PERGAMON

EJC Supplements Vol 1 No. 2 (2003) 1–12

EJC Supplements

www.ejconline.com

Angiogenesis as a new target for cancer control

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Received 10 December 2002; received in revised form 17 January 2003; accepted 21 February 2003

Abstract

Tumour angiogenesis is essential for cancer growth and metastasis. Many new pathways have been recognised in the last few years, including genes involved in embryonic development of the vasculature, e.g. the notch/delta, ephrin/eph and the HOX genes. Furthermore, vascular mimicry, intussusception and recruitment of circulating endothelial cells may contribute to tumour vessels, as well as endothelial proliferation. A close link has emerged between hypoxia and the regulation of angiogenesis, with many angiogenic factors involved in hypoxia in endothelial cells and cancer cells. Examples include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), adrenomedullin, oxygen-regulated protein 150, tumour necrosis factor (TNF) alpha, endothelins. Conversely, anti-angiogenic molecules such as thrombospondin are downregulated. The main pathway regulating these responses seems to be via the hypoxia inducible factor I alpha and its modulation by proline hydroxylases. Many oncogenes amplify the effects of the hypoxia pathway, e.g. *PTEN* mutations. Thus, a plethora of targets is now available for therapy. Most tumours express many different pathways, but some key ones are emerging. VEGF seems to be a core factor for endothelial growth, and blockade of hypoxia signalling also inhibits angiogenesis. One of the difficulties in assessing response to therapy, if only new vessels are inhibited, is that only small amounts of regression may occur. However, clinical trials are now incorporating pharmacodynamic measurements such as vascular permeability and responses have been reported for a wide range of anti-angiogenic agents, in particular the VEGF pathway inhibitors. Marked synergy with conventional treatment modalities has been shown in preclinical models and this paradigm is being followed in clinical trial development. Second generation drugs are now entering phase III trials and are likely to enter routine practice.

Keywords: Angiogenesis; Anti-angiogenesis Inhibitors; Hypoxia; Trials; VEGF

1. Introduction

Tumour angiogenesis has become an intensely investigated area over the last decade because it is an essential component of the growth and spread of cancer and is therefore potentially an important therapeutic target. Folkman has played a major role in conceptualising the role of angiogenesis and making many of the important biological and clinical discoveries [1]. The field has progressed rapidly because of the discovery of angiogenic growth factors such as the vascular endothelial growth factor (VEGF), which specifically regulates endothelial proliferation [2]. Increased understanding of these factors has made it possible to develop rational anti-angiogenic therapies. Because endothelial cells are not genetically unstable, it was thought that drug resistance would not be a problem for anti-angiogenic therapies and early preclinical data has supported this assumption [3].

However, in spite of these advances, there are few anti-angiogenic agents available in routine clinical practice and this reflects the emerging complexity of the mechanisms of angiogenesis as well as the difficulties encountered when carrying out trials of agents which do not directly target cancer cells.

There have been several recent reviews of different aspects of tumour angiogenesis [4–12]. In this review, some of the recent scientific advances will be summarised with evidence of the role of angiogenesis with regard to clinical outcome, description of some agents in clinical trials, assessment of trial methodology and analysis of problems that need to be resolved.

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doi:10.1016/S1359-6349(03)00007-7

2. Recent developments in the molecular biology of tumour angiogenesis

2.1. Pro- and anti-angiogenic factors

The most studied angiogenic factor is VEGF. It is markedly upregulated by hypoxia and high expression in cancers is associated with a poor prognosis as well as resistance to anticancer therapies. These include radiotherapy [13], immunotherapy [14] and cytotoxic drugs.

The number of pro-angiogenic factors reported continues to rise, but this may also reflect the interest that there is in this area in that pleiotrophic factors may be particularly reported for their angiogenic effects [15–25]. Some of these are listed in Table 1 and are also reviewed elsewhere [26–28]. The coagulation pathway plays a major role in angiogenesis [16]. Recently, organ-specific angiogenic factors have been reported [29]. Some of the products of glycolysis can induce angiogenesis, e.g. lactic acid and pyruvic acid [30].

A major advance in understanding was provided by Folkman's group and by Bouck [31] demonstrating natural inhibitors of angiogenesis can be produced by cancers and normal tissues and are lost during tumour development [32–34]. Tumour suppressors induce the expression of these anti-angiogenic molecules, such as thrombospondin 1, and oncogenes reduce their expression. There are several new pathways by which tumour angiogenesis may be suppressed [35,36], some reflecting genes involved in development or suppressor genes [37,38]. Thus, the development of tumour angiogenesis represents the balance of these pathways. The development of angiogenesis has been called the angiogenic switch as the balance turns to favour vascularisation at different stages of cancer [39]. Metastases may remain dormant until this switch occurs [40].

Table 1
Angiogenic and anti-angiogenic factors

Angiogenic factors	Pro-angiogenic factors
Endostatin	Vascular endothelial growth factor
Angiostatin	Fibroblast growth factor (acidic and basic)
Vasostatin	Hepatocyte growth factor
Vascular endothelial growth factor inhibitor	Tumour necrosis factor-alpha
Thrombospondin-1 and -2	Platelet-derived growth factor
Angiopoietin-2	Granulocyte-colony stimulating factor (G-CSF)
Fragment of platelet factor-4	Epidermal growth factor
Interferon alpha/beta/gamma	Angiotropin
Restin	Angiogenin
Arrestin	Interleukin-1, -6, -8
Canstatin	
PEX	Cathepsin
Derivative of prolactin	Gelatinase A/B
Proliferin-related protein	Stromelysin
SPARC cleavage product	Urokinase type plasminogen activator
Osteopontin cleavage product	Plasminogen activator inhibitor-1
Antithrombin-3 fragment	
Tissue inhibitor of metalloproteases (TIMP1, 2 and 3)	Copper
	Alpha v beta 3 integrin
Interleukin-1, -4, -10, -12 and -18	Angiopoietin 1
Angiotensin	Angiostatin 2
Angiotensin-2 (AT2 receptor)	Endothelin
Caveolin 1, 2	Erythropoietin
1,25-$(OH)_2$vitamin D_3	Nitric oxide synthase
2-Methoxyoestradiol	Platelet activating factor
EMAP-II (endothelial monocyte activating polypeptide)	Prostaglandin E
Gro-β	Thrombopoietin
IP-10 (interferon inducible protein 10)	Adrenomedullin
Maspin	Pleiotrophin
METH-1 and -2	Midkine
MIG (monokine induced by gamma interferon)	
P16	
Pigment epithelium-derived growth factor	
Prostate-specific antigen	
Protamine	
Retinoic acid	
Troponin I	
Transforming growth factor β1	

Many of the inhibitors of angiogenesis are peptides produced by proteolysis from larger precursors, e.g. angiostatin from plasminogen and endostatin from collagen XVIII. The list is rapidly expanding and many of these molecules are being developed as anti-angiogenic agents [41]. Often their direct target is not known, although a receptor has been defined for angiostatin [42]. These inhibitors may paradoxically be produced by the cancer as a result of degradation of the extracellular matrix [43], but clearly they also overproduce the angiogenic pathways, and a key angiogenic suppresser thrombospondin 1 is often downregulated.

