ANGIOGENESIS INHIBITION IN TUMOR

Bachelor’s Thesis

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Abbreviations

AAV - adeno-associated virus
Ad - adenovirus
Ang - angiopoietin
bFGF - basic fibroblast growth factor
CAR - coxsackievirusadenovirus receptor
CD13 - alanine aminopeptidase
cDNA - complementary DNA
EC - endothelial cell
ECM - extracellular matrix
EGF - epidermal growth factor
EGFR - EGF-receptor
EOMA - murine hemangioendothelioma cell line
FDA - Food and Drug Administration
FGF - fibroblast growth factor
FGF - fibroblast growth factor
FGFR - FGF receptor
Flk-1 or KDR - fetal liver kinase 1 (VEGFR-2)
flt-1 - fms-related tyrosine kinase 1 (VEGFR-1)
Flt-4 - VEGFR-3
GBM - glomerular basement membrane
GIST - imatinib-resistant gastrointestinal stromal tumor
HA - hyaluronic acid
HIF - hypoxia-inducible factor
HIV - human immunodeficiency virus
HPV - human papillomavirus
HRE - hormone response element
HS - heparan sulfate
HSV - herpes simplex virus
IL-12 - interleukin 12
IL-2R - interleukin-2 receptor
KIT (CD117) - cytokine receptor
LTR - long terminal repeat
MAPK - mitogen-activated protein kinase
MMP - matrix metalloproteinase
mTOR - mammalian target of rapamycin
MV-Edm - Edmonston vaccine strain of measles viruses
MV-ERV - Echistain-targeted measles virus vector
NRP - neuropilin receptor
PBMC - peripheral blood mononuclear cell
PC - pericyte cell
PDGF - platelet-derived growth factor
PDGFRA - platelet-derived growth factor alpha
PDGFRB - platelet-derived growth factor beta
PECAM - platelet endothelial cell adhesion molecule
Pl3k - phosphatidylinositol-3’-kinase
PIGF - placental growth factor or vascular endothelial growth factor-related protein
PTEN - phosphatase and tensin homolog
pVHL - Von Hippel-Lindau protein
RCC - renal cell carcinoma
RET - receptor for members of the glial cell line-derived neurotrophic factor (GDNF) family
rh-Endo - recombinant human endostatin
RNAi- RNA interference
RTK - receptor tyrosine kinase
scFv - single chain variable fragment
siRNA - small-interfering RNA
SMC - smooth muscle cell
Src - family of proto-oncogenic tyrosine kinases
TGF - transforming growth factor
TGF-α - transforming growth factor α
TGF-β - transforming growth factor-β
TSP1 - thrombospondin-1
VEGF - vascular endothelial growth factor
VEGFR - VEGF receptors
VHL - Von Hippel-Lindau
Introduction

Oxygenated blood and nutrients are carried throughout our bodies thanks to complex network of blood vessels. If laid end to end, the vessels from a typical adult would circle the Earth twice. That is why the process of growing new blood vessels is a fundamental biological mechanism. This process is called angiogenesis and the imbalance of this process contributes to the pathogenesis of numerous disorders.

Angiogenesis is an essential process during development-growth of a vascular system. It is also one of the earliest events in organogenesis. This process occurs also in adulthood during wound healing and restoration of blood flow to injured tissues and is regulated by sensitive interplay of growth factors and inhibitors.

Unregulated angiogenesis may lead to several angiogenic diseases and is thought to be indispensable for solid tumor growth and metastasis. In cancer, diabetic eye disease and rheumatoid arthritis, excessive angiogenesis feeds diseased tissue and destroys normal one. Conversely, unsufficient angiogenesis underlies conditions such as coronary heart disease, stroke and delayed wound healing, where inadequate blood-vessel growth leads to poor circulation and tissue death.

In this study I will advert to tumor vascular targeting because, in my opinion, it is the most important achievement in implementation of angiogenesis. I will describe normal angiogenesis and tumor vascular growth to get a better understanding of tumor vascular targeting. In addition, I introduce some exciting therapeutic tumor antiangiogenesis applications that have recently been made available.

The major attention in this work is devoted to vascular endothelial growth factor (VEGF) because it is the most important angiogenesis growth factor, and the first approved angiogenesis inhibitor for treating cancer was anti-VEGF antibody. There are also mentioned other angiogenesis growth factors and their inhibitors such as fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) etc.
1. Normal angiogenesis

1.1 Description of normal angiogenesis

Angiogenesis is a multistep process of new blood vessels formation from pre-existing vasculature. This process usually takes two forms: sprouting and unsprouting. Sprouting angiogenesis is the development of new blood vessels through proteolytic degradation of the extracellular matrix, migration/proliferation of endothelial cells (ECs), new organization of the luminal membrane, and maturation of endothelial cells to functional capillaries (Risau, 1997). New vessels can arise from postcapillary venules (angiogenesis) or from precapillary arterioles (arteriogenesis) (Liu and Deisseroth, 2006). Nonsprouting angiogenesis occurs by intussuception, which means the splitting of primary vessels by transcapillary pillars (Scappaticci, 2002).

Most normal angiogenesis occurs in the embryo. In adults, normal angiogenesis occurs during the ovarian cycle, pregnancy, and during physiologic repair processes such as wound healing or endometrial regrowth (Folkman, 1995).

In the embryo, blood vessels provide the growing organs with the necessary oxygen to develop. This close link between the blood and blood vascular systems remains important for angiogenesis throughout life, even in disease. Haematopoetic progenitors assemble into a primitive vascular labyrinth of small capillaries – a process known as vasculogenesis (Carmeliet, 2005). Already at this stage capillaries have acquired an arterial and venous cell fate, indicating that vascular-cell specification is genetically programmed and not only determined by haemodynamic force (Carmeliet, 2005). During the angiogenesis phase, the vascular plexus progressively expands by means of vessel sprouting and remolds into a highly organized and stereotyped vascular network of larger vessels ramifying into small ones (Figure 1) (Carmeliet, 2005). Nascent endothelial-cell channels become covered by pericytes (PCs) and smooth muscle cells (SMCs), which provide strength and allow regulation of vessel perfusion, a process termed arteriogenesis (Alitalo et al., 2005).
1.2 Growth factors and their receptors

Normal angiogenesis is a highly ordered process that is under tight regulation by both angiogenesis-inducing factors and angiogenesis-inhibiting factors. These factors include soluble growth factors secreted from cells, such as vascular endothelial growth factor (VEGF), angiopoetins (Ang’s), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and membran-bound molecules, such as integrins, cadherins, and ephrins (Liu and Deisseroth, 2006).
Figure 2. The Family of VEGF Molecules and Receptors.

