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Therapeutic Approaches of Angiogenesis Inhibition: Are We Tackling the Problem at the Right Level?

Arjan W. Griffioen*

A growing body of evidence now demonstrates that inhibition of angiogenesis is a promising way for treatment of disease. Although the field of angiogenesis research is strongly linked to cancer biology, many other diseases were found to be dependent on angiogenesis as well, introducing a potential benefit from antiangiogenesis treatment. Recently, the first specific angiogenesis inhibitor was approved by the Food and Drug Administration for the treatment of colorectal cancer. Currently, several compounds with angiostatic activity are approved, and many are in late-stage clinical development. Most of these are indirect inhibitors, either clearing angiogenic growth factors from the circulation or blocking the signaling pathways activated by these growth factors. Although these compounds seem to represent an efficient strategy in cancer treatment, they possess an intrinsic threat to induce resistance. Therefore, it remains to be seen whether this strategy will be the most attractive in the future. Advancing insights into fundamental mechanisms will be necessary in the development of novel anticancer strategies based on inhibition of angiogenesis. (Trends Cardiovasc Med 2007;17:171–176) *© 2007, Elsevier Inc.*

• Introduction

The hypothesis that the growth of tumors is dependent on the formation of new blood vessels, put forward by Folkman in the early 1970s (Folkman 1971), indicated that angiogenesis inhibitors might be discovered and used as therapy against cancer. Not until the early 1990s were the first specific angiogenesis inhibitors described. Over the last 15 years, considerable progress has been made in the development of therapies based on targeting tumor angiogenesis (Folkman 2006; Griffioen and Molema 2000). Currently, several angiogenesis inhibitors are approved for the treatment of cancer, and many are in late-stage clinical testing.

Angiogenesis occurs through an intricately regulated cascade of processes in growing tissues where for example conditions of hypoxia have turned on the production of angiogenic growth factors such as the families of vascular endothelial cell growth factors (VEGFs) and fibroblast growth factors. Such growth factors are sensed by endothelial cells in preexisting capillaries that subsequently produce proteases to dissolve the basement membrane and extracellular matrix, thereby allowing migration of endothelial cells into the direction of the stimulus. Endothelial cells will proliferate and form new sprouts that become functional blood vessels after the attraction of accessory

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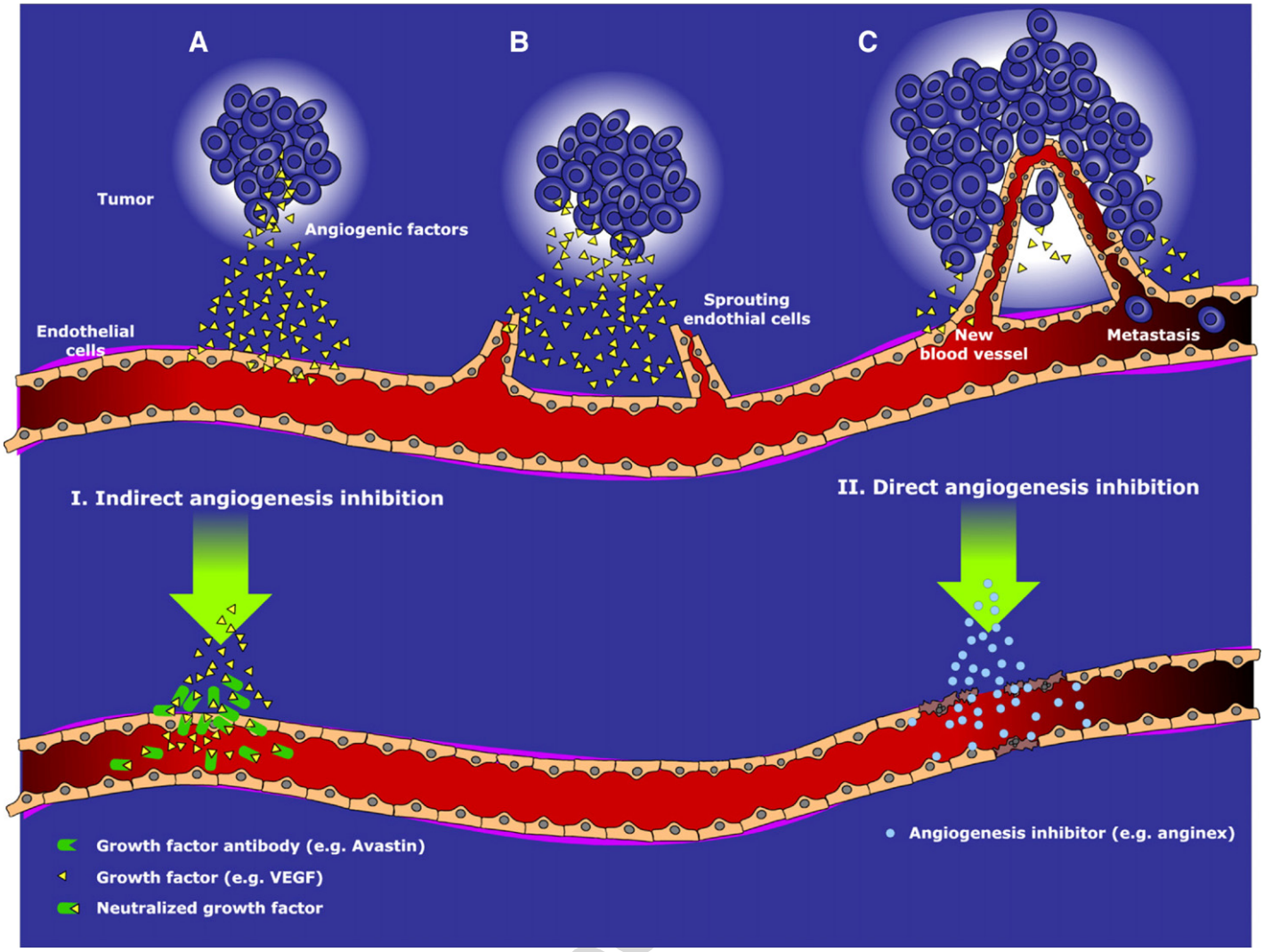

