide have received experimental form of antiangiogenic therapy.[53] However, the results from these clinical trials have not shown the dramatic antitumor effects that were expected following preclinical studies. This may be because of inadequate trial design in earlier studies.

The main problem in the development of antiangiogenic agents is that multiple angiogenic molecules may be produced by tumors, and tumors at different stages of development may depend on different factors for their blood supply. Therefore, blocking a single angiogenic molecule was expected to have little or no impact on tumor growth. However, in apparent contrast with this view, experiments with neutralizing antibodies and other inhibitors demonstrated that the blockade of VEGF alone can substantially suppress tumor growth and angiogenesis in several models.

An ideal angiogenesis inhibitor should be orally bioavailable with acceptable short-term and long-term toxicity and have a clinically useful antitumor effect. Moreover, carefully constructed clinical trials with valid end points need to be executed. Finally, cancer genomics and proteomics are likely to identify novel tumor-specific endothelial targets and accelerate drug discovery.

Acknowledgements

This study was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC, regional funds), Milan, Italy; the Ministry for Education, the Universities and Research (FIRB 2001, PRIN 2005 and Project CARSO no. 72/2), Rome, Italy; and Fondazione Italiana per la Lotta al Neuroblastoma, Genoa, Italy.

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