Most cancers express multiple angiogenic factors that do not seem to necessarily be correlated with each other [44]. Preclinical models have confirmed this and shown for individual cell lines that each factor may have a role, with their relative importance varying from one cell line to another. Similar results are not available for anti-angiogenic pathways, so whether some are more important or more commonly downregulated is not clear although, as mentioned above, most data are available for thrombospondin 1.

2.2. The role of oncogenes in tumour angiogenesis

An emerging role for oncogenes as key regulators of angiogenesis has been highlighted particularly for the ras signalling pathway [45,46]. Many other oncogene signalling pathways also upregulate angiogenesis, mostly by increasing VEGF production. Examples include the phosphatidylinositol-3 (PI3) kinase pathway [47], epidermal growth factor (EGF) receptor [48] and erbB2 [49]. The VEGF promoter is responsive to these pathways in addition to hypoxia and there may be synergy between these mechanisms [50] (see below).

Increased proteolysis, a hallmark of cancer can lead to the release of stromal growth factors, many of which are angiogenic, e.g. basic fibroblast growth factor (FGF), hepatocyte growth factor and transforming growth factor (TGF) beta. Many oncogenes induce the expression of proteases, particularly the urokinase plasminogen activator system.

The *in vivo* effects of some anticancer drugs targeting oncogenes are often greater than expected from their growth inhibitory effects *in vitro* and some, such as EGFR and erbB2 inhibitors, reduce tumour angiogenesis *in vivo*, which may contribute to their antitumour effect.

2.3. Endothelial stem cells and vasculogenesis: vascular mimicry and intussusception

Recent studies have shown the presence of circulating endothelial precursor cells in umbilical cord blood and the peripheral blood of adults [51–54]. In animal models, they have been shown to integrate into the tumour neovasculature and contribute to tumour growth [55]. Because these endothelial cells target the tumour vasculature, they have been used to deliver gene therapy specifically to tumours. Factors stimulating endothelial cell release from the bone marrow include VEGF, trauma and burns [56]. The relative importance of this pathway to human tumour vasculature is not yet known, but circulatory endothelial cells may be more susceptible to anti-angiogenic drugs than the established vasculature [57]. In addition to the progenitor cells, differentiated endothelial cells can be detected in peripheral blood and levels are higher in cancer patients than controls [58]. Whether these may be markers for tumour angiogenesis and reflect the functional state of the tumour vasculature is unknown, but they may be of value to monitor anti-angiogenic therapy.

Another mechanism for forming new vessels from pre-existing ones is intussusceptive microvascular growth [59]. This divides existing vessels by insertion of tissue folds and columns of intestinal tissue into the vessel lumen.

Vascular mimicry is a process by which the tumour cells themselves form vessels. This was first reported in uveal melanomas, but has been reproduced *in vitro* and reported in other tumours [60]. However, its importance for delivering oxygen to tumours has not been shown.

2.4. Differentiation pathways for blood vessel patterning

The mechanisms involved in the embryonic development of vessels, their growth and migration and the establishment of vascular structures are being elucidated. In many cases, these pathways will be switched off in adult tissue, but reactivated in proliferating endothelial cells. Examples include the Id gene family of transcription factors that seem essential for tumour angiogenesis [61] and the homeobox genes [62]. The former family of basic helix loop, helix transcription factors comprises three members and there is redundancy in the system. Knocking out the function of any one still allows tumour angiogenesis, but double knockouts have severely impaired tumour angiogenesis.

Homeobox genes were first characterised as causing mutations in Drosophila whereby one body segment, normally formed, replaced another. They control body plan and development. Mutations in the *HOX A3* gene cause multiple defects in the cardiovascular system. Only one homeobox gene is so far known to be restricted to endothelial cells, *HOX A9EC*. Other members of the family *HOX D3* control invasion of migratory behaviour and *HOX B3* promotes capillary vascular density. These genes are likely to play essential roles in tumour angiogenesis.

The notch receptors and their ligands (delta, jagged) are expressed in the endothelium as well as most other

tissues [63]. Typically, notch signalling is involved in differentiation, with activated notch tending to block differentiation, allowing cells to continue to proliferate and then respond to more downstream differentiation signals. Mutations in notch 3 cause vascular abnormalities and infarcts. An endothelial-specific notch ligand has recently been reported, delta 4, and is upregulated in tumours and mouse tumour vasculature [63]. Soluble forms of delta 4 inhibited endothelial proliferation, although the mechanism is not clear, e.g. dominant-negative, apoptosis, differentiation. Expression patterns for delta 4 were similar to notch 4 that is predominantly expressed in endothelial cells and is also an oncogene for mouse mammary carcinoma when constitutively activated.

The branching pattern of blood vessels is remarkably similar to that of peripheral nerves and advances in understanding neural migration has shown similarities in the regulation of the two systems [64,65]. Neuropilins have a role in both processes. The 165 amino acid neuropilin 1 protein specifically binds to VEGF (the latter having at least four splice variants with different properties) and may enhance and modify VEGF signalling. Another ligand for neuropilin 1, semaphorin III, antagonises VEGF.

In a similar manner, the ephrin/Eph pathway signals to the developing neural system, provides guidance to developing neurones, but has been shown to be involved in arterial/venous differentiation [64,65]. Eph proteins are a large family of tyrosine kinases and ephrins are their ligands. EphB4 is enriched in veins, ephrin B2, in arterial endothelium. These pathways are being studied for their role in tumour angiogenesis and for antivascular therapy.

2.5. *Endothelial-specific genes*

Clearly genes specifically upregulated on the surface of tumour endothelial cells, but not expressed on normal vessels, would be an attractive target for therapy. Delacroix and colleagues [66] microdissected blood vessels from primary human colorectal cancers and used serial analysis of gene expression to profile the transcription of these vessels. They found 46 genes differentially upregulated in the tumour vasculature, 10-fold or more compared with normal tumour vessels. They also found that many were upregulated in vessels from several cancer types, in both the primary and metastases. They fell into several major gene categories—proteases, extracellular matrix proteins, lectins and nine novel expression sequence tags (ESTs) of unknown function.

Another approach has been to use genomics and cDNA libraries to electronically profile endothelial-specific genes. This yielded four endothelial-specific genes that are currently being profiled in human tumour endothelium [67].

The cell-surface products may also yield useful secreted or shed markers of tumour angiogenesis, or be suitable for vascular imaging.

2.6. *Interactions of hypoxia and angiogenesis*

Tissue hypoxia is one of the major physiological differences between cancer and normal cells, and tumour hypoxia is associated with a poor prognosis and resistance to radiation therapy [68]. Tumours use and adapt hypoxia-induced pathways essential for normal development, growth and wound healing. Hypoxia stress induces the expression of many different tumorigenic pathways, including those that regulate cell proliferation, angiogenesis and cell death.

Direct evidence of hypoxia in human cancers has been shown most convincingly by the pioneering work of Vaupel and colleagues who performed studies involving oxygen electrodes (reviewed in Ref. [69]). They showed that low tumour oxygen tension was associated with an increased metastasis and poor survival of patients suffering from squamous tumours of the head and neck, cervical or breast cancers. Angiogenesis, or new blood vessel formation, is one of the most important responses to hypoxia.