The major mediator of tumor angiogenesis is vascular endothelial growth factor A (VEGF-A, also called VEGF), specifically the circulating isoforms of VEGF — VEGF$_{121}$ and VEGF$_{165}$. These isoforms signal through VEGF receptor 2 (VEGFR-2), the major VEGF signaling receptor that mediates sprouting angiogenesis (called kinase-insert domain–containing receptor [KDR] in humans and fetal liver kinase 1 [flk-1] in mice). The role of VEGFR-1 in sprouting angiogenesis is much less clear. VEGF is expressed in most types of human cancer, and increased expression in tumors is often associated with a less favorable prognosis. Induction of or an increase in VEGF expression in tumors can be caused by numerous environmental (epigenetic) factors such as hypoxia, low pH, inflammatory cytokines (e.g., interleukin-6), growth factors (e.g., basic fibroblast growth factor), sex hormones (both androgens and estrogens), and chemokines (e.g., stromal-cell–derived factor 1). Other causes include genetic inductive changes such as activation of numerous different oncogenes or loss or mutational inactivation of a variety of tumor-suppressor genes. The binding of VEGF to VEGFR-2 leads to a cascade of different signaling pathways (Shibuya and Claesson-Welsh, 2006), two examples of which are shown, resulting in the up-regulation of genes involved in mediating the proliferation and migration of endothelial cells and promoting their survival and vascular permeability. For example, the binding of VEGF to VEGFR-2 leads to dimerization of the receptor, followed by intracellular activation of the mitogen-activated protein kinase (MAPK) pathway and subsequent initiation of DNA synthesis and cell growth, whereas activation of the phosphatidylinositol 3’-kinase (PI3K) pathway leads to increased endothelial-cell survival. Activation of src can lead to actin cytoskeleton changes and induction of cell migration. VEGF receptors are located on the endothelial-cell surface; however, intracellular (“intracrine”)–signaling VEGF receptors (VEGFR-2) may be present as well, and they are involved in promoting the survival of endothelial cells. The detailed structure of the intracellular VEGFR-2 in endothelial cells is not yet known, but it is shown as the full-length receptor that is normally bound to the cell surface. Binding of VEGF-C to VEGFR-3 mediates lymphangiogenesis. VEGF$_{165}$ can bind to neuropilin (NRP) receptors, which can act as coreceptors with VEGFR-2 (horizontal arrow) to regulate angiogenesis. EGFR denotes epidermal growth factor receptor, flt-1 fms-like tyrosine kinase 1, PIGF placental growth factor, PTEN phosphatase and tensin homologue, S–S disulfide bond, and VHL von Hippel–Lindau.

(Robert S. Kerbel, 2008, Tumor angiogenesis)
1.2.1 VEGF and its receptor

Vascular endothelial growth factor was originally described as a homodimeric 34–42 kDa protein that increased vascular permeability in the skin (Senger et al., 1983). The ligands of the VEGF family include VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E (Figure 2). All five ligands have different roles in the process of angiogenesis and lymphangiogenesis. All members of the VEGF family stimulate cellular responses by binding to tyrosine kinase receptors (the VEGFRs) on the cell surface, causing them to dimerize and become activated through transphosphorylation. Each of the VEGF-family ligands binds to one or more of three known VEGF receptors (VEGFR): VEGFR-1 (also known as flt-1), VEGFR-2 (Flk-1 or KDR) and VEGFR-3 (Flt-4). VEGFR-1 organises blood vessels, and has a high affinity for VEGF-A and VEGF-B. VEGFR-2 activates blood vessel proliferation by binding to VEGF-A, VEGF-C, VEGF-D, and VEGF-E. VEGFR-2 is expressed in lymphatic endothelial cells (Bergsland, 2004; Björndahl, 2005; Bussolati et al., 2001; Mäkinen et al., 2001). VEGFR-3 is expressed also on the vascular endothelium, but is mainly restricted to the lymphatic endothelium. VEGF-C and VEGF-D bind to VEGFR-3, and this is critical to the growth, migration and survival of lymphatic endothelial cells, resulting in lymphangiogenesis (George et al., 2001; Hanrahan et al., 2003; Liu et al., 2006; Onogawa et al., 2004; Parr and Jiang, 2003; White et al., 2002).

Figure 3. Interactions of VEGF ligands and VEGF receptors

VEGF ligands, shown in purple boxes, bind to their associated receptors, leading to receptor dimerization and subsequent signal transduction. For example, 2 VEGFR-2 receptors with bound VEGF ligands would dimerize and be activated, causing phosphorylation of the receptor tyrosine kinase domains and a subsequent cellular signaling cascade that leads to angiogenesis. Of the primary receptors, VEGFR-2 is thought to mediate the majority of tumor angiogenic effects. (Hicklin DJ, Ellis LM. J Clin Oncol. 2005;23:1011-1027).
1.2.2 bFGF

Basic fibroblast growth factor, also known as bFGF or FGF2, is a member of the fibroblast growth factor (FGF) family. In normal tissue, basic fibroblast growth factor is present in basement membranes and in the subendothelial extracellular matrix of blood vessels. It stays membrane-bound as long as there is no signal peptide. It has been hypothesized that, during both wound healing of normal tissues and tumor development, the action of (HS) heparan sulfate-degrading enzymes activates bFGF, thus mediating the formation of new blood vessels; Witmer et al., 2001). FGFs use a dual receptor system to activate signal transduction pathways (Klagsbrun and Baird, 1991; Ornitz et al., 1992; Rapraeger et al., 1991; Yahon, 1991). The primary component of this system is a family of signal-transducing FGF receptors (FGFRs) that contain an extracellular ligand-binding domain and an intracellular tyrosine kinase domain (Basilico and Moscatelli, 1992). The second component of this receptor system consists of HS proteoglycans or heparin-like molecules that are required in order for FGF to bind to and activate the FGFR (Ornitz et al., 1992; Yahon, 1991).

