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Tumor angiogenesis: past, present and the near future

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The concept of treating solid tumors by inhibiting tumor angiogenesis was first articulated almost 30 years ago. For the next 10 years it attracted little scientific interest. This situation changed, relatively slowly, over the succeeding decade with the discovery of the first pro-angiogenic molecules such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF), and the development of methods of successfully growing vascular endothelial cells in culture as well as *in vivo* assays of angiogenesis. However, the 1990s have witnessed a striking change in both attitude and interest in tumor angiogenesis and anti-angiogenic drug development, to the point where a remarkably diverse group of over 24 such drugs is currently undergoing evaluation in phase I, II or III clinical trials. In this review I will discuss the many reasons for this. These features, together with other recent discoveries have created intense interest in initiating and expanding anti-angiogenic drug discovery programs in both academia and industry, and the testing of such newly developed drugs, either alone, or in various combinations with conventional cytotoxic therapeutics. However, significant problems remain in the clinical application of angiogenesis inhibitors such as the need for surrogate markers to monitor the effects of such drugs when they do not cause tumor regressions, and the design of clinical trials. Also of concern is that the expected need to use anti-angiogenic drugs chronically will lead to delayed toxic side effects in humans, which do not appear in rodents, especially in short-term studies.

Introduction

Roughly a decade before the inaugural issue of *Carcinogenesis* was published, Dr Judah Folkman articulated several hypotheses regarding what he felt was the critical importance of tumor angiogenesis in the development and metastatic spread of tumors, and how therapeutic inhibition of such angiogenesis might be exploited as a new and novel means of treating cancer (1,2). His ideas were based not only on his own work, but also on some studies of a small number of insightful investigators such as Algire and Chalkley (3), Greenblatt and Shubik (4) and Warren (5). Indeed, it was Shubik who first coined the term 'tumor angiogenesis' (4). The fundamental points of Folkman's visionary hypotheses can be summarized as follows: (i) most primary solid tumors probably go through a prolonged state of avascular, and apparently dormant, growth in which the maximum size attainable is ~1–2 mm in diameter. Up to this size, tumor cells can obtain the necessary oxygen and nutrient supplies they require for growth and survival by simple passive diffusion; (ii) these microscopic tumor masses can, in some way, eventually switch on angiogenesis by recruiting surrounding mature host blood vessels to begin sprouting new blood vessel capillaries which grow toward, and eventually infiltrate the tumor mass, thus setting in motion the potential for relentless expansion of the tumor mass and hematogenous metastatic spread as well; (iii) the angiogenic switch was initially hypothesized to be triggered by the ectopic production and elaboration by tumor cells of a growth factor (6) called 'tumor angiogenesis factor' (TAF) (7); (iv) it ought to be possible to affect tumor growth by blocking tumor angiogenesis, e.g. by somehow preventing TAF production (or its biologic function) or directly targeting the vascular endothelial cells of newly formed, immature blood vessels. The latter possibility presupposed that such tumor vessels and their constituent endothelial cells would be sufficiently different, phenotypically speaking, from the endothelial cells of more mature vessels such that a sufficient therapeutic index could be achieved; (v) this kind of a therapeutic approach, if successful, would not be curative in the usual sense, i.e. it would not eradicate all tumor cells, but instead would either prevent any new expansion of tumor mass or, at best, perhaps cause sustained regressions of established solid tumors to a size of ~1–2 mm diameter where survival is possible without a blood vessel supply. Such a therapeutic approach was designated as 'dormancy-inducing' and meant to control the disease in a chronic fashion.

These theories are widely accepted today; indeed, as *Carcinogenesis* approaches its twentieth anniversary, tumor angiogenesis is clearly one of the most exciting and visible areas of cancer research and therapeutics in clinical oncology (8). Such was not the case in 1980. Back then there was, relatively, only a handful of investigators working on tumor angiogenesis, and virtually no companies interested in developing anti-angiogenic drugs. What has changed this situation so dramatically such that there are now over 20 anti-angiogenic drugs being tested in various phase clinical trials, as shown in Table I (9–12), and numerous others in preclinical development?

Past and present

What follows are 10 of the most significant reasons (with no particular significance attached to their order) for the explosive growth in tumor angiogenesis research development of anti-angiogenic drugs for cancer treatment.

1. The discovery of basic fibroblast growth factor (bFGF) as the first pro-angiogenic molecule

Despite the logic of Folkman's ideas (1,2,7), sceptics awaited the precise identification of the hypothetical TAF molecule. This did not occur until the mid 1980s, when Shing *et al.* (13)

Abbreviations: α-SMA, α-smooth muscle action; bFGF, basic fibroblast growth factor; TAF, tumor angiogenesis factor; VEGF, vascular endothelial growth factor.