A key transcription factor that activates the expression of many of these genes is the hypoxia inducible factor HIF complex (HIF) (Fig. 1). It is a heterodimer of alpha and beta subunits. There is a second HIF gene (*HIF-2alpha*), also induced by hypoxia. Stable forms of HIF-1alpha and HIF-2alpha translocate to the nucleus where they interact with the aryl hydrocarbon nuclear translocator (ARNT, the beta subunit), which is essential for binding to DNA. HIF is a transcription factor that binds to hypoxia response elements (HREs), typically a sequence of 5′ TACGTG 3′, in hypoxia-responsive genes such as VEGF. Under oxygenated conditions, HIF-1alpha is bound to the tumour-suppressor Von Hippel Lindau (VHL) protein. This interaction causes HIF-1alpha to become ubiquitinated and targeted to the proteasome, where it is degraded [70,71]. Mutations in VHL that are associated with renal cancer and haemangioblastomas prevent ubiquitination, resulting in an accumulation of HIF-1alpha and activation of hypoxia response genes [72,73].

With the production of antibodies to the HIFs it has been shown that the majority of tumours switch on this pathway selectively when compared with normal tissues [74,75]. It was found that more rapidly proliferating tumours and those with p53 expression often had higher levels of HIF staining [76].

Recently, two groups have reported that a prolyl hydroxylase (a tetramer containing two hydroxylase units and two protein disulphide isomerase subunits) is part of the mechanism by which cells sense hypoxia and regulate HIF-1alpha [77,78] The enzyme, which requires

Angiogenesis pathways and targets for therapy
This figure summarises the main mechanisms involved in tumour angiogenesis and the sites of action of drugs that are currently being assessed clinically. Based on figure from Fox et al. 1991 [92].

1. Oncogenes
The switch to an angiogenic phenotype is related to activation of oncogenes and loss of tumour suppressor genes. Oncogenes activate transcription of vascular endothelial growth factor (VEGF) and other angiogenic pathways. These genes include *ras* and epidermal growth factor receptor (EGFR) amongst many others (Thus drugs targeting them also block angiogenesis).

2. Thymidine Phosphorylase
This enzyme is upregulated in many cancers and produces angiogenic metabolites (inhibitors are due to go into trial) a prodrug activate is on the market-capecitabine).

3. Urokinase and metalloproteases
Multiple metalloproteases are induced (and inhibitory drugs are now in phase III). The urokinase pathway may be a more potent pathway activating plasmin. (and various inhibitors including antibodies and urokinase receptor blockers are active preclinically). Proteolysis of the stroma also results in the release of stored angiogenic factors from the matrix.

4. VEGF and basic FGF receptors
VEGF receptors are upregulated on tumour endothelium. Synergy with basic fibroblast growth factor (FGF) in vessel growth.

5. Anti-angiogenic peptides
Production of these peptides from endogenous proteins provides a balance to angiogenesis and tumours also produce these, including endostatin and angiostatin. The receptors are poorly characterised.

6. Platelets
They are often increased in cancer (possibly via interleukin 6 (IL6) stimulation) and are a major source of VEGF.

7 Tissue factor
Procoagulant pathways are also increased in tumour vessels. Antithrombotic drugs such as fragmin may block this pathway.

8. Cell-adhesion molecules, Integrins
Integrins $alpha_v beta_3$ and $alpha_v$ $beta_5$ are critical for attachment and survival of growing endothelium. Antibodies and peptides blocking their function are in trial.

9. Cytoskeleton and vascular targeting
The growing cells are very sensitive to tubular inhibitors such as combretastatin. Many differentiation pathways are potential targets, e.g. notch.
Endothelial cells acquire an activated phenotype and change their repertoire of antigens such as upregulating endoglin. Procoagulants or other toxins can be targeted to antigens upregulated on the tumour endothelium.

10. Hypoxia
Hypoxia activates hif-1alpha to induce many angiogenic peptides including VEGF, platelet-derived growth factor b (PDGFb) and adrenomedullin. Hypoxia can be used to drive gene therapy or selectively activate prodrugs

11. Macrophages, leucocytes, and mast cells
Macrophages and other inflammatory cells are attracted by many cytokines and produce VEGF amongst many other pro-angiogenic factors. These are currently being assessed to deliver gene therapy.

12. Circulating endothelial stem cells
These contribute to vasculogenesis and may be susceptible to low dose chemotherapy

13. Pericytes
Pericytes surrounding the capillary retract and the endothelial cells (Ecs) alter their morphology.

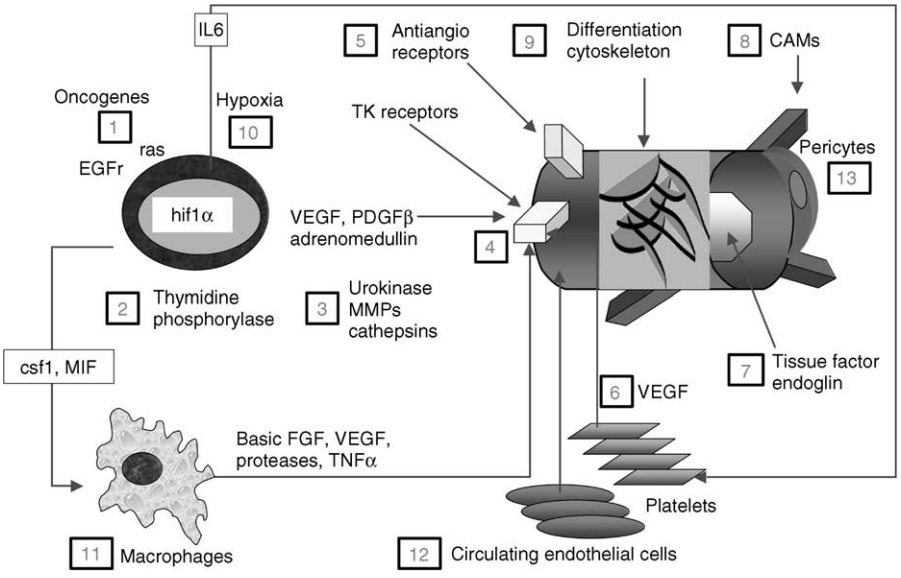

Fig. 1. Pathways of angiogenesis.

oxygen, ferrous iron and 2-oxoglutarate, covalently modifies HIF-1alpha at two proline residues, converting it to a hydroxylated form. This form of HIF-1alpha is then able to interact with the VHL protein and become degraded. Under hypoxic conditions, however, this post-translational modification no longer occurs. HIF-1alpha remains stable, and is able to regulate gene expression.

The regulation of HIF-1alpha by prolyl hydroxylase also explains previous observations that desferrioxamine, an iron chelator, can activate HIF-1alpha as it inhibits prolyl hydroxylase activity, leading to the stabilisation of HIF-1alpha. There are at least three prolyl hydroxylases that modify HIF [79,80] and further research is required to determine the expression pattern, mechanisms of regulation, and role of this enzyme in cancer pathogenesis.

