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Review Article

Concept, mechanisms and therapeutics of angiogenesis in cancer and other diseases

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Abstract

Angiogenesis supports normal physiology as well as contributing to the progression of various diseases including cancer. Determination of the key role of angiogenesis in cancer has led to much optimism for the development of targeted drugs without cytotoxic side-effects. Currently, research in angiogenesis therapy is robust, with the discovery of a growing number of pro- and anti-angiogenic molecules. More time, however, is required to be able to elucidate the complex interactions among these molecules, how they affect vasculature and their functions in different environments. As we learn more about the molecular mechanisms of angiogenesis, a number of effective methods to treat cancer and other diseases will be developed.

Introduction

Angiogenesis is the process by which new blood vessels are formed and grow from existing, quiescent vascular endothelium. The genesis of new blood vessels by either angiogenesis or vasculogenesis is an essential step in embryonic development (Lyden 2001). However, shifts in the finely balanced equilibrium between pro-angiogenic and anti-angiogenic factors that normally regulate angiogenesis are linked to the aetiology of many pathologies, such as rheumatoid arthritis, diabetic retinopathy, age-related macular degeneration and cancer (Featherstone & Griffiths 2002). In this review, we discuss how normal and abnormal blood vessel form, how they function, what key molecules are involved and how they are used for therapy.

Tumour angiogenesis

Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100–200 μm of blood vessels, the diffusion limit for oxygen. For multicellular organisms to grow beyond this size, they must recruit new blood vessels. Similarly, without blood vessels, tumours cannot grow beyond a critical size or metastasize to another organ (Carmeliet & Jain 2000). Accumulating evidence demonstrates that tumour growth and lethality are dependent on angiogenesis (Folkman 1999). For example, when angiogenesis is inhibited by the administration of molecules that specifically suppress the growth of vascular endothelial cells, tumours in animals can be limited to a dormant microscopic size at which they are essentially harmless. Clinical applications will therefore include the administration of an angiogenesis inhibitor specific for the vascular endothelium in the tumour bed, optimization of the dose and a schedule of conventional cytotoxic chemotherapy for the vascular endothelium, and even the targeting of low-dose cytotoxic chemotherapy only on the vascular endothelium in the tumour bed (Folkman 1999). It has been shown that cells in pre-cancerous tissue acquire angiogenic capacity on their way to becoming cancerous, and it has been proposed that this concept be used to design strategies to prevent cancer (Gullino 1978).

Various signals that trigger the angiogenic switch include metabolic stress (e.g. low pO_2, low pH or hypoglycaemia), mechanical stress (e.g. pressure generated by proliferating cells), immune/inflammatory response (e.g. immune/inflammatory cells that have infiltrated the tissue), and genetic mutations (e.g. activation of oncogenes or

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deletion of tumour-suppressor genes that control production of angiogenesis regulators) (Carmeliet 1999).

Formation of tumour vessels
Tumour vessels develop by sprouting or intussusception from pre-existing vessels. Circulating endothelial precursors, shed from the vessel wall or mobilized from the bone marrow, can also contribute to tumour angiogenesis (Carmeliet & Jain 2000).

Key players in the growth and maturation of vessels
Among the various molecular players involved in different mechanisms of vascular growth, members of the vascular endothelial growth factor (VEGF) and angiopoietin family have a predominant role. VEGF family members are essential in embryonic and postnatal vascular development and ischaemia-driven angiogenesis (Carmeliet 2000). All members of the family bind to cell-surface VEGF receptors to initiate a cellular response via activation of the intracellular tyrosine kinase domain. VEGFR2 (also known as FLK-1 in mice and KDR in humans) is the key receptor that triggers angiogenesis and vascular permeability (Gille et al 2001). VEGFR3 (also called Flt-4) binds VEGF-C and VEGF-D to control the growth and maintenance of lymphatic vessels. Although VEGFR3 is present on vascular endothelium, it is not clear whether it has a role in angiogenesis (Bikfalvi & Bicknell 2002). Findings such as the formation of aberrant blood vessels in the hearts of VEGF conditional-knockout mice suggest that VEGF alone is unable to direct blood vessel organization and maturation (Bikfalvi & Bicknell 2002). Other novel approaches that regulate VEGF expression are under investigation, including enzymes that generate reactive oxygen and tumour-suppressor genes (Ylikorkala et al 2001; Arbiser et al 2002).