Table I. Some endogenous inhibitors of angiogenesis

Name	Description
Thrombospondin-1 and internal fragments of thrombospondin-1	Thrombospondin is a 180 kDa, large, modular extracellular matrix protein (53)
Angiostatin	A 38 kDa fragment of plasminogen involving either kringle domains 1–3, or smaller kringle 5 fragments (58,163,164)
Endostatin	A 20 kDa zinc-binding fragment of type XVIII collagen (59)
Vasostatin	An N-terminal fragment (amino acids 1–80) of calreticulin (61)
Vascular endothelial growth factor inhibitor (VEGI)	A 174 amino acid protein with 20–30% homology to tumor necrosis factor superfamily (60)
Fragment of platelet factor 4 (PP4)	An N-terminal fragment of PP4 (63)
Derivative of prolactin	16 kDa fragment of the hormone (57)
Restin	NC10 domain of human collagen XV (165)
Proliferin-related protein (PRP)	A protein related to the pro-angiogenic proliferin molecule (166)
SPARC cleavage product	Fragments of secreted protein, acid and rich in cysteine (62)
Osteopontin cleavage product	Thrombin-generated fragment containing an RGD sequence (65)
Interferon α/β	Well known anti-viral proteins (56)
Meth 1 and Meth 1	Proteins containing *m*etalloprotease and *t*hrombospondin domains, and disintegrin domains in NH_2 termini (65)
Angiopoietin-2	Antagonist of angiopoietin-1 which binds to tie-2 receptors (39,44)
Anti-thrombin III fragment	A fragment missing C-terminal loop of anti-thrombin III (a member of the serpin family) (64)

reported that basic fibroblast growth factor (bFGF, also known as FGF-2) isolated from a chondrosarcoma could function as a tumor-derived capillary growth factor and stimulate angiogenesis in various models (14). However, a puzzling feature about bFGF is that it lacks a signal sequence for secretion and generally remains intracellular, at least in cell culture. This cast some doubt on its authenticity as a major inducer of tumor angiogenesis (15). Nevertheless, the discovery provoked new interest in the field, and there is no doubt that bFGF is recognized as a potent inducer of angiogenesis, including in the clinic for non-cancerous diseases such as hind limb ischemia and ischemic heart disease (16–18).

2. The discovery of vascular endothelial growth factor (VEGF), and VEGF receptor tyrosine kinases on activated endothelial cells

VEGF was initially termed vascular permeability factor (VPF), and still retains this term (15,19). Its first function (vascular permeability) was discovered by Dvorak and colleagues (20,21) and was first molecularly defined by Ferrara *et al*. (22,23). There are a number of families (VEGF, VEGF-B, VEGF-C, VEGF-D) of which VEGF (or VEGF-A as it is sometimes called) is the most important; there are several VEGF isoforms and a number (e.g. $VEGF_{121}$ and $VEGF_{165}$) are readily secreted. Moreover, unlike bFGF (as the name implies), VEGF is a very specific mitogen for vascular endothelial cells. It also functions as a potent pro-survival (anti-apoptotic) factor for endothelial cells in newly formed vessels (24–26) and indeed this may be one of its most significant functions. VEGF is expressed by the vast majority of cancers (20,21), often at elevated levels, and blocking its activity, e.g. by specific neutralizing antibodies to VEGF or to VEGF receptors expressed by 'activated' endothelial cells, can inhibit experimental tumor growth *in vivo*, but not *in vitro* (27,28). This is because of the highly elevated and restricted expression of two receptor tyrosine kinases, called flk-1/KDR (also known as VEGF-receptor-2) and flt-1 (also known as VEGF receptor-1), by the endothelial cells of newly formed blood vessels, which bind VEGF with high affinity (29). Thus tumor cells can 'feed' (induce) new blood vessels by producing VEGF which, in turn, can nourish the tumor cells, an insidious and self-perpetuating paracrine loop. The possibility of therapeutic disruption of this loop has stimulated intense interest in the biotech and pharmaceutical industries, as well as academic centres, using agents such as antibodies (28,30–32), VEGF-toxin conjugates (33), aptamers (30) and small molecule VEGF receptor antagonists (34,35), among others.

Another significant feature of VEGF is that levels of this growth factor in tumor cells can be significantly enhanced by hypoxia (36). This can occur through both transcriptional and post-transcriptional mechanisms, e.g. mRNA stabilization (37). Given the impact of hypoxia as a mediator of tumor progression (38), and modulator of therapeutic responsiveness to agents such as radiation, the hypoxia/VEGF connection has heightened awareness of angiogenesis among certain segments of the cancer research and medical/radiation oncology communities.

3. The discovery of the angiopoietins and their tyrosine kinase receptors

A second family of ligands and receptors specific for vascular endothelial cells have been uncovered more recently (39). In this case, it was the receptors that were discovered first, i.e. tie-2/tek and tie-1 (40,41). They remained as orphan receptors until 1996, when the ligands for one of them (tie-2), called angiopoietin-1 and angiopoietin-2 (ang-1 and ang-2), were discovered by Yancopolous and colleagues (42,43). Of considerable interest is the fact that although both ang-1 and ang-2 bind tie-2, ang-1 functions as an agonist, whereas ang-2 behaves in a contrary manner (44). Indeed, ang-2 can cause regression of newly formed vessels by endothelial cell apoptosis, unless VEGF is present, in which case the two collaborate to promote angiogenesis (45). The ligand for tie-1 remains unknown. Similar to VEGF and its receptors, the use of transgenic and gene knockout mice has been a powerful methodology to uncover the functional significance of the angiopoietin–angiopoietin receptor systems in both embryonic vasculogenesis and angiogenesis (46). By way of example, embryonic mice which are only partially VEGF deficient, i.e. express one VEGF allele, do not survive *in utero* and express major defects in vasculogenesis and angiogenesis (46). Mice made deficient in flk-1, flt-1 and tie-2 also do not survive *in utero* (46). In the case of flk-1-deficient mice, endothelial cells are not produced, whereas tie-2-deficient embryos produce endothelial cells and form primitive vascular structures but

these do not assemble into mature, stabilized vessels as a result of a failure to recruit periendothelial support cells such as pericytes and smooth muscle cells (46). These developmental biology and other gene knockout studies have been particularly important contributions to the angiogenesis field in general, and as such have had an accelerating effect on tumor angiogenesis research as well. In particular, they have helped fuel interest in developing drugs which target VEGF, VEGF receptors, or the tie-2 receptors (35,47).