1.2.3 PDGF

Platelet-derived growth factor or PDGF.

There are five different isoforms of PDGF that activate cellular response through two different receptors. Known ligands include A (PDGFA), B (PDGFB), C (PDGFC) and D (PDGFD) and an AB heterodimer and receptors alpha (PDGFRA) and beta(PDGFRB). The receptor for PDGF, PDGFR is classified as a receptor tyrosine kinase (RTK). Two types of PDGFRs have been identified alpha type and beta type PDGFRs (Matsui et al., 1989). The alpha type binds PDGF-AA, PDGF-BB and PDGF-AB while the beta type PDGFR binds with high affinity PDGF-BB and PDGF-AB (Heidaran et al., 1991).

1.2.4 PlGF

Placental growth factor or vascular endothelial growth factor-related protein is a member of the VEGF sub-family that binds to VEGFR-1 receptor (Ferrara, 2002; Hicklin and Ellis, 2005). A human cDNA coding for one such angiogenic protein was isolated from
a placental cDNA library and named as the Placenta Growth Factor. PlGF-mRNA is abundantly expressed in the placental tissue and is also present in very small amounts in heart, lung, thyroid, goitre and skeletal muscle. There are three isoforms of PlGF, which arise from alternative splicing of the PlGF-mRNA: PlGF-1, PlGF-2 and PlGF-3 (Carmeliet et al., 2001).

1.2.5 TGF-α

Transforming growth factor α is a cytokine that shares about 40% homology with epidermal growth factor (EGF) and also binds to the EGF-receptor (EGFR) to produce its biologic effects. TGF-α is expressed by tumor cells in a large number of carcinomas and has amore potent proangiogenic effect than EGF (Schreiber et al., 1986). Renal cell carcinoma cells that are deficient for Von Hippel-Lindau protein (pVHL) rely on EGFR activation mediated by HIF-induced TGF-α for proliferation and survival (Gunaratnam et al., 2003). Upon binding to TGF-α, EGFR is activated, which can then activate PI3K (Bjorge et al., 1990; Hu et al., 1992). Activation of the PI3K/AKT pathway further increases HIF-1 expression and activity (Zhong et al., 2000). Hence, TGF-α could lead to the activation of HIF-1-dependent gene transcription through the PI3K pathway.

Expression of TGF-α correlates with vascularity and stage in a number of neoplasms, including gliomas (Cai et al., 1997; Eggert et al., 2000; Li et al., 2000; Schlegel et al., 1990). Increased expression of TGF-α and EGFR has been reported in a number of neoplasms (Gerosa et al., 1989; Nistér et al., 1988; Waha et al., 1996; Yung et al., 1990)). Cells treated with TGF-α induce expression of VEGF via the transcription factor AP-2 (Detmar et al., 1994). TGF-α increases cell motility (El-Obeid et al., 1997), proliferation (Kurimoto et al., 1994), and invasiveness (Mori et al., 2000) in glioma cells. Additionally, blocking TGF-α expression inhibits cell growth in vitro and also results in a reduced tumorigenicity in vivo (Rubenstein et al., 2001; Tang et al., 1999). The direct role of TGF-α as a regulator of angiogenesis in glomerular basement membrane (GBM) formation needs to be further elucidated.
1.2.6 TGF-β

Transforming growth factor β is a cytokine with three different isoforms encoded by three separate genes. All of the TGF-β isoforms are secreted as inactive proteins associated with a latency-associated peptide (McMahon et al., 1996). TGF-β is secreted by gliomas (Leitlein et al., 2001) and has a wide variety of both tumor-suppressive and tumor-promoting effects (Van Meir, 1995; Wieser, 2001). TGF-β ligands transmit signals by binding their serine/threonine kinase receptors, thus causing the phosphorylation and activation of SMAD family proteins. Upon ligand binding, dimers of TGF-β type II receptors join and phosphorylate TGF-β type I receptor dimers, which in turn phosphorylate the SMAD proteins. Upon activation, SMADs translocate to the nucleus, where they require interaction with various DNA binding cofactors to specify the cell’s diverse responses to TGF-β (Massagué and Wotton, 2000).

HIF-1α stimulates TGF-β production in a cell-type and isoform-specific manner. HIF-responsive elements near the start site of the TGF-β3 gene were identified and demonstrated to be responsive to hypoxia and to bind HIF-1α (Schäffer et al., 2003). The invasive phenotype of trophoblasts in the low-oxygen conditions at early pregnancy is mediated by HIF-1-induced TGF-β3 (Caniggia et al., 2000). HIF-1α regulation of TGF-β1 is less clear, where depending on the cell type, TGF-β1 is either induced (Leungwattanakij et al., 2003; Norman et al., 2000) or reduced (Scheid et al., 2000; Zhang et al., 2003) in hypoxia and/or the presence of HIF-1α. To date, HREs have not been identified or examined in the TGF-β1 promoter. HUVECs express increased levels of TGF-β2 in hypoxia, but hormone response elements (HREs) in the TGF-β2 promoter have yet to be identified, so it is unclear whether this is a direct, HIF-dependent effect (Zhang et al., 2003). The relationships of TGF-β1, 2, and 3 and HIF-1α have not yet been studied in gliomas.

In gliomas, both TGF-β1 and TGF-β2 have been shown to stimulate VEGF production, and in hypoxic situations, cooperation between HIF-1α and SMAD proteins induced by TGF-β signaling leads to the induction of VEGF expression (Sánchez-Elsner et al., 2001). HRE and SMAD binding elements were identified in the VEGF gene promoter, and strong induction of VEGF resulted from the activation of both HIF-1 and SMAD3 (Sánchez-Elsner et al., 2001). TGF-β has been shown to have other proangiogenic activities in gliomas, including the upregulation of metalloproteinases, the downregulation of
metalloproteinase inhibitors (Wick et al., 2001), and the secretion of ECM (Rich et al.,
1999). Inhibition of TGF-β activity by a novel small-molecule inhibitor (SB-431542)
prevented the expression of VEGF and PAI-1 and inhibited proliferation of glioma cells in
vitro (Hjelmeland et al., 2004). Such an inhibitor could prove useful in treatment of GBM by
preventing some of the tumor-promoting effects of TGF-β.
2. Tumor angiogenesis

2.1 Description of tumor angiogenesis

In 1971, Dr Judah Folkman first proposed the hypothesis that tumor growth is angiogenesis dependent (Folkman, 1971). The growth of tumor which have been implanted in any one of several different organs and maintained by long-term perfusion stops when the tumor reaches a diameter of 3-4 mm (Folkman et al., 1966). Further growth of tumor tissue in in vivo organ cultures cannot be sustained without neovascularization of the tumor (Gimbrone et al., 1969). It is now recognized that the angiogenesis is crucial for tumor metastasis and progression. Tumor cells may begin growing around existing blood vessels (Yancopoulos et al., 1998).