Angiopoietin 1 (Ang-1) is another angiogenic factor that signals through the endothelial cell-specific Tie-2 receptor tyrosine kinase. A factor closely related to Ang-1, termed angiopoietin 2 (Ang-2), is a naturally occurring antagonist for Ang-1 and its Tie-2 receptor (Maisonpierre et al 1997). Ang-1 is thought to stabilize vessels by rendering them less sensitive to VEGF. Binding of Ang-2 to the Ang-1 receptor Tie-2 disrupts this stabilizing effect. Formation of mature vessels is thought to involve subtle interplay between the VEGFs, angiopoietins and platelet-derived growth factors (PDGFs) (Bikfalvi & Bicknell 2002).

Tumour angiogenesis and tissue factor Recently, the potent procoagulant tissue factor expressed in human breast cancer was shown to have implications for the interaction of neoplastic cells with vascular endothelium (Folkman 1996). The expression of tissue factor by vascular endothelial cells in a tumour bed suggests a linkage between angiogenesis regulators and components of the clotting system. For example, angiostatin, a cleavage product of plasminogen, is a potent angiogenesis inhibitor (O'Reilly et al 1994). The platelet-derived products, thrombospondin (Folkman 1996) and platelet factor 4, inhibit angiogenesis, whereas platelet-derived endothelial cell growth factor and PDGF-BB stimulate angiogenesis and endothelial cell proliferation, respectively. Angiogenesis can be potentiated in some experimental systems by heparin or fibrin. Fibrin and fibrinogen can bind to the $\alpha_v\beta_3$ receptor on endothelial cells and can inhibit endothelial cell motility (Cheresh et al 1989).

c-Myc in vasculogenesis and angiogenesis Genetic changes in cancer may also flip the angiogenic switch. For example, the oncogenes *v-src*, *c-jun* and *c-myc* suppress the anti-angiogenic factor thrombospondin 1. Furthermore transgenic studies have shown that transformation induced by several oncoproteins, including c-Myc, is sufficient to induce an angiogenic response and the expression of VEGF. Thus, Myc is essential for the proper regulation of many components of the angiogenic network, including both positive (VEGF, Ang-2) and inhibitory (thrombospondin 1) cytokines, as well as VEGF receptors themselves (Baudino et al 2002).

Angiogenesis and the level of plasminogen activator inhibitor type 1 (PAI-1) Tumour progression seems to be dependent on cancer cell controlled tissue remodelling, including angiogenesis, mediated to a large extent by the plasminogen activation system. Similarly, the urokinase-type plasminogen activator (uPA) system has been shown to play a crucial role in cancer metastasis. Various components of the uPA system, including uPA itself and its primary inhibitor, PAI-1, have demonstrated significant prognostic impact in breast cancer patients. Thus, the level of PAI-1 in primary tumours is one of the most informative biochemical prognostic markers in several cancer types. In several cell culture and animal model systems, PAI-1 has been found to have a pro-angiogenic effect. However, the clinical significance of the biological interaction of angiogenesis and the plasminogen activator system is unknown (Hansen et al 2003).