Expression profiling studies have highlighted many pathways that are regulated by hypoxia and by HIF-1alpha [81–84]. Pathways regulated by HIF include angiogenesis and vasodilatation, by activation of the transcription of the gene encoding VEGF and one of its receptors, VEGF receptor 1 (VEGFR1/FLT-1) This is one of the most important pathways regulated by hypoxia and HIF and may explain the association of activation of many transforming genes with the regulation of angiogenesis, since they can activate the VEGF promoter as well as interact with HIF. There is a wide range of other molecules involved in angiogenesis that are upregulated, e.g. adrenomedullin, endothelin-1,2, VEGFR1, nitric oxide synthase, placenta growth factor, transforming growth factor alpha, angiopoietin-2, Tie2, cyclooxygenase-2, histone deacetylase, hepatocyte growth factor, osteopontin, TGF beta1 and beta3, FGF3, monocyte chemotactic protein 1, basic FGF. Hypoxia can reciprocally downregulate anti-angiogenic genes such as thrombospondin 1 [85].

Apart from the direct link between HIF-1alpha and VHL, many growth factor signalling pathways also positively regulate HIF-1alpha (and probably HIF-2alpha (Fig. 1). These include pathways involving wild-type and mutated EGF [86], insulin growth factor (IGF1), the breast cancer-associated tyrosine kinase receptor HER2 [87,88]. The activation of p42/p44 MAP kinases, which phosphorylate HIF-1alpha leads to VEGF transcription. This pathway also activates HIF-2. It is likely that a large number of growth factors can activate the HIF response. Mutations in the tumour suppressor, PTEN, enhance HIF responses [89]. There are several reports on the interactions of p53 with HIF-1alpha; wild-type p53 blocking the effects of HIF, HIF stabilising p53 [90] and dephosphorylated HIF mediating the apoptotic effects of p53 [91]. Thus, oncogenes synergise with hypoxia on several levels to enhance the angiogenic pathways regulated by HIF.

3. Clinico-pathological studies of tumour angiogenesis

It is nearly a decade since the first clinical studies measuring microvessel density histologically in breast cancer were correlated with prognosis. Since then, there have been hundreds of studies analysing nearly all major groups of cancer, including sarcomas, haematological, central nervous system (CNS) and paediatric malignancies. In general, with suitably large studies, an association of high microvessel density with poor prognosis and aggressive tumour behaviour is reported (reviewed by Fox and colleagues in Ref. [92]).

However, such counts of stained blood vessels are open to various artefacts and a detailed evaluation of these problems and approach to scoring, staining and analysis is suggested [93,94]. The most important variables relate to tumour heterogeneity, quality of staining and experience of the pathologist.

Recently, more detailed evaluation of the vasculature, as new pathways have been elucidated, have shown the importance of subgroups of blood vessels in tumour behaviour. For example, assessing the number of blood vessels with VEGF bound to endothelial surface receptors, rather than the total number of vessels, provided a much better prediction of tumour behaviour [95]. Similar results were reported for the expression of CD105, endoglin, a TGF beta receptor [96,97].

Analysis of vascular differentiation, as assessed by pericyte recruitment, has shown substantial variability from one case to another with tumour type and also generic differences from one tumour to another. Eberhard and colleagues have measured the pericyte coverage index of microvessels in six different types of malignant human tumours [98], by simultaneous staining of endothelial cells (anti-CD34 or anti-von Willebrand factor (vWF)) and mural cells (anti-alpha-smooth muscle actin. The fraction of microvessels covered by pericytes ranged from 10% (median value) in glioblastomas to approximately 70% in colon carcinomas. Blood vessel remodelling, as has been shown in the postnatal vasculature of the retina [98], is only possible in the absence of a pericyte coating. This observation has important implications for anti-angiogenic treatment. Benjamin and colleagues [99] have shown that androgen ablation therapy of prostate tumours, leading to downregulation of VEGF within the tumour, induces selective regression of microvessels that were not covered by pericytes. Blood vessel maturation parameters might predict the efficacy of an anti-VEGF treatment in reducing the tumour mass in individual patients.

Serial biopsies may be a way to analyse the effects of anti-angiogenic drugs on tumour vessels, using markers of proliferation, apoptosis and differentiation [100]. In addition to comparing pretreatment and posttreatment microvessel density, the fraction of apoptotic endothelial cells directly reflects the effect of treatment on

endothelial cell survival. Apoptotic endothelial cells can be detected by combining immunohistochemistry for CD31 with the TUNEL method.

However, there is substantial tumour heterogeneity, although magnetic resonance imaging (MRI) or ultrasound-guided biopsy could target vascular areas. Of more concern is the possibility that the tumour shrinks to fit the reduced vasculature with no change in the overall vascular density, as shown in some experimental models using drugs that are currently in clinical trials [101].

As a substitute for measuring vessel density, VEGF has been measured in tumours by enzyme-linked immunosorbent assay (ELISA) or immunohistochemistry, or in the plasma and serum. Studies on primary tumours e.g. breast cancer, have shown that high VEGF expression is associated with a poor prognosis. Results for plasma and serum are more complex because platelets are a major source of VEGF which they are released in coagulation and serum VEGF is a reflection of the platelet count [102]. Nevertheless, high platelet counts are associated with a poor outcome and may contribute to tumour angiogenesis by releasing VEGF locally [103].

Overall, the clinical pathological data supports the clinical importance of angiogenesis as a major anti-cancer target.

Table 2
Anti-angiogenic drugs in trials

1. Inhibitors of matrix metalloproteases
Solimastat (BB 3644)
BAY12-9566
COL-3
CGS 27023A
AG3340
Neovastat
BMS 275291
2. Blockers of angiogenic growth factors
Anti-VEGF antibody
SU5416
SU6668
Interferon alpha
PTK 787/ZK 22584
Angiozyme
3. Inhibitors of endothelial cell migration and proliferation
Endostatin
Angiostatin
Squalamine
TNP-70
Thalidomide
2 Methoxyoestradiol
4. Blockers of endothelial cell surface proteins, e.g. integrins
Vitaxin
EMD 121974
CM 101/ZD0101
5. Copper chelating agents
Penicillamine
Tetrathiomolybdate
Captopril
6. Angiogenic blockers by unknown/unique/multiple mechanisms
CAI
Interleukin 12
IM862
7. Drugs with other main mechanisms of action
Conventional chemotherapy drugs
Antimetabolites (methotrexate)
Alkylating agents (cyclophosphamide)
Taxanes
Camptothecins and their analogues (topotecan, CPT-11)
Anthracyclines
Vinca alkaloids
Signal transduction inhibitors
Herceptin
Ras farnesyl transferase inhibitors
Hormonal ablation
Tamoxifen
Zoladex/antiandrogens

4. Anti-angiogenic drugs in clinical trials

There are many potential targets for anti-angiogenic drugs and these are highlighted in Fig. 1 and Tables 1 and 2. It is important to distinguish mechanistically between anti-angiogenesis agents and vascular targeting drugs because of the different ways of assessing them and different expected interactions with other agents.

Anti-angiogenic drugs inhibit the growth of new vessels and vascular sprouting and may not cause regression of the differentiated vasculature. The biological effects may be very dependent on tumour size, being more active on smaller tumours with a higher proportion of developing blood vessels. Biological effects may take days or weeks. Tumour regression may occur without necrosis.