4. The discovery of endogenous inhibitors of angiogenesis

Like the field of cancer genetics (48), where virtually all the emphasis in the early years was placed on dominantly acting oncogenes which act as positive acting stimulators of cell growth, research in angiogenesis over the first 15–20 years was also heavily focused on pro-angiogenic growth factor stimulators (14). Later, in the cancer genetics field, the concept of (recessive) tumor suppressor genes came to be accepted, i.e. genes which encode proteins that normally function to block cell growth (or survival), and inactivation of which (e.g. by mutation or chromosome loss) can result in a loss of this cellular 'brake' mechanism (48). It is now accepted that it is the combination of these two types of event which promote cancer development and progression (48). The same appears to be the case for (tumor) angiogenesis (49–51). Thus, a large, growing and structurally diverse family of endogenous protein inhibitors of angiogenesis has been discovered, e.g. thrombospondin-1 (52,53), interferon α/β (54–56), the 16 kDa fragment of prolactin (57), angiostatin (58), endostatin (59), vascular endothelial cell growth inhibitor (VEGI) (60), vasostatin (61), Meth-1 and Meth-2 (62) and cleavage products of platelet factor 4 (63), or anti-thrombin III (64), among many others (Table I). Some of these are internal fragments of various proteins which normally lack any anti-angiogenic activity (51,65), e.g. angiostatin is one or more fragment(s) of plasminogen (58) and endostatin is a fragment of type XVIII collagen (59), as summarized in Table I. Many of the precursor proteins are components of the extracellular matrix (ECM)/basement membranes (e.g. type XVIII collagen and thrombospondin) or members of the clotting/fibrinolytic pathways (e.g. plasminogen and anti-thrombin III) (64).

It is now thought that the tumor angiogenic switch is triggered as a result of a shift in the balance of stimulators to inhibitors (50). When the ratio is low, tumor angiogenesis is blocked or modest in magnitude; in contrast, when the ratio is high, the switch is turned to the 'on' position (50). Of considerable interest was the finding by Bouck and colleagues, that loss of wild-type *p53* gene function resulted in a loss of thrombospondin expression (52). Not only did this finding establish a possible critical link between the genetic basis of cancer and tumor angiogenesis, it also opened up the now flourishing field of endogenous angiogenesis inhibitors. Furthermore, it is now increasingly recognized that oncogenes, such as mutant *ras*, may also contribute to tumor angiogenesis by influencing (i.e. enhancing) the production of pro-angiogenic molecules such as VEGF (66–69). Such effects were slow to be uncovered in the oncogene and tumor suppressor gene fields given the predominant use of pure tumor cell culture systems to study the function of cancer causing genetic alterations.

Other tumor suppressor genes, in their non-mutant, wild-type form, such as *VHL* (von Hippel–Lindeau) and *p16*, have been shown to block the production of regulators of angiogenesis, such as VEGF (70,71), while other oncogenes, such as the erbB family, can stimulate VEGF production (67). Given the enormous interest in the molecular genetics of cancer, these relationships between cancer causing genetic changes and angiogenesis have fostered an increased awareness of the importance of angiogenesis to the development, growth and treatment of cancer.

5. The discovery of additional molecular markers in newly formed blood vessels, especially integrins and cell adhesion molecules

The discovery of VEGF receptors and their upregulation in newly formed blood vessels highlights the fact that indeed, there can be major phenotypic differences between mature, quiescent vessels, and their newly formed counterparts. Such differences are essential to avoiding unwanted toxicity to normal vessels when using anti-angiogenic drugs, and thus achieving a sufficient therapeutic index. A number of such differences are now known, and include a very significant elevation of expression in ECM-binding integrin receptors, such as $\alpha_v\beta_3$ or $\alpha_v\beta_5$ (72,73). This was first reported by Cheresh and colleagues (72) who exploited such differences using specific neutralizing antibodies or small molecule peptide antagonists to block angiogenesis, which occurs, at least in part, by induction of endothelial cell apoptosis. Other 'markers' that are upregulated in activated endothelial cells include adhesion molecules such as E-selectin (74), endoglin (75), glycoproteins such as 'prostate-specific' membrane antigen (76), the ED-B domain of fibronectin (77–79) and various proteases (80). Many of these can be exploited not only as potential therapeutic targets but also for detection of cancer by nuclear medicine based medical imaging techniques (78).

6. The development of quantitative assays for angiogenesis

It is of course difficult to evaluate the function of angiogenic growth regulators in the absence of reliable assays. This was a particularly serious problem in the early days of angiogenesis research since it had not been possible to successfully grow vascular endothelial cells in long term culture until Folkman's group established the appropriate conditions in 1980, and used them to elucidate the functional/sequential steps in angiogenesis (81). Since then a number of semi-quantitative or quantitative assays have appeared that involve sprouting angiogenesis in tumor (cell) free systems (82), such as the corneal micropocket assay, the subcutaneous 'Matrigel plug' assay (83) and assays involving growth of cut sections (slices) of blood vessels in three dimensional gels of an ECM-associated material such as collagen (84). The development of these systems has been a boost to the discovery of new stimulators and inhibitors of angiogenesis, especially the latter (84).