Integrin signalling and angiogenesis Among the various members of the integrin family, a family of adhesion receptors, $\alpha_v\beta_3$, has been found to play a significant role in the process of angiogenesis. Integrin $\alpha_v\beta_3$ is a promiscuous receptor in as much as it is capable of interacting with a number of extracellular matrix proteins, including fibrinogen and thrombospondin, as well as other proteins with different biological functions, including fibroblast growth factor 2 (FGF-2) and metalloproteinase 2. In addition, $\alpha_v\beta_3$ has been shown to associate with activated platelet-derived growth factor, insulin and VEGF receptors to facilitate optimal activation of cell proliferative signalling pathways (Giancotti & Ruoslahti 1999) and to prevent apoptosis (Giancotti & Ruoslahti 1999; Chandrakumar et al 2001). Integrin $\alpha_v\beta_3$ is minimally expressed on resting or normal blood vessels, but is significantly upregulated on vascular cells within human tumours or in response to certain growth factors in-vitro (Brooks et al 1994). FGF-2 and tumour necrosis factor α (TNF-α) stimulate $\alpha_v\beta_3$ expression on developing blood vessels in chick embryo chorioallantoic membrane (CAM)

(Brooks et al 1994) and on the rabbit cornea (Friedlander et al 1995a). Endothelial cells exposed to growth factors, or those undergoing angiogenesis in tumours, wounds or inflammatory tissue, express high levels of $\alpha_v\beta_3$ (Friedlander et al 1995a). In fact, recent studies suggest that $\alpha_v\beta_3$ may serve as a useful diagnostic or prognostic indicator of tumours (Sipkins et al 1998).

Copper and angiogenesis In an attempt to isolate a peptide, endothelial-stimulating growth factor, a high concentration of copper salts was noted (McAuslan & Reilly 1980). More recently, copper was found to stimulate directly the in-vitro proliferation of endothelial cells (Hu 1998). In a series of experiments at the National Cancer Institute USA, it was discovered that availability of copper in-vivo is critical to the initiation and development of angiogenesis. Furthermore, copper repletion switches angiogenesis back on when a copper-sufficient diet is restored. This mechanism could explain the inhibition of angiogenesis observed in the brains of animals by copper reduction (Brem et al 1990).

Cu/Zn superoxide dismutase plays a role in angiogenesis Hypoxia causes secretion of VEGF, which acts to increase the immediate availability of oxygen from capillaries through increased vascular permeability as well as inducing formation of new vessels (Shewiki et al 1992). Angiogenesis involves intense endothelial cell cytokine-dependent proliferation and hypoxia/re-oxygenation. Superoxide dismutase 1 (SOD-1) is a key enzyme in dismutation of the potentially toxic superoxide radicals into hydrogen peroxide and dioxygen (Fridovich 1978). Because angiogenesis is characterized by proliferating endothelial cells and re-oxygenation, there is the possibility that altered activity of SOD-1 will effect the angiogenic process. This assumption is consistent with recent findings demonstrating that the anti-angiogenic compound, 2-methoxy oestradiol (Huang et al 2000), is a SOD-1 inhibitor (Fotsis et al 1994). Recently, it was speculated that upregulation of SOD-1 will increase the ability of endothelial cells to confront an increased level of reactive oxygen species during angiogenesis, resulting in increased angiogenesis. Inhibition of SOD-1 will diminish this ability, resulting in inhibition of angiogenesis (Marikovsky et al 2002).

Cyclooxygenase-2 (COX-2) expression and angiogenesis Inflammatory mediators are also thought to contribute to the process of angiogenesis. In fact, some of the inflammatory prostaglandins, products of arachidonic acid metabolism, are pro-angiogenic (Jackson et al 1997). The cells involved in both rheumatoid arthritis and malignant tumour include fibroblasts, vascular endothelial cells, immune cells and platelets. The in-vivo processes, including hypoxia, growth factor production, cytokine-activated COX-2 induction, prostaglandin production, VEGF-mediated vascular permeability and neovascularization, are strikingly similar. The angiogenic vasculature within and adjacent to tumours expresses COX-2 in addition to the neoplastic epithelial cells of human cancers including colon (Anderson et al 1996), lung, breast, prostate, pancreatic, and head and neck squamous cell carcinoma (Sano et al 1995; Tucker et al 1999).