In contrast, vascular targeting implies the acute shutdown of most of the tumour vasculature; with an immediate reduction in blood flow, often leading to infarction or tumour necrosis [104]. However, because oxygen can diffuse from adjacent normal tissues, a viable outer rim will survive. It can be seen that these approaches are potentially complementary. Both can synergise with conventional or novel therapies that target proliferation for antitumour effects. This is the way they are most likely to be used in the future.

Gene therapy provides another generic approach including targeting to vessels, tissue specific promoters or simply expressing anti-angiogenic proteins systemically that are difficult to synthesise otherwise [105,106]. Promoters switched on by hypoxia may provide a generic way to activate transcription [107].

The use of conventional chemotherapy drugs in prolonged frequent schedules may have selective effects against endothelial cells and is termed metronomic therapy [108–110].

Besides small molecules, antibodies are also showing activity when targeted against angiogenic growth factors or antigens upregulated on tumour vessels such as integrin alpha v beta3 and beta 5 [111,112]. VEGF remains a major target and blocking it can also block responses to other angiogenic factors [113].

Although it may seem counter-intuitive, anti-angiogenic drugs also potentiate radiotherapy, possibly by decreasing abnormal blood supply and allowing better perfusion, or reducing survival factors for tumours produced by the endothelium, as well as reducing repopulation of the tumour between doses of radiation [114].

Improvements in imaging, particularly dynamic MRI, but also specifically labelled agents that can target the vasculature, are becoming increasingly applied in clinical trials. MRI contrast labelled peptides or antibodies to detect a_vb_3 integrins, VEGF antibodies and positron emission tomography (PET) [115]or MRI scanning [116–119] to measure blood flow are investigational approaches. Labelled anti-angiogenic angiostatin has been used recently [120]. The problems are that the techniques to assess the anti-angiogenic drugs are themselves not validated and need to be shown to correlate with an anti-angiogenic effect.

Because of the low response rate of anti-angiogenic drugs as single agents and their synergy in preclinical models with other modalities [121], the aim in most drug development is to proceed to randomised phase III trials combining anti-angiogenic therapy with an optimum first-line regimen for metastatic disease [122].

5. Problems for anti-angiogenic therapy

Although some encouraging results are now being reported, there are specific problems in developing a routine anti-angiogenic therapy for all cancers. Some have already been described above, e.g. multiple angiogenic pathways and variable differentiation of blood vessels.

There is evidence that different angiogenic pathways may be involved at early and later stages of tumour growth, for example in breast cancer basic FGF expression is lower in larger tumours [123]. In a preclinical model of pancreatic endocrine cancer, four different anti-angiogenic drugs were tested at different stages of progression from premalignant to cancer, early cancer growth and growth and regression of larger masses [101]. The drugs included a metalloprotease inhibitor, AG470, endostatin and angiostatin. No one drug worked at of the all the stages; some were better for early and some for later stages. This implies differences in mechanisms of angiogenesis at each stage and may be relevant for extrapolation of anti-angiogenic therapy to adjuvant treatment—i.e. would the mechanisms be the same?

Non-angiogenic deposits are also becoming recognised in primary lung cancers [124] and lung secondaries from breast cancers [125], brain, liver and lymph nodes [126]. In primary non-small cell lung carcinomas, a novel growth pattern was observed. In the 'alveolar' growth pattern, tumour cells filled the alveolar spaces, entrapping, but not destroying, the alveolar septa with the co-opted blood vessels. In the tumour cell nests, no associated desmoplastic stroma or new blood vessels were present. This putatively non-angiogenic growth pattern was also observed in lung metastases from breast cancer. The lung tumours expressing this growth pattern were more often poorly differentiated than tumours expressing other growth patterns.

Holash and colleagues [127] have studied the early vascularisation of gliomas in a rat model. Although the prevailing view was that malignancies and metastases initially reside as small avascular masses, 1 week after implantation of tumour cells, well-vascularised tumours were observed in their model. The tumour cells had co-opted the blood vessels of the surrounding brain tissue. There was no angiogenic response in the tumours. As a consequence of tumour growth in the absence of angiogenesis, the centre of the tumours became hypovascular. Later on, frank vessel regression led to tumour cell death. This tumour cell loss and hypoxia resulted in intense angiogenesis at the tumour margins.

More recently, changing paradigms on mechanisms of action of targets have modified the interpretation of results and plans for certain types of anti-angiogenic drugs. For example, metalloprotease inhibitors are clearly anti-angiogenic in preclinical models, but in clinical trials have not yet yielded similar results [128]. After they were developed, it was recognised that proteases may generate anti-angiogenic molecules such as angiostatin [43,129], hence inhibiting them may provide variable results, depending on which pathways predominate in an individual tumour and the design of the inhibitor.

Resistance to anti-angiogenic drugs can occur, as shown by the elegant experiments of Bouck [130]. In these experiments, tumour cell lines were engineered to express the angiogenesis inhibitor, thrombospondin 1. Tumours, that grew in spite of this although they were more slowly growing than in the controls, when re-implanted, grew at the control rate despite still expressing the anti-angiogenic factor at the same level. These tumours were producing more angiogenic factors such as VEGF or changed their responsiveness to other inhibitory factors such as TGF beta.

6. Conclusions

Anti-angiogenic therapy is beginning to come to fruition, but not with as dramatic results as has been shown in the preclinical models. Nevertheless, clear responses

and regressions and stabilisation of rapidly progressive diseases with changes in vascular permeability are being reported and phase III trials are underway. Clinical trials co-ordinators and companies have learned rapidly over the last few years how to address the problems and it is likely that drugs, particularly VEGF, endothelin antagonists and thrombospondin mimics [131], will find a routine place in therapy.