7. Recognition of the prognostic significance of tumor angiogenesis

Another major finding which attracted interest in the field was reported by Weidner *et al.* (85) who found that the greater the degree of angiogenesis detected in a primary tumor, the worse the prognosis. This established a direct relationship between metastasis and angiogenesis. It was first shown in breast cancer and subsequently a large and diverse array of other tumors, including melanomas, gliomas, lung, bladder and prostate cancers, and many others (86). Tumor vascularity is measured by staining tissue sections with antibodies specific (or highly specific) for antigens expressed by vascular endothelial cells such as factor VIII (von Willibrand factor), CD-31 or CD-34

(86) and then counting (under high power) the number of highlighted vessels in so-called vascular 'hotspots' i.e. localized areas where there are unusually high numbers of vessels, as detected first under lower power magnification (85).

Aside from the prognostic implications of this work, it also served to highlight the extent to which tumor masses can become 'contaminated' by blood vessels. Many such vessels are very small and deformed, containing bizarre tortuosities, corkscrew structures, blind ends and abnormal branching characteristics, thus making many of them almost impossible to detect in a normal hematoxylin and eosin tissue section. Consequently, the degree of tumor angiogenesis had been underestimated, and hence less appreciated, prior to publication of this type of work. In this regard, endothelial cells are a rich source of biologically active substances such as cytokines and proteases (87,88) which can themselves affect the behavior of adjacent cancer cells independent of classic angiogenesis, i.e. of providing oxygen and nutrients.

Detection of blood vessels in tissue sections has recently been modified by Benjamin *et al*., so that it is now possible to discriminate between newly formed immature vessels and those that are more established and mature (26). It is based on the use of antibodies to α-smooth muscle action (α-SMA), which appears to stain the latter type of vessel as a result of mature vessels attracting a 'coat' of periendothelial support cells, i.e. pericytes and smooth muscle (α-SMA-antigen-positive) cells (26). Of interest is the finding that anti-angiogenic therapeutic procedures, such as blockade of tumor cell VEGF production, results not only in a drop in the vessel count, but also a change in the ratio of immature/mature vessels because of the relative vulnerability of the immature vessels to this, and most other, forms of anti-angiogenic therapy.

8. Lack of acquired resistance to direct-acting anti-angiogenic drugs

It was first proposed in 1991 by Kerbel (89) that anti-angiogenic therapy might bypass a major problem encountered in virtually all strategies used to treat cancer, especially chemotherapy and hormonal ablation/antagonist therapies, namely acquired drug resistance. The underlying rationale was that the cellular target of anti-angiogenic drugs is a normal, and hence genetically stable, host cell, i.e. the vascular endothelial cells which line newly formed blood vessels in tumors. Because acquired resistance is largely a consequence of the small and large scale genetic instabilities associated with cancer cells (e.g. gene amplification, chromosomal translocations, chromosome loss, simple point mutations, etc.), it should not develop when using certain anti-angiogenic therapeutic strategies (89), just as heritable, and acquired resistance does not emerge among the descendants of drug-sensitive normal host cells exposed to chemotherapy, e.g. dividing bone marrow, gut mucosal or hair follicle cells (90). Clinical 'experiments of nature' provided the first indication that, indeed, acquired resistance may not be inevitable when using certain anti-angiogenic drugs. Thus, long term (eg. 1 year), daily treatment (using interferon α2β) of infants with life-threatening hemangiomas can result in complete regression of these benign blood vessel tumors without any evidence of the development of acquired resistance to this drug (56). In contrast, chronic exposure of malignant cancer cells to the same type of drug is known to result in acquired interferon resistance among variants of the treated cancer cells (54)

The first preclinical/experimental evidence for circumventing acquired drug resistance came from long term, cyclic exposure of tumor-bearing mice with the anti-angiogenic protein drug known as endostatin (91). Unlike conventional cyclophosphamide therapy, using maximum tolerated doses administered in an intermittent fashion, no resistance to endostatin was ever seen and the tumors eventually stopped growing after a certain number of cycles of therapy (91). This work probably attracted more attention than perhaps any other single study on tumor angiogenesis, if not cancer research, over the last decade (92,93).

Given the great importance (and effort) attached to developing drugs, or therapeutic strategies, to reverse (or actually prevent) acquired drug resistance, e.g. the use of P-glycoprotein antagonists such as cyclosporin analogues to block multidrug resistance and the fact that such efforts have not met with much clinical success, at least thus far (94–96), the results obtained using endostatin have certainly increased interest in the pharmaceutical industry for using this type of therapeutic approach as a means of treating cancer. Indeed, it is also possible to use conventional cytotoxic drugs as potent direct-acting 'resistance-free' cytotoxic anti-endothelial agents by such methods as peptide-based targeting strategies (97) or altering the dosing and schedules of the drug so as to create an 'anti-angiogenic' schedule of chemotherapy (98,99).