Prostaglandin-dependent mechanisms of angiogenesis What is the underlying mechanism that links growth-factor-induced angiogenesis to COX-2 expression? Basic fibroblast growth factor (bFGF), a growth factor produced by many tumours, can induce COX-2 messenger RNA expression and protein in several cell lines, including bone-derived endothelial cells, rat gastric mucosal epithelium and rheumatoid arthritis synovial cells (Sasaki et al 1998; Chan et al 1999; Kage et al 1999). In tumour cells, COX-2-derived prostaglandins upregulate the production of growth factors, including VEGF, which can act directly on endothelial cells, and bFGF, which stimulates COX-2 upregulation in fibroblasts. COX-2-derived prostaglandins in fibroblasts stimulate VEGF production, which acts on endothelial cells in a paracrine fashion to again upregulate COX-2 and facilitate vascular permeability and angiogenesis. Prostaglandin E2 by itself has been shown to stimulate angiogenesis in-vivo (Goddard et al 1992). VEGF is a potent stimulator of angiogenesis and, importantly, induces the vascular permeability, which is essential for new vessel formation (Ziche et al 1982). VEGF expression is predominantly found in tumour-invading fibroblasts (Chiarugi et al 1998).

Chaotic architecture and function of tumour vessels Because of an imbalance of angiogenic regulators, such as VEGF and angiopoietins, tumour vasculature is highly disorganized; vessels are tortuous and dilated, with uneven diameter, excessive branching and shunts. These conditions modulate the production of angiogenic stimulators and inhibitors, and select for cancer cells that are more malignant and metastatic. In addition, hypoxia may select for clonal expansion of cells that have lost their apoptotic response to hypoxia (Fukumura et al 1998).

High vascular permeability of tumour vessels Tumour vessel walls have numerous openings, widened inter-endothelial junctions and a discontinuous or absent basement membrane. In addition, the endothelial cells are abnormal in shape, growing on top of each other and projecting into the lumen. These effects make tumour vessels leaky. Vascular permeability and angiogenesis depend on the type of tumour and the host organ where the tumour is growing in part because each organ has different stromal cells, which produce different pro- and anti-angiogenic molecules (Hobbs et al 1998).

Tumour dormancy

Owing to a balance between cell proliferation and apoptosis, human tumours can remain dormant for years. As a result of their longer half-life, the systemic concentration

of angiogenic inhibitors may exceed that of stimulators and inhibit growth of metastases at distal sites. This hypothesis formed the basis of the discovery of endogenous inhibitors of angiogenesis (O'Reilly et al 1994; Fidler 1995). It has been shown that production of angiogenesis inhibitors similar to angiogenesis stimulators is dependent on the site of the primary tumour (O'Reilly et al 1997). The production of these inhibitors may also change during the course of therapy. For example, radiation has been shown to increase the production of various angiogenic molecules, including endostatin, in tumours (Gohoni 1999).

Angiogenesis in non-neoplastic diseases

In non-neoplastic diseases, vessels do not grow, but rather abnormally remodel. Abnormal deposition of extracellular matrix or vascular congestion impairs delivery of oxygen and causes hypoxia in diabetes, Alzheimer's disease and asthma. Recent discoveries have shown that hypoxia activates hypoxia-inducible transcription factors, which induce expression of several angiogenic factors (Carmeliet & Jain 2000). Hypoxia-driven angiogenesis can cause blindness in premature newborns and in diabetic patients (Hartford et al 2000), and haemorrhagic rupture of atherosclerotic plaques. In chronic obstructive lung disease, hypoxia causes irreversible loss of vessels and thickening of the vascular muscular coat, with resultant life-threatening pulmonary hypertension. This vascular remodelling has been ascribed to an imbalance between vasodilators (nitric oxide) and vasoconstrictors (endothelin-1) (Carmeliet & Jain 2000).