Tumor angiogenesis, the growth of new vessels from preexisting vascular beds (Figure 4), is regulated in part by local changes in the relative balance between soluble and insoluble molecules that elicit either pro- or antiangiogenic effects on endothelial and perivascular cell proliferation, differentiation, migration, and/or tube formation (Tlsty and Coussens, 2006).

Figure 4. Angiogenesis during cancer development.

(a–c) Immunoreactivity of CD31-positive endothelial cells in tissue sections reveals vascular (hematogenous and lymphatic) architecture in (a) normal mouse skin, (b) skin from HPV16 transgenic mice with dysplasia, and (c) in a squamous cell carcinoma. In normal skin, vessels are located deep in the dermal compartment (arrows). As
neoplastic skin develops, vessels density and branching increases at sites where nascent vessels are juxtaposed to skin basement membrane (arrows). Stromal vascular structures in carcinomas are prominent (arrows) where their dilated nature is obvious. (d–f) Fluorescent angiography (Eichten et al., 2005; Thurston et al., 2000) and whole-mount confocal microscopy of (d) normal mouse skin, (e) skin from HPV16 transgenic mice with dysplasia, and (f) a squamous cell carcinoma reveal three-dimensional organization and architecture of hematogenous vasculature. In premalignant and malignant tissue, angiogenic activation of blood vessels results in highly branched, dilated, and tortuous networks. The black line indicates skin basement membrane (a, b) and epithelial-stromal interfaces (c). Bar: 100 µm (a–c); 200 µm (d–f). (Thea D. Tlsty, Lisa M. Coussens, Tumor stroma and regulation of cancer development, 2006)

Blood and lymphatic vessels comprise two interdependent vascular networks in all tissues. Whereas blood vessels deliver blood cells, plasma proteins, and oxygen to tissues, lymphatics, composed of lymph-forming capillaries and collecting vessels, continually regulate interstitial fluid pressure by draining interstitial fluid and debris to maintain tissue homeostasis (Eichten et al., 2007). When tissues are acutely damaged, activation of both vascular systems occurs as part of innate repair programs; once complete, both systems return to their homeostatic states. In contrast, sustained activation of one or both vascular networks is associated with some chronic disorders, such as rheumatoid arthritis (Folkman, 1995) and psoriasis (Braverman and Sibley, 1982; Detmar et al., 1994), and contributes to disease pathogenesis.

In pathological angiogenesis, such as psoriasis, ophthalmic and rheumatic diseases, inflammation is often involved, and the release of proangiogenic factors and proteases by the infiltrating inflammatory cells provide the signals for aberrant angiogenesis (Arbiser, 1996; Carmeliet and Jain, 2000; Risau, 1997). On the other hand, tumor angiogenesis involves an "angiogenic switch" that shifts the balance to more pro- than antiangiogenic signals and often occurs at an early, premalignant stage (Bergers and Benjamin, 2003; Carmeliet and Jain, 2000; Hanahan and Folkman, 1996). The induction of angiogenesis in tumors can divide roughly into two: epigenetic and genetic (Figure 2). Epigenetic factors like hypoxia, low pH, inflammatory cytokines (e.g., interleukin-6), growth factors (e.g., bFGF), sex hormones (both androgens and estrogens), and chemokines induce an increase in VEGF expression. The genetic factors include genetic inductive changes such as amplification of growth factor receptors (HER2, EGFR), activation of numerous different oncogenes or loss or mutational inactivation of a variety of tumor-suppressor genes (Carmeliet and Jain, 2000). Without adequate vascular perfusion, the high proliferation rate in hyperplastic/dysplastic lesions is balanced by increased differentiation, apoptosis, and/or necrosis, and so tumor volume is limited. Developing hypoxia in a growing tumor mass upregulates VEGF expression by tumor cells and when VEGF levels become high enough to overcome endogenous
antiangiogenic signals, angiogenesis is initiated (Bergers and Benjamin, 2003; Hanahan and Folkman, 1996).

Many tumors have been found to express various pro-angiogenic growth factors, such as FGFs and VEGF (Bergers and Benjamin, 2003). Loss of the tumor suppressor gene, p53, which frequently occurs in human cancers, can also contribute to the angiogenic switch by enhancing HIF-1α levels and thereby induction of VEGF expression, as well as by down-regulating the expression of the angiogenesis inhibitor, TSP-1 (Bergers and Benjamin, 2003; Ravi et al., 2000). TSP-1 expression has been found to be inversely correlated with malignant progression in melanoma, breast, and lung cancer cell lines (Bergers and Benjamin, 2003).

2.1.1 The VEGF and VEGF-receptor family in tumor angiogenesis

Much attention has been focused on the VEGF family of growth factors and the receptor tyrosine kinases that mediate their proangiogenic effects (Ferrara, 2002; Hicklin and Ellis, 2005) (Fig. 3). The major mediator of tumor angiogenesis is VEGF-A, usually referred to as VEGF. VEGF signals mainly through VEGF receptor 2 (VEGFR-2), which is expressed at elevated levels by endothelial cells engaged in angiogenesis and by circulating bone marrow–derived endothelial progenitor cells (Kerbel, 2008). The role of VEGF receptor 1 (VEGFR-1) activation is in inducing molecular changes that mediate epithelial to mesenchymal transition (EMT) which plays a role in cell motility by altering the cell phenotype and morphology. VEGF-A and VEGF-B binding to VEGFR-1 leads to characteristic changes of EMT, including loss of polarity, increased intercellular separation, and the presence of pseudopodia (Shibuya and Claesson-Wel, 2006).

In addition, VEGFR-1 is involved in the induction of matrix metalloproteinases (MMPs) (Hiratsuka et al., 2002) and in the paracrine release of growth factors from endothelial cells (LeCouter et al., 2003).