Nevertheless, it should be noted that some anti-angiogenic drugs work by blocking a particular redundant tumor cell property, such as VEGF production, and thus may be subject to inactivation over time by classic acquired resistance mechanisms since, for example, tumor cells can produce a number of different pro-angiogenic growth factors. Therefore, rare cellular variants producing a spectrum of different pro-angiogenic molecules will likely be selected by the therapy. This could be an important factor in determining the best anti-angiogenic drugs to use, given that treatments using such drugs are probably going to be chronic in nature. In other words, it may be useful to think in terms of 'direct-acting' anti-angiogenic agents, such as endostatin, and those that are 'indirect acting', such as a drug that blocks a pro-angiogenic growth factor produced by tumor cells, or even its relevant endothelial cell receptor tyrosine kinase (90). An indirect acting anti-angiogenic agent, such as an anti-VEGF neutralizing antibody, could be converted to a direct-acting agent by such procedures as conjugating the antibody with a toxic radionuclide or a toxin molecule such that binding of the antibody to endothelial cells results in direct endothelial cell death. In addition, there are reports showing that the majority of the VEGF produced in a tumor mass appears to come from the normal host stromal cells infiltrating the tumor, rather than the tumor cell population itself (100). Use of VEGF blocking antibodies in such situations may not be compromised by the rapid development of acquired drug resistance (90).

Finally, with respect to the issue of drug resistance, anti-angiogenic therapies may also circumvent what may be a major mechanism of intrinsic drug resistance, namely insufficient drug penetration into the interior of a tumor mass due to high interstitial pressure gradients within tumors (101). Targeting the tumor vasculature (rather than the tumor cell population) would avoid the necessity of having to obtain intra-tumor drug delivery (101).

9. The discovery of the impact of angiogenesis on 'liquid' hematologic malignancies

Another remarkable recent development, and one that is clearly counter-intuitive as well, is the recent realization that so-called

'liquid' hematologic malignancies are angiogenesis dependent (102–104), i.e. this is not just a property of solid tumors. The discovery was based on findings such as elevated levels of pro-angiogenic growth factors, i.e. bFGF and VEGF, in the serum and urine of patients with acute lymphatic leukemia and multiple myeloma (102). Similarly, a sharp increase in bone marrow angiogenesis, as measured by means of vessel density in vascular hot spots, has also been detected in such patients (102). The newly formed blood vessels detected in the marrow of patients with acute lymphoytic leukemia or multiple myeloma could be a rich source of growth factors and cytokines, as well as survival factors, for tumor cells that arise in this tissue.

This work has already resulted in the initiation of early phase I clinical trials to test putative anti-angiogenic drugs such as thalidomide (105) in multiple myeloma patients, the results of which look very encouraging (106). If this holds up and is found to be a consequence of an anti-angiogenic effect it would provide major impetus to a large segment of the medical oncology community to become much more actively engaged in angiogenesis research and anti-angiogenic therapies to treat these types of cancer.

10. The discovery of the 'accidental' anti-angiogenic effects of various conventional or new anti-cancer drugs and treatment strategies

A number of discoveries led to the provocative conclusion that the use of anti-angiogenic drugs and therapies—in the clinic—is, in reality, probably not a new development. Oncologists may have been using them without realizing it for decades, albeit in a less than optimal manner. This is based on preclinical data showing that various cytotoxic drugs such as taxanes (107–109), topoisomerase inhibitors (110), purine analogue anti-metabolites (111), radiation therapy (112) and hormonal ablation therapeutic procedures (26,113,114) can all result in either direct or indirect killing of vascular endothelial cells of newly formed tumor blood vessels. Denekamp and colleagues were the first to have the insight to point out these 'accidental' anti-vascular effects using various rodent tumor models and a diverse spectrum of anti-cancer agents ranging from cytokines/biological response modifiers and ionizing radiation to chemotherapeutic drugs and photodynamic therapy (115). Denekamp coined the term 'vascular targeting' to describe this effect (115). The possibility of optimizing the anti-vascular targeting effects of chemotherapeutic drugs by altering the drug doses and schedules used for therapy has been elegantly shown by Folkman and colleagues (98), as well as Klement *et al*. (99).

More recently, much interest has been aroused by findings which relate to how withdrawal of androgens may cause regression of hormone-sensitive cancers, such as prostate cancer, at least in part through an anti-angiogenic mechanism (26,113). The basis for this intriguing possibility is that androgens are powerful inducers of VEGF in hormone-sensitive tissues (114) and VEGF, in turn, is a potent powerful survival factor for endothelial cells of newly formed immature blood vessels (24), perhaps by upregulating bcl-2 (116,117) or activating the PI3 kinase/Akt/PKB survival/signalling pathway (118,119) in endothelial cells. Hence, acute withdrawal of tumor cell VEGF, which could occur in patients or animals with androgen-dependent tumors after androgen ablation therapy, can result in rapid apoptosis of the endothelial cells comprising such immature tumor vessels (113). This, in turn, results in a secondary, but much more massive, wave of apoptotic cell death in the cords of tumor cells surrounding the regressive/dying vessels (113). This secondary cell death process leads to the regression of tumor mass (113). Unfortunately such tumors will eventually recur due to the emergence of hormone refractory tumor cell variants (113). Thus, this would be another example of an indirect acting anti-angiogenic therapy, or drug, to which resistance may develop.