References

1. Folkman J. What is the evidence that tumours are angiogenesis dependent? *J Natl Cancer Inst* 1990, **82**, 4–6.
2. Ferrara N. Vascular endothelial growth factor and the regulation of angiogenesis. *Recent Prog Horm Res* 2000, **55**, 15–36.
3. Boehm T, Folkman J, Browder T, Oreilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug-resistance. *Nature* 1997, **390**, 404–407.
4. Ellis LM, Liu WB, Ahmad SA, *et al*. Overview of angiogenesis: biologic implications for antiangiogenic therapy. *Semin Oncol* 2001, **28**, 94–104.
5. Webb CP, Woude GFV. Genes that regulate metastasis and angiogenesis. *J Neurooncol* 2000, **50**, 71–87.
6. Maio M, Altomonte M, Calabro L, Fonsatti E. Bioimmunotherapeutic targets on angiogenetic blood vessels in solid malignangies. *Front Biosci* 2001, **6**, D776–D784.
7. Robert EG, Hunt JD. Lipid messengers as targets for anti-angiogenic therapy. *Curr Pharm Des* 2001, **7**, 1615–1626.
8. Eatock MM, Schatzlein A, Kaye SB. Tumour vasculature as a target for anticancer therapy. *Cancer Treat Rev* 2000, **26**, 191–204.
9. Sato Y. Molecular mechanism of angiogenesis—transcription factors and their therapeutic relevance. *Pharmacol Ther* 2000, **87**, 51–60.
10. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J* 1999, **13**, 9–22.
11. Hamby JM, Showalter HDH. Small molecule inhibitors of tumor-promoted angiogenesis, including protein tyrosine kinase inhibitors. *Pharmacol Ther* 1999, **82**, 169–193.
12. Mousa SA. Anti-integrins as a potential therapeutic target in angiogenesis. *Expert Opin Ther Pat* 1999, **9**, 1237–1248.
13. Geng L, Donnelly E, McMahon G, Lin PC, *et al*. Inhibition of vascular endothelial growth factor receptor signaling leads to reversal of tumor resistance to radiotherapy. *Cancer Res* 2001, **61**, 2413–2419.
14. Ohm JE, Carbone DP. VEGF as a mediator of tumor-associated immunodeficiency. *Immunol Res* 2001, **23**, 263–272.
15. Ambartsumian N, Klingelhofer J, Grigorian M, *et al*. The metastasis-associated Mts1(S100A4) protein could act as an angiogenic factor. *Oncogene* 2001, **20**, 4685–4695.
16. Browder T, Folkman J, PirieShepherd S. The hemostatic system as a regulator of angiogenesis. *J Biol Chem* 2000, **275**, 1521–1524.
17. Cao RH, Brakenhielm E, Wahlestedt C, Thyberg J, Cao YH. Leptin induces vascular permeability and synergistically stimulates angiogenesis with FGF-2 and VEGF. *Proc Natl Acad Sci USA* 2000, **98**, 6390–6395.
18. Funasaka T, Haga A, Raz A, Nagase H. Tumor autocrine motility factor is an angiogenic factor that stimulates endothelial cell motility. *Biochem Biophys Res Commun* 2001, **284**, 1116–1125.
19. Hu YL, Tee MK, Goetzl EJ, *et al*. Lysophosphatidic acid induction of vascular endothelial growth factor expression in human ovarian cancer cells. *J Natl Cancer Inst* 2001, **93**, 762–768.
20. Pola R, Ling LE, Silver M, *et al*. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med* 2001, **7**, 706–711.
21. Shimo T, Kubota S, Kondo S, *et al*. Connective tissue growth factor as a major angiogenic agent that is induced by hypoxia in a human breast cancer cell line. *Cancer Lett* 2001, **174**, 57–64.
22. Vikhanskaya F, Bani MR, Borsotti P, *et al*. p73 overexpression increases VEGF and reduces thrombospondin-1 production: implications for tumor angiogenesis. *Oncogene* 2001, **20**, 7293–7300.
23. Liu CY, Paradis H, Gendron R, *et al*. AngX/CDT-6, a novel factor that enhances angiogenesis. *Mol Biol Cell* 2000, **11**, 66A.
24. Marneros AG, Olsen BR. The role of collagen-derived proteolytic fragments in angiogenesis. *Matrix Biol* 2001, **20**, 337–345.
25. NangiaMakker P, Honjo Y, Sarvis R, *et al*. Galectin-3 induces endothetial cell morphogenesis and angiogenesis. *Am J Pathol* 2000, **156**, 899–909.
26. Talks KL, Harris AL. Current status of antiangiogenic factors. *Br J Haematol* 2000, **109**, 477–489.
27. Jones N, Iljin K, Dumont DJ, Alitalo K. Tie receptors: New modulators of angiogenic and lymphangiogenic responses. *Nat Rev Mol Cell Biol* 2001, **2**, 257–267.
28. Powers CJ, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. *Endocr-Relat Cancer* 2000, **7**, 165–197.
29. LeCouter J, Kowalski J, Foster J, *et al*. Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature* 2001, **412**, 877–884.
30. Lee MS, Moon EJ, Lee SW, Kim HS, Kim KW, Kim YJ. Angiogenic activity of pyruvic acid in in vivo and in vitro angiogenesis models. *Cancer Res* 2001, **61**, 3290–3293.
31. Lawler J, Miao WM, Duquette M, Bouck N, Bronson RT, Hynes RO. Thrombospondin-1 gene expression affects survival and tumor spectrum of p53-deficient mice. *Am J Pathol* 2001, **159**, 1949–1956.
32. O'Reilly MS, Boehm T, Shing Y, *et al*. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997, **88**, 277–285.
33. O'Reilly MS, Holmgren L, Shing Y, *et al*. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994, **79**, 315–328.
34. de F-F, Nicholson AC, Feige JJ, Van MEG. Thrombospondins and tumor angiogenesis. *Trends Mol Med* 2001, **7**, 401–407.
35. Kim MS, Kwon HJ, Lee YM, *et al*. Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat Med* 2001, **7**, 437–443.
36. Larsson H, Akerud P, Nordling K, Raub SE, Claesson WL, Bjork I. A novel anti-angiogenic form of antithrombin with retained proteinase binding ability and heparin affinity. *J Biol Chem* 2001, **276**, 11996–12002.
37. Schwarte-Waldhoff I, Volpert OV, Bouck NP, *et al*. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc Natl Acad Sci USA* 2000, **97**, 9624–9629.
38. Glienke J, Fenten G, Seemann M, Sturz A, Thierauch KH. Human SPRY2 inhibits FGF2 signalling by a secreted factor. *Mech Dev* 2000, **96**, 91–99.
39. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996, **86**, 353–364.
40. Holmgren L, Oreilly MS, Folkman J. Dormancy of micrometastases- balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat Med* 1995, **1**, 149–153.
41. Sage EH. Pieces of 8- bioactive fragments of extracellular proteins as regulators of angiogenesis. *Trends Cell Biol* 1997, **7**, 182–186.