Figure 1. The angiogenic cascade and examples of angiogenesis inhibition. A growing tissue, either a rheumatoid pannus, an atherosclerotic plaque, or a tumor, will produce angiogenic growth factors upon occurrence of hypoxia **(A)**. These factors activate nearby endothelial cells of preexisting capillaries to produce matrix proteases (MMPs) for the breakdown of the extracellular matrix. This will allow the endothelial cells to start migrating toward the stimulus **(B)**. Endothelial cells will proliferate and form a tube that can carry blood but is initially very leaky. After attracting accessory cells and the formation of a new basement membrane and a firm extracellular matrix, a new functional blood vessel is formed **(C)**. The **lower panel** shows the difference between indirect inhibition of angiogenesis by neutralization of (activity of) tumor-derived growth factors; the mechanism of Avastin, ZD6474, and SU11248 (I); and inhibition by a direct effect on tumor endothelial cells by, for example, anginex and caplostatin (II).

cells such as pericytes and the formation of a new rigid extracellular matrix (Griffioen and Molema 2000) (Figure 1). This angiogenesis cascade provides opportunities for intervention in every single step separately, and inhibitors for each of these steps have been discovered and are being developed in clinical studies. Antiangiogenesis compounds can specifically and directly inhibit the proliferation of endothelial cells (TNP-470/caplostatin, platelet factor-4 [PF4]) or interfere directly with the migratory activity of these cells (endostatin, integrin antagonists). Alternatively, they can inhibit the production or activity of metalloproteinases (MMPs), inducing a hampered mobility of endothelial cells. However, the best developed angiogenesis inhibitors are the ones that act indirectly, either by clearing angiogenic growth factors from the circulation, blocking the corresponding growth factor receptors, or by intervention in the intracellular signaling pathways activated by these growth factors.

Next to a critical role in the formation of tumors, angiogenesis is also a key step in cardiovascular diseases and rheumatoid arthritis, as well as in a large array of other diseases such as neovascular eye diseases, psoriasis, and endometriosis.