In addition to conventional therapeutic agents, some of the newer generation of anti-cancer drugs making their way into the clinic (and which were never designed with the intent of inhibiting angiogenesis) may affect tumor growth, partly by suppression of tumor angiogenesis. An example is the so-called signal transduction inhibitor therapies which target the products of mutant oncogenes, such as *ras*, or overexpressed proto-oncogenes such as the EGF receptor tyrosine kinase and the erbB2/Her-2 receptor tyrosine kinase (69). The drugs in question include small molecule inhibitors of mutant *ras* (or a related target) such as *ras* farnesyltransferase inhibitors (Ras FTIs) and monoclonal neutralizing antibodies to the EGF receptor, such as C225, or to erbB2/Her2, e.g. Herceptin (69). Their potential ability to block angiogenesis (69) is based on the discovery that oncogene activation can lead to induction or upregulation of pro-angiogenic growth factors such as VEGF, as first reported by Grugel *et al.* (120) and Rak *et al.* (66). Thus, treatment using drugs which block oncoprotein function may result in downregulation of VEGF, and perhaps other pro-angiogenic growth factors (66,67). This could contribute to the drug's overall therapeutic effect *in vivo*, where angiogenesis is required, but not *in vitro*, where angiogenesis is irrelevant for tumor cell growth and survival, a possibility which could help explain why such drugs may lack cytotoxic properties *in vitro* but sometimes express such an effect *in vivo* (69). Similar reasoning is evident with a number of other drugs and therapeutic modalities such as interleukin-12 based immunotherapy (121).

Finally, with respect to the effects of traditional/conventional therapeutics, mention should be made of the findings of Teicher and colleagues, where it has been shown preclinically that the combination of an anti-angiogenic drug (or drugs) (such as TNP-470) with a conventional cytotoxic agent, such as cisplatinum, taxol or cyclophosphamide, can significantly improve the anti-tumor efficacy of the cytotoxic drug (122). This observation is counterintuitive since it might be expected that anti-angiogenic agents would decrease blood flow into tumors and, hence, limit drug delivery to tumors. However, just the opposite may be the case (123), and this may be due to a transient normalization of the abnormal structure of tumor vessels mediated by certain angiogenesis inhibitors, e.g. neutralizing anti-VEGF antibodies (124), thus leading to the possibility of a temporary increase in blood flow and, hence, drug delivery (123). Alternatively, there is the potential for a cytotoxic drug to function directly on endothelial cells of newly formed tumor vessels; this effect may be exaggerated by the inclusion of an anti-angiogenesis drug which compromises endothelial cell survival mechanisms (99). Regardless of the actual mechanism, these combination therapy effects, which have also been observed with radiation therapy and angiogenesis inhibitors (112), could play a significant role in the clinical evaluation and effects of angiogenesis inhibitors, as discussed below.

Phase I

Drug	Sponsor	Mechanism
COL-3	Collagenex, NCI	Synthetic MMP inhibitor; tetracycline derivative
Squalamine	Magainin	Inhibits Na/H exchanger
Combretastatin	Oxigene	Apoptosis in proliferating endothelium
PTK787/ZK2284	Novartis	Blocks VEGF receptor signaling
Endostatin	NCI/EntreMed	Induction endothelial cell apoptosis *in vivo*
CAI	NCI	Inhibitor of calcium influx
PTK787/2K22584	Novartis	Small molecule inhibitor of VEGF receptor

Phase II

CGS-27023A	Novartis	Synthetic MMP inhibitor
TNP-470	TAP Pharm.	Fumagilin analogue; inhibits endothelial proliferation
Thalidomide	Celgene	Unknown
SU5416	Sugen	Blocks VEGF receptor signaling
Vitaxin	Ixsys	Antibody to integrin on endothelial surface
Interleukin-12	Genetics Inst.	Induces IFN-gamma and IP-10
EMD121974	Merck, Germany	small molecule integrin antagonist

Phase III

Marimastat	British Biotech	Synthetic MMP inhibitor
AG3340	Agouron	Synthetic MMP inhibitor
Neovastat/AE941	Aeterna	Natural MMP and VEGFR inhibitor
Anti-VEGF Ab	NCI	Monoclonal antibody to VEGF
Interferon-alfa	Commercially available	Inhibition of bFGF production
IM862	Cytran	unknown mechanism

Fig. 1. Angiogenesis inhibitors in clinical trials. From National Cancer Institute Database (updated August 1999).

The future: opportunities to be seized, problems to overcome

Figure 1, which shows various anti-angiogenic drugs currently in clinical development, highlights the obvious fact that we shall soon have some kind of indication of the potential value of this kind of new therapeutic approach to treat cancer. While this is an exciting time, there are a number of problems which could hamper progress in the field and overall enthusiasm for the concept of anti-angiogenic therapy. Tackling these problems presents some challenging opportunities; success in doing so would help foster a new era of cancer therapeutics in the clinic. Some of the problems and opportunities are discussed below.