Monocytes, macrophages, platelets, mast cells and other leukocytes release angiogenic factors, including VEGF, Ang-1, bFGF, PDGF and TNF-α (Alon et al 1995; Pinedo et al 1998). Some of these factors attract wound cells and release additional angiogenic factors (Seljelid et al 1999). Haematopoietic cells release inhibitors such as platelet factor 4 and thrombospondin, and cause proteolytic conversion of plasminogen to angiostatin and collagen XVIII to endostatin (O'Reilly et al 1994; Fidler 1995).

Angiogenesis may contribute to excess accumulation of body fat in obese individuals. Pre-adipocytes migrate to sites of neovascularization and adipose tissue is highly angiogenic (Coussens et al 1999). VEGF, bFGF (induced by insulin) and leptin (a central mediator in obesity) have been identified as mediators of angiogenesis in adipose tissue (Silverman et al 1988).

Strategies for therapeutic angiosuppression

According to the Angiogenesis Foundation, at least 184 million patients in Western nations alone could benefit from some form of anti-angiogenic therapy, and at least 314 million could benefit from some form of pro-angiogenic therapy. To date, it is one of the most heavily invested areas of medical research ever. More than 30 anti-angiogenic molecules are now in clinical development, mainly for the treatment of cancer, with many others undergoing preclinical evaluation (Featherstone & Griffiths 2002).

Antagonists of angiogenic growth factors

Two approaches have been adopted: (i) the use of anti-VEGF antibody (Kim et al 1993); and (ii) the development of specific inhibitors of VEGF receptor kinase. Anti-VEGF antibodies have few side-effects, such as asthenia, fever, arthralgia, cough, dyspnea and rash (Bikfalvi & Bicknell 2002), and humanized forms are now in Phase III clinical trials for the treatment of solid tumours.

Because of the difficulties and expense of using biological agents in the clinic, several low molecular weight inhibitors of tyrosine kinase activity of KDR (VEGFR2) have been identified and are undergoing clinical evaluation. Thalidomide is being used clinically as an anti-angiogenic molecule on a limited "off-label" basis. It acts by blocking the activity of angiogenic growth factors such as bFGF (D'Amato et al 1994) and VEGF (Liu et al 1999). It is effective orally and has been found useful in the treatment of malignant recurrent gliomas, especially when given in combination with carboplatin. An open label Phase II study of thalidomide at a dose of 100 mg daily in androgen-independent prostate cancer was conducted to evaluate its efficacy and tolerability. The results indicated that thalidomide could decrease prostate-specific antigen level in patients with androgen-independent prostate adenocarcinoma, suggesting the potential for improved disease control (Drake et al 2003).

Inhibitors of endothelial-specific integrin signalling

Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ are arginine/glycine/aspartic acid-dependent adhesion receptors, which play a critical role in angiogenesis. Hence, selective dual $\alpha_v\beta_3$ and $\alpha_v\beta_5$ antagonists may represent a novel class of angiogenesis and tumour growth inhibitors. One of these compounds is SCH 221153. In cell-based assays, SCH 221153 blocked the adhesion of endothelial cells to immobilized vitronectin and FGF-2. SCH 221153 also inhibited angiogenesis induced by FGF-2 in the CAM assay. Finally, SCH 221153 exerted significant inhibition of tumour growth induced by intradermal or subcutaneous injection of human melanoma LOX cells in severe combined immunodeficient mice (Chandrakumar et al 2001). Furthermore, antagonists of $\alpha_v\beta_3$, including cyclic arginine/glycine/aspartic acid peptides and monoclonal antibodies, significantly inhibited angiogenesis induced by cytokines and solid tumour fragments (Brooks et al 1994). Importantly, recent findings suggest that these anti-angiogenic effects may be attributable to the ability of these antagonists to induce apoptosis in proliferating blood vessels. Remarkably, $\alpha_v\beta_3$ antagonists had very little effect on pre-existing blood vessels, indicating the usefulness of targeting this receptor for therapeutic benefit without adverse side-effects. It has been shown that antibody antagonists of $\alpha_v\beta_3$ inhibit FGF-2-stimulated angiogenesis, and antagonists of integrin $\alpha_v\beta_5$ inhibit VEGF-induced angiogenesis,

in the corneal and CAM models (Friedlander et al 1995a, b). These results suggest that FGF-2 and VEGF may activate different angiogenic pathways that require $\alpha_v\beta_3$ and $\alpha_v\beta_5$, respectively. Therefore, dual antagonists of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ may be useful in blocking tumour-induced angiogenesis (Chandrakumar et al 2001).