Cardiovascular diseases are still the leading cause of death in Western societies. Angiogenesis has been shown to be an essential process in atherosclerosis. Neovascularization has been shown to be associated with atherosclerotic plaque growth (Moulton 2006; Moulton et al. 1999). In addition, an association has been found with plaque instability (Virmani et al. 2005). Expression of VEGF-A increases during atherogenesis, and very recently, VEGF-A has been shown to induce a more vulnerable plaque phenotype by promoting leukocyte recruitment (Lucerna et al. 2007). Treatment of ApoE−/− mice with

Table 1. Examples of direct angiogenesis inhibitors and drugs that (indirectly) block activators of angiogenesis

Compound	*Class*	*Clinical studies/ FDA approval*
Direct angiogenesis inhibitors		
ABT-510	Thrombospondin-1 mimetic	Phase II
Anginex, 0118	α-Chemokine like compounds	–
Angiostatin	Plasminogen fragment	–
Endostatin	Collagen XVIII fragment	Approved (China)
NGR-TNF	CD13	Phase I
PPI-2458	Fumagillin class compound	Phase I
Tumstatin	Collagen XV fragment	–
Indirect angiogenesis inhibitors		
AMG-706	(PTK inhibitor)	Phase II
Avastin	(Anti-VEGF Ab)	Approved
Iressa/Gefitinib	(PTK inhibitor)	Approved
Tarceva/Erlotinib	(PTK inhibitor)	Approved
Sunitinib/SU11248	(PTK inhibitor)	Approved

an anti-VEGFR1 antibody reduced atherosclerosis in ApoE−/− mice by inhibiting leukocyte infiltration (Luttun et al. 2002). These results suggest that angiogenesis inhibition is a therapeutic option for atherosclerosis. The adverse side effects of Avastin (Genentech, San Francisco, CA) reported in patients with cancer (Ratner 2004) may dictate that this compound not be used on patients who have cardiovascular diseases in the near future. Identification of downstream VEGF(R) signaling mechanisms involved in atherosclerosis will allow the development of more specific and effective therapeutic interventions.

- **Direct Vs Indirect Angiogenesis Inhibitors**

The first angiogenesis inhibitor approved by the Food and Drug Administration (FDA) was Avastin, neutralizing anti-VEGF monoclonal antibody (Ferrara et al. 2004; Wang et al. 2004). Avastin was approved for colorectal cancer in combination with chemotherapy (5-fluorouracil) in 2004 and since October 2006 for first-line treatment of unresectable, locally advanced or metastatic nonsquamous non–small cell lung cancer, in combination with carboplatin and paclitaxel. Avastin is currently in some 300 clinical studies for many other cancer types, including solid tumors as well as leukemia and lymphoma, and for neovascular eye diseases such as age-related macular degeneration (an overview of clinical studies is available at http://www.clinicaltrials.gov). Next to systemic neutralization of VEGF, antagonists of growth factor receptors and inhibitors of growth factor receptor signaling are currently being developed. The latter