Most types of human cancer cells express VEGF, often at elevated levels; this is a likely consequence of the numerous and diverse genetic and epigenetic ways in which VEGF can be induced (Kerbel and Folkman, 2002). Hypoxia, a characteristic of solid tumors (Semenza, 2003) is an important inducer of VEGF. Its effect is mediated through the hypoxia-inducible transcription factors 1α (HIF1α) and 2α (HIF2α) (Semenza, 2003).
Hypoxia-inducible factors (HIFs) are transcription factors that respond to changes in available oxygen in the cellular environment, in specific, to decreases in oxygen, or hypoxia; Wang et al., 1995). In mammalian cells at least two isoforms of the a-subunit (HIF-1α and HIF-2α) exist, each of which is regulated by cellular levels of dioxygen, and contains a basic helix-loop-helix domain (Semenza, 1999; Wang et al., 1995).

### 2.2 Matrix metalloproteinases

The matrix metalloproteinases (MMP) are a family of zinc-containing endopeptidases that are able to degrade the extracellular matrix (ECM) and allow angiogenesis and tumor invasion, with each MMP acting on a different substrate (Murphy and Docherty, 1992; Stetler-Stevenson et al., 1993).

As ECM degrading proteinases, they are secreted both by tumour and stromal cells (Sun and Zhang, 2006). MMP activity is balanced by tissue inhibitors of metalloproteinases (TIMPs) (Ponton et al., 1991; Stetler-Stevenson et al., 1989; Uría et al., 1994). Based on substrate specificities and sequence characteristics, the classic MMP family members can be divided into at least four subgroups; collagenases, gelatinases, stromelysins, and matrilysins. So far, 23 different MMPs, MMP-1, -2, -3, -5, -7, -8, -9, -10, -11, -12, -13, 14, -15, -16, -17, -18, -19, -20, -21, -23, -25, -26, and -28, and four tissue inhibitors of metalloproteinases (TIMPs), TIMP-1, -2, -3 and -4, have been cloned (Chakraborti et al., 2003; Momohara et al., 2004; Stetler-Stevenson et al., 1996).

MMPs play a major role in physiological and pathological processes such as embryonic development, differentiation, apoptosis, immune surveillance, wound healing, tumour angiogenesis and invasion and metastasis (Lukashev and Werb, 1998).

MMPs are also important for endothelial invasion occurring during neovascularization. Application of a blocking peptide that prevents the interaction of MMP2 with its substrates has been shown to reduce angiogenesis (Sun and Zhang, 2006). When tumour cells are introduced into MMP2 knockout mice, the tumours that develop are less vascularized and exhibit reduced growth compared to the tumours in wild-type animals (Egeblad and Werb, 2002).
3. Tumor vascular targeting

3.1 Background

In 1971, it was hypothesized that inhibition of angiogenesis (antiangiogenesis) would be an effective strategy to treat human cancer, and active search for angiogenesis inducers and inhibitors began (Folkman, 1971). Antiangiogenesis treatment could represent an effective therapeutic strategy with which to suppress both primary tumor growth and tumor metastasis. Tumor growth can be inhibited by blocking tumor-derived angiogenic signals, or by directly targeting the tumor vascular endothelial cells (Liu and Deisseroth, 2006). Since the vasculature of tumor tissue is different from normal vasculature, it is possible to develop therapeutic agents that specifically target tumor vasculature.

3.2 Tumor vascular targeting with viral vectors

The use of viral vectors for tumor vascular targeting therapy is a promising strategy based on the unique properties of viral vectors. In order to circumvent the potential problems of antiviral neutralizing antibodies, poor access to extravascular tumor tissue, and toxicities to normal tissue, viral vectors need to be modified to target the tumor endothelial cells (Liu and Deisseroth, 2006).

The use of viral vectors for delivery of antiangiogenesis genes as well as cytotoxic agents represent an effective strategy for anticancer treatment because:
1. most gene therapy vectors can be easily manufactured and purified to high titers;
2. they are stable and can be stored long periods of time, thus permitting the production of thousands of single-use therapeutic vials;
3. they can be engineered to bind to markers on tumor vascular cells;
4. vectors can be engineered to selectively express genes in tumor vascular endothelial cells for long periods of time;
5. therapeutic viral vectors can be engineered to directly destroy tumor vascular endothelial cells once they bind (Liu and Deisseroth, 2006).
A variety of viral vectors are being studied for possible use in tumor vascular targeting therapy, including adenoviral vectors, adeno-associated viral vectors, retroviral vectors, lentiviral vectors, measles virus, and herpes simplex viral vectors.

### 3.2.1 Adenoviral vectors

The adenoviral vector is a popular vector for gene therapy because of its high gene transfer efficiency and high levels of transgene expression. However, the transient expression of the transgene, the presence of neutralizing antibodies in human subjects, the inflammatory and immune response elicited by the vector, and its receptor-independent uptake by the reticuloendothelial system (RES) and the liver are its main drawbacks. Song et al (Song et al., 2005) constructed the transcriptionally targeted adenoviral vector AdhVEGFR2-iCaspase-9, in which inducible caspase (iCaspase-9) was under the regulation of the VEGFR2 promoter. The human VEGFR2 promoter induced reporter gene expression primarily in proliferating human dermal microvascular endothelial cells (HDMECs). The activation of the iCaspase-9 mediates apoptosis of neovascular endothelial cells, and overcomes the prosurvival effect of VEGF or bFGF (Song et al., 2005).

### 3.2.2 Adeno-associated viral vectors

The adeno-associated virus (AAV) is a single-strand DNA virus and is replication defective. In the absence of a helper virus like the adenovirus, the wild-type AAV genome can integrate into the host-cell chromosomal DNA to maintain a latent state. There are at least 8 serotypes of AAV.

The AAV-mediated delivery of antiangiogenesis factors, such as a truncated form of the VEGF receptor Flk-1, or antisense mRNA against VEGF, can be used as an antitumor strategy (Davidoff et al., 2002; Nguyen et al., 1998). However, AAV-2 has a relatively poor tropism for endothelial cells, which makes directly targeting the vector to endothelial cells difficult. This poor transductional efficiency is partly due to the sequestration of AAV-2 with the extracellular matrix around endothelial cells, which prevents cell binding and entry, and the degradation of the internalized AAV-2 particles in the proteasome (Nicklin et al., 2001). Recent studies have shown that AAV1 or AAV5 serotypes can transduct endothelial cells.
efficiently, and that sialic acid residues are required for rAAV1 transduction in endothelial cells (Chen et al., 2005).