Naturally occurring angiogenesis inhibitors

Endostatin, a 20-kDa C-terminal fragment of collagen XVIII, specifically inhibits endothelial proliferation and potently inhibits angiogenesis and tumour growth. Primary tumours regress to dormant microscopic lesions. Furthermore, the concept of dormancy therapy (a novel anticancer strategy in which malignant tumours are suppressed by prolonged blockade of angiogenesis) is being extended to endostatin using cycles of therapy. Systemic administration of human endostatin potently inhibits the growth of Lewis lung carcinoma, T241 fibrosarcoma and B16F10 melanoma in mice. An almost complete inhibition of tumour growth was observed without detectable toxicity and drug resistance. Tumours were then allowed to regrow. Following resumption of endostatin, after multiple treatment cycles, the tumours did not reappear after discontinuation of therapy (Boehm et al 1997).

Angiostatin is a 38-kDa circulating endogenous, anti-angiogenic protein (O'Reilly et al 1996; Gately et al 1997; Sim et al 1997). Angiostatin binds ATP synthase on the surface of human endothelial cells and induces apoptosis in endothelial and tumour cells (Claesson-Welch et al 1998; Moser et al 1999), but it does not affect growth-factor-induced signal transduction (Claesson-Welch et al 1998). Angiostatin inhibits matrix-enhanced plasminogen activation to account, in part, for its angiosuppressive property (Stack et al 1999). Of great interest, angiostatin is generated by sulfhydryl donors (e.g. D-penicillamine and captopril), which may explain, in part, their angiosuppressive properties (Gately et al 1997).

Remarkably, no drug resistance occurs with endostatin or angiostatin. When angiostatin and endostatin are combined, the tumours do not recur once treatment is suspended.

Tumour vasculature directed drug targeting

The concept of vascular targeting is related to angiogenesis inhibition but involves a different approach. Rather than inhibiting the formation of new vessels, drugs are aimed at destroying the existing vasculature, with consequent tumour regression (Ruoslahti 2002). However two advances have led to a recent resurgence of interest in vascular targeting: (i) the development of novel, low molecular weight drugs that are selectively toxic to tumour vasculature; and (ii) advances that enable either direct analysis of tumour vasculature or bioinformatic approaches to identify novel targets (Bikfalvi & Bicknell 2002). In general, tumour vasculature directed therapies either aim at the specific interference with endothelial cell behaviour during the angiogenic process, or at the direct induction of tumour endothelial cell death or tumour blood flow inhibition (Molema 2002).

Vascular targeting induced tumour endothelial cell killing

Angiogenesis-related carrier molecules conjugated to toxin molecules represent the angiotoxin therapeutic system, for example, angiotoxins consisting of VEGF as a carrier molecule for the selective killing of tumour endothelial cells. On binding to VEGF receptor overexpressed on tumour neovasculature, the VEGF–VEGF receptor complex is internalized. This makes VEGF protein of use as a carrier for the intracellular delivery of pharmacologically active drugs or toxins. Both chemical conjugates of diphtheria toxin and VEGF protein (Oslon et al 1997), and fusion proteins of VEGF165 or VEGF121 and diphtheria toxin translocation and enzymatic domain (Arora et al 1999) have been reported.