42. Troyanovsky B, Levchenko T, Mansson G, Matvijenko O, Holmgren L. Angiomotin: an angiostatin binding protein that regulates endothelial cell migration and tube formation. *J Cell Biol* 2001, **152**, 1247–1254.
43. Gately S, Twardowski P, Stack MS, *et al*. The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. *Proc Natl Acad Sci USA* 1997, **94**, 10868–10872.
44. Relf M, Lejeune S, Scott PAE, *et al*. Expression of the angiogenic factors vascular endothelial-cell growth-factor, acidic and basic fibroblast growth-factor, tumor-growth factor-beta-1, platelet-derived endothelial-cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. *Cancer Res* 1997, **57**, 963–969.
45. Rak J, Yu JL, Klement G, Kerbel RS. Oncogenes and angiogenesis: signaling three-dimensional tumor growth. *J Invest Dermatol Symp Proc* 2000, **5**, 24–33.
46. Rak J, Mitsuhashi Y, Sheehan C, *et al*. Oncogenes and tumor angiogenesis: differential modes of vascular endothelial growth factor up-regulation in ras-transformed epithelial cells and fibroblasts. *Cancer Res* 2000, **60**, 490–498.
47. Wen SH, Stolarov J, Myers MP, *et al*. PTEN controls tumor-induced angiogenesis. *Proc Natl Acad Sci USA* 2001, **98**, 4622–4627.
48. Ciardiello F, Bianco R, Damiano V, *et al*. Antiangiogenic and antitumor activity of anti-epidermal growth factor receptor C225 monoclonal antibody in combination with vascular endothelial growth factor antisense oligonucleotide in human GEO colon cancer cells. *Clin Cancer Res* 2000, **6**, 3739–3747.
49. Kumar R, Yarmand BR. The role of HER2 in angiogenesis. *Semin Oncol* 2001, **28**, 27–32.
50. Sanchez ET, Botella LM, Velasco B, Corbi A, Attisano L, Bernabeu C. Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. *J Biol Chem* 2001, **276**, 38527–38535.
51. Rafii S. Circulating endothelial precursors: mystery, reality, and promise. *J Clin Invest* 2000, **105**, 17–19.
52. Asahara T, Masuda H, Takahashi T, *et al*. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 1999, **85**, 221–228.
53. Shi Q, Rafii S, Wu MH, *et al*. Evidence for circulating bone marrow-derived endothelial cells. *Blood* 1998, **92**, 362–367.
54. Lin Y, Weisdorf DJ, Solovey A, Hebbel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest* 2000, **105**, 71–77.
55. Davidoff AM, Ng CYC, Brown P, *et al*. Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice. *Clin Cancer Res* 2001, **7**, 2870–2879.
56. Shintani S, Murohara T, Ikeda H, *et al*. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 2001, **103**, 2776–2779.
57. Ito H, Rovira II, Bloom ML, *et al*. Endothelial progenitor cells as putative targets for angiostatin. *Cancer Res* 1999, **59**, 5875–5877.
58. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood* 2001, **97**, 3658–3661.
59. Djonov V, Schmid M, Tschanz SA, Burri PH. Intussusceptive angiogenesis—its role in embryonic vascular network formation. *Circ Res* 2000, **86**, 286–292.
60. Folberg R, Hendrix MJC, Maniotis AJ. Vasculogenic mimicry and tumor angiogenesis. *Am J Pathol* 2000, **156**, 361–381.
61. Benezra R. Role of Id proteins in embryonic and tumor angiogenesis. *Trends Cardiovasc Med* 2001, **11**, 237–241.
62. Myers C, Charboneau A, Boudreau N. Homeobox B3 promotes capillary morphogenesis and angiogenesis. *J Cell Biol* 2000, **148**, 343–351.
63. Villa N, Walker L, Lindsell CE, Gasson J, Iruela-Arispe ML, Weinmaster G. Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mech Dev* 2001, **108**, 161–164.
64. Cheng N, Brantley DM, Chen J. The ephrins and Eph receptors in angiogenesis. *Cytokine Growth Factor Rev* 2002, **13**, 75–85.
65. Adams RH, Klein R. Eph receptors and ephrin ligands. essential mediators of vascular development. *Trends Cardiovasc Med* 2000, **10**, 183–188.
66. Carson WEB, Watkins DN, Nanda A, Vogelstein B, Kinzler KW, Croix BS. Cell surface tumor endothelial markers are conserved in mice and humans. *Cancer Res* 2001, **61**, 6649–6655.
67. Huminiecki L, Bicknell R. In silico cloning of novel endothelial-specific genes. *Genome Res* 2000, **10**, 1796–1806.
68. Giordano FJ, Johnson RS. Angiogenesis: the role of the microenvironment in flipping the switch. *Curr Opin Genet Dev* 2001, **11**, 35–40.
69. Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* 2001, **93**, 266–276.
70. Ohh M, Park CW, Ivan M, *et al*. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol* 2000, **2**, 423–427.
71. Kamura T, Sato S, Iwai K, CzyzykKrzeska M, Conaway RC, Conaway JW. Activation of HIF1 alpha ubiquitination by a reconstituted von Hippel-Lindau (VHL) tumor suppressor complex. *Proc Natl Acad Sci USA* 2000, **97**, 10430–10435.
72. Maxwell PH, Wiesener MS, Chang GW, *et al*. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 1999, **399**, 271–275.
73. Stebbins CE, Kaelin Jr WG, Pavletich NP. Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. *Science* 1999, **284**, 455–461.
74. Talks KL, Turley H, Gatter KC, *et al*. The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *Am J Pathol* 2000, **157**, 411–421.
75. Zhong H, De Marzo AM, Laughner E, *et al*. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* 1999, **59**, 5830–5835.
76. Bos R, Zhong H, Hanrahan CF, *et al*. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. *J Natl Cancer Inst* 2001, **93**, 309–314.
77. Jaakkola P, Mole DR, Tian YM, *et al*. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* 2001, **292**, 468–472.
78. Ivan M, Kondo K, Yang H, *et al*. HIF{alpha} targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* 2001, **292**(5516), 449–451.
79. Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 2001, **294**(5545), 1337–1340.
80. Epstein AC, Gleadle JM, McNeill LA, *et al*. Elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 2001, **107**, 43–54.
81. Denko N, Schindler C, Koong A, Laderoute K, Green C, Giaccia A. Epigenetic regulation of gene expression in cervical cancer cells by the tumor microenvironment. *Clin Cancer Res* 2000, **6**, 480–487.
82. Koong AC, Denko NC, Hudson KM, *et al*. Candidate genes for the hypoxic tumor phenotype. *Cancer Res* 2000, **60**, 883–887.
83. Wykoff CC, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. Identification of novel hypoxia dependent and independent target genes of the von Hippel-Lindau (VHL) tumour suppressor