### 3.2.3 Retroviral vectors

The retrovirus buds from the plasma membrane of infected cells and therefore contains a lipid covering within which is found homodimers of linear single-stranded RNA.

Retroviral vectors have been engineered to bind to specific cell types (Kasahara et al., 1994). Retroviral vectors are suitable for targeting endothelial cells in the tumor neovasculature because of their intrinsic selectivity for proliferating cells (Richardson et al., 2004). Several specific promoters, such as Flt-1, ICAM-2, and KDR have been used in the development of transcriptionally targeted retroviral vectors to tumor endothelial cells (Richardson et al., 2004). A retroviral vector in which the herpes simplex virus thymidine kinase (HSV-TK) gene was driven by a hybrid endothelial specific PPE-1 long terminal repeat (LTR) has been constructed. Treatment of xenograft tumor models by this vector combined with chemotherapeutic agents resulted in widespread vascular disruption and tumor-cell apoptosis. In this report, they also demonstrated that vascular targeting combined with appropriate chemotherapy is more effective than either therapy alone (Mavria et al., 2005).

### 3.2.4 Lentiviral vectors

The lentivirus is a subclass of retroviruses. Lentiviral vectors are derived from the human immunodeficiency virus (HIV) by removal of the nonessential regulatory genes and sequences through which homologous recombination could lead to the recombination of therapeutic vector with HIV. Unlike retroviral vectors, lentiviral vectors can integrate their cDNA into both dividing and nondividing cells. Thus they can also transduce terminally differentiated cells such as neurons, macrophages, and hematopoietic stem cells (Barker and Planelles, 2003). Lentiviral vectors expressing angiotatin, and endostatin have been developed for antiangiogenesis therapy (Pfeifer et al., 2000; Shichinohe et al., 2001).
3.2.5 Measles viral vectors

The measles virus is an enveloped negative strand RNA virus. The Edmonston vaccine strain of measles viruses (MV-Edm) has oncolytic effects against Hodgkin and non-Hodgkin lymphomas (Grote et al., 2001). The great advantage of the measles virus is that the tumor cells have no defense against the mechanism through which it destroys cells, and that the measles virus can be easily engineered to target tumor vasculature by inserting single-chain antibodies (scFv) into the coat proteins (Liu and Deisseroth, 2006). The major obstacles for clinical use of the measles viral vectors include neutralizing antibodies to the vector in human subjects and difficulties in production for gene therapy trials.

There have been developed an Echistatin-targeted measles virus vector (MV-ERV), which binds the αvβ3 integrin receptor with a high affinity (Hallak et al., 2005). The MV-ERV vector has potential use in gene therapy for targeting tumor-associated vasculature for the treatment of solid tumors (Hallak et al., 2005).

3.2.6 Herpes simplex viral vectors

The herpes simplex virus (HSV) is an enveloped, double-strand DNA virus. The toxicity of the vector limits its use. One of the major obstacles to the clinical use of HSV therapeutically is the inflammatory response it induces at the site of initial injection, and at sites distant from the initial infection of neurons at which it is released through axonal transfer (Wood et al., 1994).

Several reports indicated that endothelial cells are susceptible to HSV-1 infection (Holbach et al., 1998). An oncolytic HSV vector has been shown to infect and kill tumor endothelium and thus exert antiangiogenic effects in vitro and in vivo (Benencia et al., 2005). Oncolytic HSV vectors which expressed IL-12 have also been shown to enhance the therapeutic effect of the vector on squamous-cell carcinoma (SCC) through antiangiogenic mechanisms (Wong et al., 2004). One of the major obstacles to the clinical use of HSV therapeutically is the inflammatory response it induces at the site of initial injection, and at sites distant from the initial infection of neurons at which it is released through axonal transfer (Wood et al., 1994). It is important to note the deaths that occurred among test mice following treatment with amplicon vectors derived from HSV(Kucharczuk et al., 1997).
3.3 Recombinant CD44-HABD as an angiogenesis inhibitor

CD44 is a transmembrane receptor for hyaluronic acid (HA) (Aruffo et al., 1990) that is functional in HA metabolism (Culty et al., 1992; Kaya et al., 1997), cell migration (Thomas et al., 1992) and cell adhesion (Lesley et al., 1993). The observed tumor growth-and angiogenesis-inhibiting effect of CD44-HABD is independent of HA binding since a non-HA-binding mutant was equally effective (Päll et al., 2004). Furthermore, CD44-HABD blocked cell proliferation in an endothelial cell-specific manner and showed no effect on proliferation of tumor cells or untransformed epithelial or fibroblast cells. Therefore, recombinant CD44-HABD represents a new type of direct angiogenesis inhibitor based on a cell surface receptor (Päll et al., 2004).

CD44-HABD might prevent cell–cell contact of tumor cells precluding the development of a solid mass or it might alter the expression of stromal factors essential for tumor growth. Whatever the mechanism, the observed inhibition of tumor growth opens up the possibility that CD44-HABD may be developed into a drug for treatment of human cancer (O'Reilly et al., 1997).

3.4 Endostatin

Endostatin is a naturally-occurring 20-kDa C-terminal fragment derived from type XVIII collagen; Päll et al., 2004). It is reported to serve as an antiangiogenic agent, similar to angiostatin and thrombospondin. Endostatin is a broad spectrum angiogenesis inhibitor and may interfere with the pro-angiogenic action of growth factors such as bFGF/FGF-2 and VEGF (Folkman, 2006).

Endostatin was first isolated from the supernatant of an in vitro culture of EOMA cells, a murine hemangioendothelioma cell line (Boehm et al., 1997; O'Reilly et al., 1997). Human and murine endostatin specifically inhibit the proliferation and migration of capillary endothelial cells and can induce apoptosis of proliferating endothelial cells. However, no direct effect on the growth of numerous tumor cell lines has been seen (O'Reilly et al., 1997).