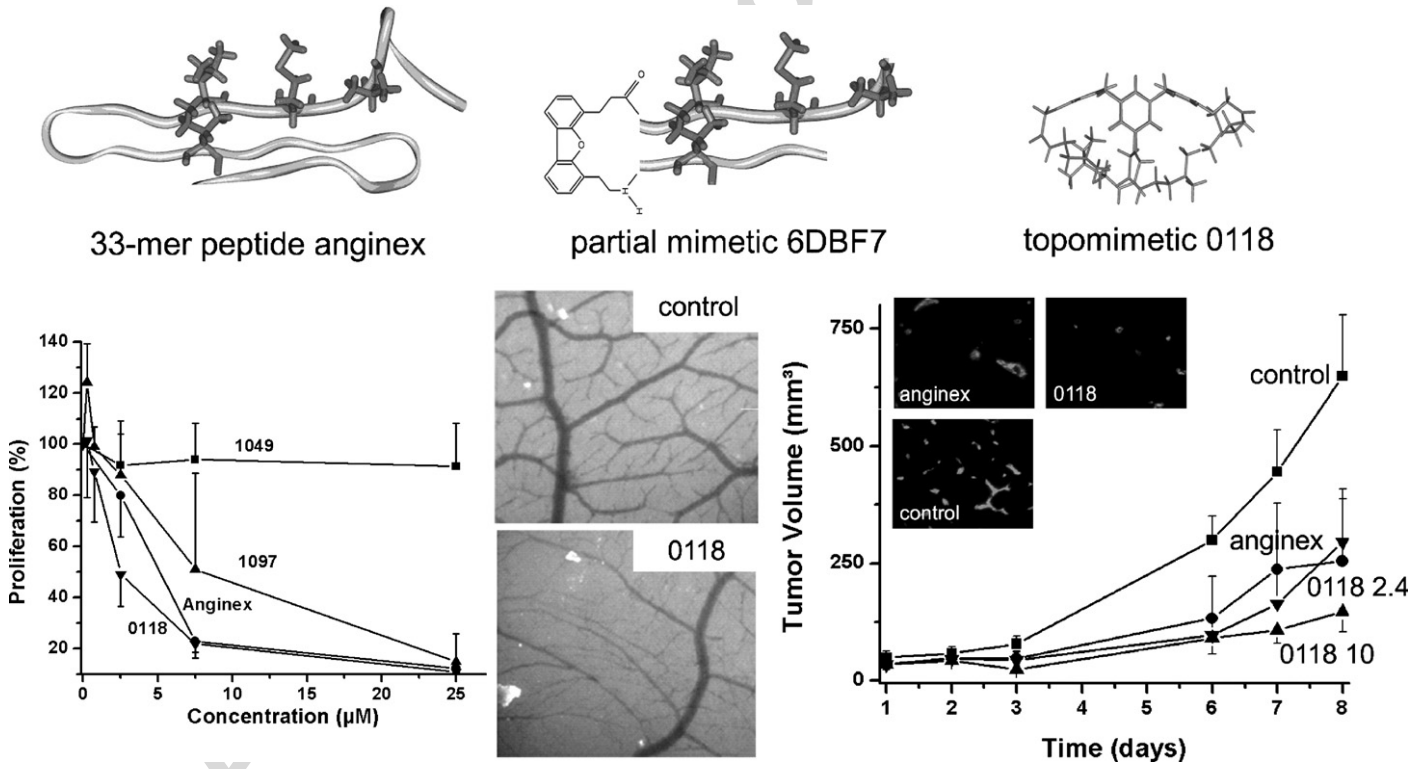

Figure 2. Development and activity of anginex and related compounds. **Top panels** show the development of a small molecule of anginex. **Left panel** shows parent anginex. **Middle panel** represents the partial mimetic with 2 peptide chains attached to the dibenzofuran turn-mimetic. **Right panel** shows the calixarene-based mimetic 0118. **Lower left panel** shows the antiproliferative activity of anginex and 0118 together with several calixarene-based control compounds. **Middle panels** show the inhibition of angiogenesis in the chorioallantoic membrane of a chicken embryo. In the **lower right panel**, tumor growth inhibition is shown in an ovarian carcinoma xenograft mouse model by anginex (*inverted triangles*) and 0118 (*circles*, 2.4 mg/kg; *triangles*, 0118 10 mg/kg); *squares* represent control mice. The insets in this figure demonstrate the inhibition of angiogenesis by microvessel density assessment.

category of compounds is represented by the rapidly growing number of receptor tyrosine kinase inhibitors. Kinases have turned out to be one of the richest target mines for oncology drug discovery and development. A clear benefit of this approach is that it is now possible to hit several kinases at once. For example, ZD6474, which targets epidermal growth factor receptor and VEGFR2, was approved by FDA for thyroid cancer in November 2005, followed by SU11248, which inhibits fms-like tyrosine kinase 3 (Flt3), Kit, VEGFR, and platelet-derived growth factor receptor, that was approved for treatment of advanced renal cell carcinoma and gastrointestinal stromal tumors in January 2006.

The previously mentioned antiangiogenesis compounds/strategies can be seen as indirect inhibitors because they intervene in signals that are derived from the tumor cells and stromal cells. In fact, it may even be stated that these strategies are based on treatment of the tumor cells, just like many other conventional and investigational anticancer treatment strategies do, such as chemotherapy and antibody-based therapies with Herceptin and Rituximab (Collins and Workman 2006).

Although this category of indirect angiogenesis inhibitors seems to outperform direct angiogenesis inhibitors in clinical studies, it is argued that therapy based on these strategies may suffer from several drawbacks. First, most tumors express several different angiogenic growth factors, suggesting that blocking only one or a few may not be sufficient. In favor of this argument, one might add that a common profile of growth factors does not seem to exist. Even for the most common growth factor, VEGF, it is estimated only to be expressed in approximately 60% of all tumor types. Second, the intrinsic selection by growth factor inhibition may force the genetically unstable tumor cells to drift toward the production of alternative proangiogenic factors. This may ultimately lead to drug resistance, which is the major problem in conventional treatment of cancer.