by mRNA differential expression profiling. *Oncogene* 2000, **19**, 6297–6305.
84. Lal A, Peters H, St Croix B, *et al*. Transcriptional response to hypoxia in human tumors. *J Natl Cancer Inst* 2001, **93**, 1337–1343.
85. Laderoute KR, Alarcon RM, Brody MD, *et al*. Opposing effects of hypoxia on expression of the angiogenic inhibitor thrombospondin 1 and the angiogenic inducer vascular endothelial growth factor. *Clin Cancer Res* 2000, **6**, 2941–2950.
86. Clarke K, Smith K, Gullick WJ, Harris AL. Mutant epidermal growth factor receptor enhances induction of vascular endothelial growth factor by hypoxia and insulin-like growth factor-1 via a PI3 kinase dependent pathway. *Br J Cancer* 2001, **84**, 1322–1329.
87. Zhong H, Chiles K, Feldser D, *et al*. Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* 2000, **60**, 1541–1545.
88. Blancher C, Moore JW, Talks KL, Houlbrook S, Harris AL. Relationship of hypoxia-inducible factor (HIF)-1α and HIF-2α expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines. *Cancer Res* 2000, **60**, 7106–7113.
89. Zundel W, Schindler C, Haas-Kogan D, *et al*. Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev* 2000, **14**, 391–396.
90. An WG, Kanekal M, Simon MC, Maltepe E, Blagosklonny MV, Neckers LM. Stabilization of wild-type p53 by hypoxia-inducible factor 1-alpha. *Nature* 1998, **392**, 405–408.
91. Suzuki H, Tomida A, Tsuruo T. Dephosphorylated hypoxia-inducible factor 1alpha as a mediator of p53-dependent apoptosis during hypoxia. *Oncogene* 2001, **20**, 5779–5788.
92. Fox SB, Gasparini G, Harris AL. Angiogenesis: pathological prognostic and growth-factor pathways and their link to trial design and anticancer drugs. *Lancet Oncol* 2001, **2**, 278–289.
93. Vermeulen PB, Gasparini G, Fox SB, *et al*. Second international concensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. *Eur J Cancer* 2002, **38**(12), 1564–1579.
94. Fox SB, Harris AL. Markers of tumor angiogenesis- clinical-applications in prognosis and anti-angiogenic therapy. *Invest New Drugs* 1997, **15**, 15–28.
95. Koukourakis MI, Giatromanolaki A, Thorpe PE, *et al*. Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvessel density in non-small cell lung cancer. *Cancer Res* 2000, **60**, 3088–3095.
96. Miller DW, Graulich W, Karges B, *et al*. Elevated expression of endoglin, a component of the TGF-beta-receptor complex, correlates with proliferation of tumor endothelial cells. *Int J Cancer* 1999, **81**, 568–572.
97. Kumar S, Ghellal A, Li C, Byrne G, Haboubi N, Wang JM, Bundred N. Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. *Cancer Res,* 1999, **59**, 856–861.
98. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* 1999, **125**, 1591–1598.
99. Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 1999, **103**, 159–165.
100. Mundhenke C, Thomas JP, Wilding G, *et al*. Tissue examination to monitor antiangiogenic therapy: a phase I clinical trial with endostatin. *Clin Cancer Res* 2001, **7**, 3366–3374.
101. Bergers G, Javaherian K, Lo KM, Folkman J, Hanahan D. Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. *Science* 1999, **284**, 808–812.
102. Jelkmann W. Pitfalls in the measurement of circulating vascular endothelial growth factor. *Clin Chem* 2001, **47**, 617–623.
103. Pinedo HM, Verheul HM, D'Amato RJ, Folkman J. Involvement of platelets in tumour angiogenesis? *Lancet* 1998, **352**, 1775–1777.
104. Tozer GM, Prise VE, Wilson J, *et al*. Combretastatin A-4 phosphate as a tumor vascular-targeting agent: early effects in tumors and normal tissues. *Cancer Res* 1999, **59**, 1626–1634.
105. Chen QR, Zhang L, Gasper W, Mixson AJ. Targeting tumor angiogenesis with gene therapy. *Mol Genet Metab* 2001, **74**, 120–127.
106. Varda BN, Shaish A, Gonen A, *et al*. Tissue-specific gene therapy directed to tumor angiogenesis. *Gene Ther* 2001, **8**, 819–827.
107. Dachs GU, Patterson AV, Firth JD, *et al*. Targeting gene-expression to hypoxic tumor-cells. *Nat Med* 1997, **3**, 515–520.
108. Browder T, Butterfield CE, Kraling BM, *et al*. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* 2000, **60**, 1878–1886.
109. Gately S, Kerbel R. Antiangiogenic scheduling of lower dose cancer chemotherapy. *Cancer J* 2001, **7**, 427–436.
110. Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, Bohlen P, Kerbel RS. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* 2000, **105**, R15–R24.
111. Halin C, Neri D. Antibody-based targeting of angiogenesis. *Crit Rev Ther Drug Carrier Syst* 2001, **18**, 299–339.
112. Hicklin DJ, Witte L, Zhu ZP, *et al*. Monoclonal antibody strategies to block angiogenesis. *Drug Discov Today* 2001, **6**, 517–528.
113. Tille JC, Wood J, Mandriota SJ, *et al*. Vascular Endothelial growth factor (VEGF) receptor-2 antagonists inhibit VEGF- and basic fibroblast growth gactor-induced angiogenesis in vivo and in vitro. *J Pharmacol Exp Ther* 2001, **299**, 1073–1085.
114. Dicker AP, Williams TL, Grant DS. Targeting angiogenic processes by combination rofecoxib and ionizing radiation. *Am J Clin Oncol* 2001, **24**, 438–442.
115. Weber WA, Haubner R, Vabuliene E, *et al*. Tumor angiogenesis targeting using imaging agents. *Q J Nucl Med* 2001, **45**, 179–182.
116. Dennie J, Mandeville JB, Boxerman JL, *et al*. Nmr imaging of changes in vascular morphology due to tumor angiogenesis. *Magn Reson Med* 1998, **40**, 793–799.
117. Pham CD, Roberts TPL, Vanbruggen N, Melnyk O, *et al*. Magnetic-resonance-imaging detects suppression of tumor vascular-permeability after administration of antibody to vascular endothelial growth-factor. *Cancer Invest* 1998, **16**, 225–230.
118. Sipkins DA, Cheresh DA, Kazemi MR, Nevin LM, Bednarski MD, Li KCP. Detection of tumor angiogenesis in-vivo by alpha(v)beta(3)-targeted magnetic-resonance-imaging. *Nat Med* 1998, **4**, 623–626.
119. Vanzijl PCM, Eleff SM, Ulatowski JA, *et al*. Quantitative assessment of blood-flow, blood-volume and blood oxygenation effects in functional magnetic-resonance-imaging. *Nat Med* 1998, **4**, 159–167.
120. Lee KH, Lee SY, Paik JY, *et al*. Tumor imaging with I-123 labeled angiostatin, a novel anti-angiogenesis targeted radiopharmaceutical. *J Nucl Med* 2001, **42**, 253P.
121. Teicher BA, Holden SA, Ara G, *et al*. Potentiation of cytotoxic cancer therapies by tnp-470 alone and with other anti-angiogenic agents. *Int J Cancer* 1994, **57**, 920–925.
122. Burke PA, DeNardo SJ. Antiangiogenic agents and their promising potential in combined therapy. *Crit Rev Oncol Hematol* 2001, **39**, 155–171.
123. Smith K, Fox SB, Whitehouse R, Taylor M, *et al*. Upregulation of basic fibroblast growth factor in breast carcinoma and its relationship to vascular density, oestrogen receptor, epidermal

growth factor receptor and survival. *Ann Oncol* 1999, **10**, 707–713.
124. Pezzella F, Pastorino U, Tagliabue E, *et al*. Non-small-cell-lung carcinoma tumor-growth without morphological evidence of neo-angiogenesis. *Am J Pathol* 1997, **151**, 1417–1423.
125. Pezzella F, Manzotti M, DeBAcco A, *et al*. Evidence for novel non-angiogenic pathway in breast cancer metastasis. *Lancet* 2000, **355**, 1787–1788.
126. Naresh KN, Nerurkar AY, Borges AM. Angiogenesis is redundant for tumour growth in lymph node metastases. *Histopathology* 2001, **38**, 466–470.
127. Holash J, Wiegand SJ, Yancopoulos GD. New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* 1999, **18**, 5356–5362.
128. Raza SL, Cornelius LA. Matrix metalloproteinases: pro- and anti-angiogenic activities. *J Invest Dermatol Symp Proc* 2000, **5**, 47–54.
129. O'Reilly MS, Wiederschain D, Stetler-Stevenson WG, Folkman J, Moses MA. Regulation of angiostatin production by matrix metalloproteinase-2 in a model of concomitant resistance. *J Biol Chem* 1999, **274**, 29568–29571.
130. Filleur S, Volpert OV, Degeorges A, *et al*. In vivo mechanisms by which tumors producing thrombospondin 1 bypass its inhibitory effects. *Genes Dev* 2001, **15**, 1373–1382.
131. Dawson DW, Volpert OV, Pearce SF, *et al*. Three distinct D-amino acid substitutions confer potent antiangiogenic activity on an inactive peptide derived from a thrombospondin-1 type 1 repeat. *Mol Pharmacol* 1999, **55**, 332–338.