The difficulties associated with clinical evaluation of anti-angiogenic drug efficacy

The nature of most anti-angiogenic drugs makes it difficult to evaluate their potential efficacy in early phase clinical trials since, with few known exceptions (91,126–128), they do not cause overt or rapid tumor regression, as can conventional cytotoxic agents (129). Thus, obvious tumor shrinkage observed over a relatively short period of time is unlikely to be encountered. Indeed, if one uses the example of chronic interferon $\alpha 2\beta$ administration of either life-threatening hemangiomas (56) or giant cell tumors of the mandible (130), the effects of the therapy are not initially detected for several months, which is then followed only by a very gradual regression of these benign tumors. This necessitates a reappraisal of the design and needs of early phase clinical trials to test such agents in a number of ways (127,129). For example, it will probably result in increased emphasis on combining anti-angiogenic agents with conventional cytotoxic drugs since, as discussed above, preclinical evidence suggests that the efficacy of a conventional cytotoxic drug can be improved by combination with an angiogenesis inhibitor (122). Indeed, a number of anti-angiogenic clinical trials currently in progress have been designed to compare the effects of a particular cytotoxic agent alone compared with this same agent in combination with an angiogenesis inhibitor. Clearly, the success of Herceptin in improving the effects of cytotoxic chemotherapy in a proportion of advanced-stage breast cancer patients has given this strategy of evaluating cytostatic drugs much added credibility (131). This could allow conventional endpoints such as tumor shrinkage and prolongation of survival of very sick patients to be used, albeit indirectly, as a convenient means of more rapidly assessing the merit of anti-angiogenic drugs.

Another possible resolution to this problem could come with the increased use of improved 'anti-vascular targeting' strategies which can cause acute tumor regressions, as shown in various preclinical models. For example, certain tubulin-binding agents (109) such as combretastin A-4 can cause such an effect (125,132,133) as can antibodies which target tissue factor to newly formed blood vessels, thus causing an intravascular thrombogenic response in such vessels (134). These drugs kill endothelial cells of newly formed blood vessels by different mechanisms (133), thus causing a vascular collapse and the subsequent death of much larger numbers of tumor cells. Clearly the problem here will be to develop drugs which have this ability to cause such a dramatic 'tumor infarction' (134) without major, perhaps even life-threatening, toxic side effects. In this regard, a potentially significant development in the near future could be the use of genomics based technologies to uncover a large number of highly (or even totally) specific molecular markers for the activated endothelial cells of newly formed blood vessels. This could make antibody based therapeutics safer and more effective.

What will also be required are vastly improved means to definitively show that a cytostatic anti-angiogenic agent has the desired biological (i.e. anti-angiogenic) effect *in vivo*, in the absence of acute tumor regression, when the agent is cytostatic and is tested in the absence of a chemotherapeutic drug. This is difficult, to say the least, even in experimental animal models, let alone patients. In the experimental animal situation, tumors can be removed and examined for such

changes as the extent of vascularization, vascular structure, endothelial cell viability or apoptosis, as well as for markers of angiogenic activity, e.g. VEGF expression. Taking serial biopsies of metastatic tumors may not be a particularly practical or desirable approach in the clinical situation. As such, reliable surrogate markers of tumor angiogenesis found in serum or urine may be necessary. At present few, if any (at least of a reliable nature), such markers exist. The use of various non-invasive medical imaging strategies (e.g. MRI, doppler ultrasound) to monitor changes in tumor blood flow, vascular structure and permeability may be the most fruitful approach and, indeed, there are considerable research efforts (and some successes) underway in this area (135–139).

The prospect of delayed toxicity associated with long-term anti-angiogenic therapy

Another obvious problem is that toxic effects associated with chronic anti-angiogenic therapy may not show up in short term early phase clinical trials or animal models, but rather, only after very protracted courses of therapy using these new drugs. The development of spastic diplegia in some infants or children who had been treated previously and successfully over 1 year with anti-angiogenic (interferon $\alpha 2\beta$) therapy for their life threatening hemangiomas (140) is an example of such delayed toxic side effects. This undoubtedly will increase the need for very highly specific or completely specific targets expressed by blood vessels on tumors. The growing inter-relationship between the clotting and fibrinolytic pathways, and angiogenesis (64) raises the possibility of bleeding/coagulation disorders in patients who receive certain anti-angiogenic drugs, as well as causing or exacerbating existing cardiovascular defects in older patients. Such problems may not show up in short-term preclinical studies utilizing mice which are <2 or 3 months of age (the standard practice) as opposed to much older mice. In addition, there is the obvious concern about affecting physiologic forms of angiogenesis in various situations. Thus, wound healing may be adversely affected in a cancer patient who is receiving anti-angiogenic drugs, as would 'reproductive angiogenesis', e.g. corpus luteum development in adult females, and development of the vasculature in developing embryos. Growth in neonates may also be compromised by angiogenesis inhibitor therapies (141). However, given the unique structural features of the tumor vasculature, some angiogenesis inhibitors may selectively block tumor angiogenesis without actually affecting other physiological forms of angiogenesis. This possibility could turn out to be an important factor in selecting the optimal angiogenesis inhibitors for clinical development and their use in cancer patients.