Endostatin was shown to inhibit, in a potent and dose dependent manner, the growth of a wide variety of human and murine primary and metastatic tumors growing in mice (Boehm
et al., 1997; O'Reilly et al., 1997). Prolonged therapy with high doses of endostatin induced a virtually complete blockade of tumor angiogenesis and caused established tumors to regress to microscopic lesions (Boehm et al., 1997). Examination of these dormant lesions revealed decreased angiogenesis with little or no change in the rate of tumor cell proliferation but significantly increased tumor cell apoptosis as compared with untreated controls. No resistance to therapy or toxic effects was observed in mice even after prolonged therapy with endostatin (Boehm et al., 1997; Sim et al., 2000). These striking results in experimental mouse models provided the impetus to initiate clinical trials of endostatin in patients with cancer. There are going on phase III clinical trials for endostatin in combination with chemotherapy for non-small cell lung cancer (website, 1).

3.5 siRNA inhibitors for modulation of angiogenesis

RNAi is an endogenous mechanism for the potent and specific inhibition of gene expression, which can be diverted to act on cellular genes by introducing siRNA agents. Their success as a powerful research tool, illustrated by the many advances, is fueling enthusiasm for siRNA as a novel modality for antiangiogenesis therapeutics, a large unmet clinical need (Lu et al., 2005).

There are two distinct approaches using siRNA to achieve antiangiogenesis activity for cancer treatments:
1. activating endogenous or exogenously delivering anti-angiogenesis factors;
2. delivering inhibitors to reduce the activities of endogenous pro-angiogenesis factors.

siRNA is a particularly useful inhibitor with both potency and sequence-specific selectivity that has been demonstrated to induce a phenotypic response in cell-culture studies of antiangiogenesis. However, their therapeutic potential will only be realized when the in vivo antiangiogenesis efficacy of the siRNA agents can be achieved with clinically feasible delivery systems (Lu and Woodle, 2005).

Using intratumoral delivery of VEGF-targeted siRNA, the inhibition of tumor growth was observed in MDA MB-435 and MCF-7 human xenograft breast cancer models (Xie, 2004). In tumors expressing both thrombospondin-1 (TSP1) and VEGF, the effect of TSP1 to reduce vascularization and tumor growth was restored using the systemic administration of aqueous VEGF siRNA but failed to show signs of target inhibition (Filleur et al., 2003). In a
different study, intratumoral delivery of VEGF siRNA with a cationic carrier resulted in dramatically suppressed angiogenesis and growth (Takei, 2004).

It has been recognized that the local delivery of antitumor agents is limited to only a few tumor types with clinical relevance. Therefore, systemic administration of siRNA will provide the greatest clinical benefit as a treatment, especially for disseminated metastatic cancer. To that end, VEGF-pathway siRNA agents with in vivo validated activity were further evaluated by systemic administration to mice bearing neuroblastoma tumors using a ligand-directed nanoparticle carrier, and exhibited the inhibition of target expression, angiogenesis and tumor growth after repeated dosing (Schiffelers et al., 2004). The observed efficacy was achieved with a potency amenable to clinical application, further strengthening the promise of siRNA as a therapeutic modality.

3.6 Approved angiogenesis inhibitors

There are several angiogenesis inhibitors now approved for clinical use in oncology. Some of them are antibodies, targeting VEGF; some small molecules, targeting tyrosine kinase receptors or mTOR pathway.

3.6.1 Avastin

Avastin is the first U.S. Food and Drug Administration (FDA) and European Medicines Agancy approved therapy designed to inhibit angiogenesis. Avastin is the anti-VEGF antibody bevacizumab.

Avastin is a therapeutic antibody that is believed to work by targeting and inhibiting the function of VEGF that stimulates angiogenesis. Preclinical models have shown that anti-VEGF agents like Avastin may work by causing the following changes to occur in the blood vessels supporting tumor growth (tumor vasculature):

1. Regression of existing microvessels — helps arrest tumor growth and reduce tumor size;

2. "Normalization" of surviving mature vasculature — makes the tumor vasculature more conducive to effective anti-cancer therapy;
3. Inhibition of vessel growth and neovascularization (e.g., the sprouting of new microvasculature from existing vessels).

Avastin in combination with intravenous 5-fluorouracil–based chemotherapy, is indicated for first- or second-line treatment of patients with metastatic carcinoma of the colon or rectum. The effectiveness of Avastin in metastatic breast cancer is based on an improvement in progression-free survival. Avastin is not indicated for patients with breast cancer that has progressed following anthracycline and taxane chemotherapy administered for metastatic disease (web site, 1)

3.6.2 Sofarenib

Sorafenib is an orally available inhibitor of vascular endothelial growth factor receptors, PDGFR-β, and RAF kinases. Recently approved by the Food and Drug Administration, sorafenib (Bayer Pharmaceuticals, West Haven, CT) is an agent with established single-agent efficacy in metastatic renal cell carcinoma (RCC).

Sorafenib has established efficacy in RCC and is well tolerated. The spectrum of kinases inhibited by sorafenib goes far beyond VEGFRs and is unique compared with other agents in this class (Flaherty, 2007). The contribution of these various targets to the activity of sorafenib in RCC is not known. Based on its toxicity profile and target spectrum, sorafenib is well suited to inclusion in combination regimens (Flaherty, 2007).

There is among 17 inhibitors of VEGF receptor 2 (VEGFR2 or KDR) in clinical testing (Flaherty, 2007).

3.6.3 Sunitinib

Sunitinib is an oral, small-molecule, multi-targeted RTK inhibitor that was approved by the FDA for the treatment of renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumor (GIST) on January 26, 2006. Sunitinib was the first cancer drug simultaneously approved for two different indications (Le Tourneau et al., 2007).
Sunitinib inhibits cellular signaling by targeting multiple RTKs. These include all PDGFR and VEGFR, which play a role in both tumor angiogenesis and tumor cell proliferation. The simultaneous inhibition of these targets therefore leads to both reduced tumor vascularization and cancer cell death, and ultimately tumor shrinkage. Sunitinib also inhibits KIT (CD117), the RTK that drives the majority of GISTs. In addition, sunitinib inhibits other RTKs including RET, CSF-1R, and flt3; US Food and Drug Administration, ).