A significant part of research on angiogenesis inhibition has been dedicated to the VEGF signal transduction cascade. Furthermore, inhibitors of bFGF, metalloproteinase, antagonists of $\alpha_v\beta_3$ integrins, and small molecular weight inhibitors of enzymes associated with endothelial cell function (e.g. COX-2 and mitogen-activated protein kinase inhibitors) are under development or have entered clinical testing (Molema 2002). Low molecular weight compounds that target the vasculature include the combrestatins, which are currently in Phase I trials. They act by selectively disrupting the tubulin cytoskeleton without showing peripheral neuropathy or neurotoxicity following chronic administration (Bikfalvi & Bicknell 2002). Recently, it was observed that the immunosuppressive drug, rapamycin, strongly inhibits VEGF-driven angiogenesis in animal tumour models at relatively low doses. Selective delivery of this drug into the tumour neovascular endothelium may dissociate the desired anti-angiogenic effects from the immunosuppressive effects that unfavourably affect tumour growth (Guba et al 2002). In another study, an attempt was made to inhibit the production of angiogenic factors in hypoxic tumour cells using a novel hypoxia-dependent 2-nitroimidazole, KIN-841, which covalently binds and is cytotoxic to hypoxic tumour cells, and simultaneously disrupt the endothelial cell function using the same agent, to achieve angiogenesis inhibition (Shimamura et al 2003). It was observed that KIN-841 inhibited the proliferation of endothelial cells under normoxia. Its inhibitory effect on endothelial cells was more potent than that on tumour cells. Under hypoxic conditions, KIN-841 inhibited the proliferation of tumour cells more potently than under normoxia.

Highs and lows

The rationale behind the frequent administration of chemotherapeutic drugs at low doses (high-time or metronomic dosing) is to prevent time for repair of damage to the tumour vasculature, thereby deriving increased therapeutic benefit. Logically, haematopoietic cells and gut epithelial tissues should also sustain more damage because of the lack of recovery time between cycles of chemotherapy, but, interestingly, such side-effects, at least in the short-term, seem to be much less severe. An explanation

provided for this shows that cycling endothelial cells are inherently more sensitive than other cells to continuous low-dose therapy, and suggests that this might therefore be an optimal way of delivering certain types of anti-angiogenic therapies, especially those using conventional chemotherapeutic drugs. In addition, it was observed that treated endothelial cells had a higher level of apoptosis than the cancer cell lines or fibroblasts. So, prolonged exposure times, once an effective dose of drug has been reached, are crucial for cell kill in high-time chemotherapy regimens and might be selective for endothelial cells. This type of schedule might create an anti-angiogenic therapeutic window, and could be used to treat tumours that are resistant to the very drugs that are used for low-dose chemotherapy or to decrease host toxicity without reducing efficacy (Bocci et al 2002).

Emerging concepts in angiogenesis research

In the case of gene therapy based strategies, genes encoding dominant negative forms of endogenous inhibitors of enzymes involved in pro-angiogenic signalling cascades can affect endothelial cell behaviour. Furthermore, genes encoding anti-angiogenic proteins such as platelet factor 4, thrombospondin, and Tie-2 (Ang-1 receptor) have been applied for anti-angiogenic effects (Molema 2002). New targets in tumour endothelium have been identified by comparing libraries of genes (e.g. serial analysis of gene expression) constructed from isolated endothelial cells from tumours and normal tissue (St Croix et al 2000). By applying serial analysis of gene expression to endothelium isolated from normal human colon and tumour colon tissue, a number of genes specifically expressed by the tumour vasculature have been identified. Subsequent investigation of a selection of these tumour endothelial markers (TEM) with regard to protein structure identified TEM1, TEM5 and TEM8 as potential targets for the development of anti-angiogenic therapies (Carson-Walter 2001). Furthermore, the rapid development of tools for in-silico analysis of gene expression, supported by the online availability of data via the Cancer Genome Anatomy Project (Molema 2002), offers additional opportunities to identify new genes of interest for vascular targeting. Systemic evolution of ligands by exponential enrichment is based on in-vitro selection and amplification to identify novel nucleic acid sequences that bind to target molecules of interest. The resulting aptamers are theoretically suitable for application in diagnosis and therapeutics. Aptamers typically have high affinity for their target proteins, ranging from 0.05 to 10 nM. Therapeutically, a liposome/aptamer approach to neutralize VEGF in chorioallantoic membrane angiogenesis has been reported (Willis et al 1998).