The need for better animal tumor/therapy models

Most preclinical studies on tumor angiogenesis and anti-angiogenic therapy usually employ rapidly growing transplantable mouse tumors, or human tumor xenografts, which are usually grown as a solid, localized tumor in the subcutaneous space. For several reasons this approach almost certainly exaggerates the anti-tumor responses observed using anti-angiogenic drugs, as well as other types of anti-tumor agents. First, in such experimental situations, unlike the clinic, distant visceral metastases are usually not the focus of the treatment, and it is precisely such secondary tumors (which are often much harder to treat) which are ultimately responsible for cancer's lethality. Hence, more emphasis should be placed upon evaluating anti-angiogenic therapies on metastases growing in such sites as the lungs, liver, brain and bone, especially since it cannot be assumed that an angiogenesis inhibitor that is effective in the particular organ site, e.g. the lungs, will be equally effective in other sites. For example, there appear to be organ-specific endothelial markers and the vasculature of a tumor mass can differ depending on the site of growth (97,131,142–144). Hence some anti-angiogenic drugs, depending on their target, may show large differences in anti-tumor efficacy as a function of organ site (145). In this regard, new and improved metastasis models are being developed, such as the use of firefly luciferase on green fluorescent protein-tagged tumor cell lines which greatly enhance detection of microscopic and macroscopic metastases in many different organ sites (146,147). In addition, the use of orthotopically transplanted tumors may be preferable for these studies, not only to induce or enhance the incidence of metastases but also because the response of a tumor mass growing ectopically (e.g. a colon tumor transplanted into the subcutaneous space) may be abnormal compared with the same tumor growing in a physiologically relevant site (147–150).

Another problem associated with the use of such rapidly growing transplantable tumor models is that, by definition, they will contain an extremely high proportion of newly formed immature vessels, and it is just such vessels which appear to be especially vulnerable to the effects of most anti-angiogenic drugs (26). Presumably, spontaneous, slow-growing tumors in humans, which may have been incubating for years before their detection, would have a much lower proportion of immature vessels, and thus would be much less responsive to most forms of anti-angiogenic therapy. Consequently, the use of spontaneous tumors in rodents should probably be used in conjunction with the more traditional transplantable tumor models, when testing anti-angiogenic drugs, as shown by several groups such as Hanahan and colleagues (151,152) and Dexter *et al.* (153) using various transgenic 'oncomouse' models. Such mice are harder to work with in many ways, and clearly less economical (154) but the results obtained using such models may ultimately provide a more faithful picture of what will occur later in the clinic (153), although this has yet to be proved.

A final point worth raising concerns the relative proportions of 'new' (immature) vessels versus 'older' mature vessels in tumors, and stems from the recent work of Benjamin *et al.* (26) and Holash *et al.* (45). The latter group has reported that tumor cells may actually parasitize ('co-opt') pre-existing host organ blood vessels in sites of metastases or in vascularized organs such as the brain in order to initiate blood-vessel-dependent tumor growth (45) as opposed to classical angiogenesis mechanisms. These co-opted vessels then actually regress as a result of induction of apoptosis of the constituent endothelial cells, a process apparently mediated by angiopoietin-2 (45). Subsequently, this is followed by induction of angiogenesis at the periphery of the growing tumor mass by a co-operative interaction of VEGF and angiopoietin-2 (45). Thus, there may be a dynamic equilibrium established between regression of 'established' vessels and the formation of new ones, a process which may favour a relatively constant and very high ratio of immature to mature vessels in spontaneous human tumors. In this respect it is interesting to note that Bejnamin, Keshet and colleagues analyzed highly malignant human glioblastomas obtained from patients using antibodies to α-SMA to distinguish between mature (α-SMA-positive) and immature (α-SMA-negative) vessels (26), and found very high (e.g. $>80\%$)

proportions of immature vessels, compared with normal brain. These studies await confirmation but, if correct, could bode well for the clinical use of anti-angiogenic drugs which target newly formed immature vessels.

Summary and conclusions

Like anti-cancer 'signal transduction inhibitor drugs,' anti-angiogenic drugs are the recent outcome of the fruits of a prolonged period of basic research, mostly undertaken over the last 20 years. Both are hopefully about to make a significant impact in clinical oncology, if Herceptin serves as a reliable guide (131). Research in tumor angiogenesis and anti-angiogenic drugs has also significantly increased awareness of the importance of tumor–host interactions and the tumor microenvironment with respect to tumor development, disease progression, and response to therapy. In this regard, the endothelial cell compartment of a tumor mass might well receive almost as much therapeutic attention in the future as the tumor cell compartment itself has in the past. Thus, when thinking of new targets, or anti-cancer strategies, which have only been investigated in the context of the tumor cell *per se*, some consideration should now be given to the host endothelial cell of tumor vessels. For example, developing and testing anti-cancer drugs which modulate apoptosis (155) should be analyzed for their ability to induce apoptosis in the endothelial compartment of a tumor mass, and not just the tumor cell population itself. Such a shift in cell focus has begun to take place in the gene therapy field, where targeting a tumor's vasculature may help overcome many of the problems encountered thus far in trying to use gene vectors for anti-cancer cell therapy, such as delivery of the vector/gene into the target tumor cells (156,157).

It is also heartening to note the spinoffs of the research on angiogenesis factors and inhibitors that was initiated almost exclusively in cancer research laboratories such as Folkman's. Exciting clinical results in the area of 'therapeutic angiogenesis' are being obtained using bFGF, VEGF or the genes which encode these proteins, to stimulate vessel growth in ischemic heart disease and peripheral vascular disease (16–18,158,159). Conversely, inhibitors of angiogenesis are now being used to treat a variety of 'angiogenic diseases' characterized by unwanted or over-exuberant angiogenesis such as diabetic retinopathy and age-related macular degeneration, and perhaps even endometriosis (160) and atherosclerotic plaques (161,162). Successes in treating such diseases with drugs which modulate angiogenesis is bound to increase the efforts in the area of cancer, and vice versa.

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