3.6.4 Temsirolimus


Temsirolimus (CCI-779) is an inhibitor of mammalian target of rapamycin (mTOR) kinase, a component of intracellular signaling pathways involved in the growth and proliferation of cells (Fingar et al., 2004; Schmelzle and Hall, 2000) and the response of such cells to hypoxic stress (Hudson et al., 2002). Temsirolimus binds to an abundant intracellular protein, FKBP-12, and in this way forms a complex that inhibits mTOR signaling (Harding, 2003; Skotnicki et al., 2001). The disruption of mTOR signaling suppresses the production of proteins that regulate progression through the cell cycle (Hay and Sonenberg, 2004; Yu et al., 2001) and angiogenesis (Del Bufalo et al., 2006; Thomas et al., 2006). The inhibition of angiogenesis by temsirolimus is clinically relevant because unregulated angiogenesis is prominent in renal-cell carcinoma (Pantuck et al., 2003).
Conclusion

Tumor angiogenesis is different from normal angiogenesis with the exception that tumor expresses its own vascular growth factors what afford tumor to develop. If there is no blood vessels, that provide tumor with nutrients and oxygen, the growth of tumor stops when the it reaches a diameter of 3-4 mm.

As the tumor growth is highly depending on angiogenesis, therefore there has been over many tens of years already interest of researches to investigate the possibilities tumor vascular inhibition. Vessels growth could be inhibited by blocking angiogenesis signals or precluding activation of their receptors.

One potent treatment options of tumor angiogenesis targeting is using vectors. They take affects intracellularly and vectors could be modified in different way. It can be done by modifying viral capsid proteins to be attached specifically to tumor ECs or by using endothelial specific promoters to trigger death signals in ECs. The problems with viral vectors are mainly that there can be induced immune response against viral proteins which eliminates their effect. In viral vectors used pathogenic viruses are specific to certain cell lines, so they target only marked cells.

It is also possible to impede EC moving and cells interactions to inhibit tumor growth. One of this type inhibitor is CD44-HABD. As the specific target for this protein is still under debate but its tumor antiangiogenesis influence clearly shown in preclinical experiments, there must be found the specific biomarkers to evaluate the effect of this agent on ECs, and maybe the dynamic changes of some proliferation markers in tumor cells (with immunohistochemistry).

There are approved anti-angiogenesis drugs already used in oncology, although the experience is not that wide. It come more clear that different angiogenesis inhibitors having different targets have also different side effects, and pharmacodynamic changes. There is of great importance to find the methods for specifying tumors which can be cured with this or that agent, and maybe even elaborate some surrogate markers for nondirect assessment of the drug inhibiting effect on tumors. Such first test have been done for Avastin evaluating
proangiogenic markers in serum of treated patients and in circulating endothelial cells and circulating progenitor cells (Jain, 2008).

Anyway, the tumor vascular targeting is very exciting and perspective research area given already some fruits of real clinical benefits in improving progression free and overall survival of part of advanced cancer patients.
Veresoonte arengu pärssimine kasvajas

Olga Šapran

Resümee

Angiogenees on protsess, mille käigus moodustuvad veresoone. Normaalne angiogenees leiab aset embrüogeenesis, millal olemasolevatest veresoonte algetest areneb angiogeneesiti toimel kogu veresoonkond. Täiskasvanud organismis toimub normaalne angiogenees haavade paranemisel, menstruaaltsükli eri faasides ja raseduse jooksul, loote arenemise ajal.


Normaalne angiogenees on kõrgelt organiseeritud protsess, mis on reguleeritud mõlema angiogeneesi-esilekutsuvate ja angiogeneesi-inhibeerivate faktorite koostoimel. Need faktorid sisaldavad rakus sekreteeritud lahustuvaid kasvufaktoreid nagu vaskulaarne endoteelne kasvufaktor (VEGF), angioopoetinid (Ang’s), fibroblasti kasvufaktor (FGF), trombotsüüt-päritolu kasvufaktor (PDGF), transformeeritud kasvufaktor-β (TGF-β) ja membraan-seoselised molekulid nagu integriinid, kadhberinid ja efriinid.

VEGF on lahustuv kasvufaktor (valk), mis käivitab veresoonte moodustumise. Vähkkasvajad toodavad seda valku tunduvalt enam kui tavaline kude. VEGF-i toime vähkkasvaja kasvule on järgmine: (1) VEGF käivitab veresoonte moodustumise kasvajas. Angiogeneesi käivitumine on vähkkasvajale vältimatu vajalik, sest veresooned transportivad sellele hapnikku ja toitaineid. Angiogenees võimaldab vähirakkudel vereringet pidi ka mujale...
organismi tervetesse elunditesse jõuda; (2) VEGF võib häirida organismi immuunkaitset. Arvatakse, et vähirakud ekspresseerivad VEGF-I, et takistada endi vastu tekinud immuunvastust; (3) VEGF toimel suureneb kasvaja veresoonte läbilaskvus ja endoteeli rakud muutuvad liikuvamateks.


Üheks paljulubavaks ravimeetodiks peetakse viiruse vekitoreid. Nende kasutamine antiangiogeneesi geenide toimetamiseks on hea selle pooles, et: (1) enamik geeniteraapia vekitoreid saab kergelt toota ja puhastada; (2) nad on stabiilsed ja neid saab hoida pikka aega, see võimaldab toota tuhandeid ühekordseid terapeutilisi viaale; (3) neid saab luua nii, et nad seonduks spetsiifiliselt vähi vaskulaarrakkude markeritele; (4) vekitoreid saab moodustada nii, et nad ekspresseriks selektiivselt geene vähi vaskulaarendoteeli rakkudes pikka aega; ja (5) terapeutilised vektorid saavad olla moditseeritud nii, et hävitavad vähi endoteeli raku kohe kui seonduvad sellega. Valik viiruse vekitoreid, mis on potentsiaalsed vähivastases teraapias on: adenoviiruse vektorid, adeno-assotsieeritud viiruse vektorid, retroviiruse vektorid, lentiviiruse vektorid, leetrite viirus ja herpes simplex viiruse vektorid.

On ka teisi meetodeid, millest olulisemal kohal kasutusel olevad inhibiitorid nagu Avastin, mis on esimene heaks kiidetud anti-angiogeneesi inhibitor. Tema järelsaid heaskiitu ka Sofarenib, Sunitinib, Temsirolimus ja teised.

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References

A) Article


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B) Web site