Angiogenesis inhibition using targeted gene therapy

There are two strategies for anti-angiogenic gene therapy: (i) strategies aiming at the selective delivery of genes into the angiogenic endothelium to inhibit endothelial cell function; and (ii) strategies aiming at the production of anti-angiogenic proteins at the sites in the body distant from the tumour. Adenoviruses have been studied for the application in anti-angiogenic gene therapy. Specificity of transgene expression may be obtained by combining transductional and transcriptional targeting in one approach (Molema 2002). It should be noted that the choice of the transgene to be expressed by gene therapy may be quite difficult to make based on studies with the original protein (Kuo et al 2001). Furthermore, the prolonged production of anti-angiogenic proteins obtained by gene therapy may be of therapeutic value as it leads to prolonged exposure of tumour vasculature to anti-angiogenic proteins and therefore may improve therapeutic efficacy (Kisker et al 2001). The observation that tumour angiogenesis is associated with recruitment of endothelial precursor cells via the circulation has led to the use of genetically modified endothelial cells to deliver genes at sites of active neovascularization. In addition to ligand-modified viral vectors for selective delivery of anti-angiogenic genes, non-viral vehicles such as liposomes and artificial virus-like envelopes can also be endowed with targeting ligands specific for tumour vascular endothelium (Molema 2002).

Clinical trials involving angiostatin and endostatin require large quantities of bioactive recombinant proteins, which are difficult to produce. This difficulty may be resolved by tumour-specific in-vivo delivery and expression of these anti-angiogenic genes. Hypoxic regions are characteristic of solid tumours in rodents and many types of human tumours. The hypoxic regions of solid tumours provide some species of anaerobic bacteria such as *Bifidobacterium* and *Lactobacillus* with a suitable environment in which to germinate and grow. Both *Bifidobacterium bifidum* and *Bifidobacterium longum* can selectively germinate and grow in the hypoxic regions of solid tumours after intravenous injection. It is currently being investigated if the genus *Bifidobacterium* can be used to achieve tumour-specific gene delivery. This would provide a means of transporting antitumour gene expression directly into tumours. A strain of *Bifidobacterium adolescentis* as a delivery system to transport the endostatin gene into hypoxic tumours has been studied (Xi et al 2003). *B. adolescentis* with the endostatin gene was injected into mice bearing Heps liver cancer. At 168 h after the third injection of *B. adolescentis* with the endostatin gene, *B. adolescentis* was only found in the tumours and no bacilli were found in other normal tissues. Results indicate a strong inhibition of both angiogenesis and hypoxic tumour growth in the treated group, suggesting that *B. adolescentis* might be a highly tumour-specific gene delivery vector for transporting anticancer genes into tumours in cancer gene therapy (Xi et al 2003).

Conclusion

This review highlights the complexity of angiogenesis at a molecular level. Nevertheless, the field of angiogenesis research is at an exciting stage, with the identification of key targets such as VEGF and its receptor KDR, and the rapid increase in endothelial-specific markers in tumours. Angiogenesis inhibitors, when administered as protein

therapeutics as well as in gene therapy studies, strongly affect tumour outgrowth. A prerequisite for these newer strategies is the specific delivery of the effector molecules to the tumour vasculature. It is important to note that no anti-angiogenic product has yet gained market approval because different tumours may induce the expression of different target molecules, complicating the development of one therapeutic strategy. However, it can be anticipated that the first products to appear on the market are likely to be approved not as single agents, but as a part of a combination regimen with other traditional chemotherapeutic agents.

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