Another strategy of angiogenesis inhibition is by using directly acting angiostatic compounds. These agents have a direct effect on the endothelium, affecting cellular regulatory pathways, independently of the tumor cells. Among these compounds are agents that (i) inhibit the proliferation of tumor endothelial cells or induce apoptosis in these cells, (ii) prevent the migration or tube formation of endothelial cells, and (iii) inhibit the activity of MMPs in activated endothelial cells. It is likely that a therapy based on these inhibitors will reduce the risk of developing drug-induced resistance. The reason that this category of direct agents is lagging behind, regarding their translation to the clinic, may be the lack of sufficient knowledge regarding the mechanism of action of these compounds. Without trying to be complete, Table 1 gives examples of direct and indirect angiogenesis inhibitors.

• Development of New Angiogenesis Inhibitors

Although many laboratories searched for novel angiogenesis inhibitors by screening for endogenous molecules, we applied de novo design of stable and water soluble β-sheet forming peptides (Mayo et al. 1996). Based on the fact that many endogenous angiogenesis inhibitors shared a common structure of β-sheet forming domains (Dings et al. 2003a), we designed a series of β-sheet forming peptides analogous to the 3-dimensional structure of the α-chemokines PF4 and interleukin-8 as well as of the neutrophil cytokine bactericidal/permeability increasing protein. From a large library of peptides, we selected one peptide that shared strong angiostatic activities with PF4. The inhibitory activity of this 33-mer peptide called anginex in in vitro bioassays was stronger than that of any other angiogenesis inhibitor tested (Griffioen et al. 2001). The activity of anginex was found to be mediated by the prevention of attachment of activated endothelial cells to the extracellular matrix, a process called anoikis, and by subsequent induction of apoptosis in such activated endothelial cells. In vivo, anginex was found to display an effective antiangiogenesis and antitumor activity in many mouse and rat tumor models (Dings et al. 2003b; van der Schaft et al. 2002) (Figure 2).

In very recent research, we completed an effort to design a small molecule copying the activity of anginex. To achieve that, we investigated, by alanine scanning and overlapping small peptide production, the structure and minimal size of a peptide with similar activity as anginex. It was evident that the β-sheet structure in anginex is necessary for its activity. Making use of a β-sheet-inducing dibenzofuran (DBF)-turn mimetic and attaching two short key amino acid sequences from anginex, thereby producing a new antiangiogenic molecule called 6DBF7, we were able to minimize the number of amino acids to 13. 6DBF7 has similar activities in vivo as parent anginex. In a mouse xenograft model for ovarian carcinoma, 6DBF7 is observed to reduce tumor growth by up to 80% (Mayo et al. 2003).

The next step in designing a full nonpeptidic mimetic was the selection of a molecular scaffold that approximates the molecular dimensions and surface topology of spatially related, key amino acid residues in the antiangiogenic peptide anginex. Because the molecular dimensions of a 2-stranded β-sheet are nicely mimicked in calix[4]arene, we produced a library of molecules mimicking the amphipathic antiparallel β-sheet in anginex, by adding hydrophobic and basic chemical groups to the calix[4]arene scaffold. From structural biology and synthetic chemistry perspectives, calixarene provides a very good scaffold off of which it is easy to position chemical groups that mimic the character and surface topology of key amino acid side chains in anginex. This research resulted in the development of *0118*, an angiostatic molecule that markedly inhibits tumor growth, in several preclinical models resulting in full stasis of tumor development (Dings et al. 2006) (Figure 2). The beneficial features of this molecule, that is, easy synthesis, stability, and potential oral availability, make *0118* an ideal compound for translational research and development into clinical studies.

• Target Discovery

As mentioned above, the development of effective angiogenesis inhibitors depends partially on the understanding of action mechanisms and cellular targets on angiogenically activated endothelial cells. Knowledge of the mechanisms and/or receptors of angiostatic molecules allows improvement of compounds and therapies. For several direct angiogenesis inhibitors, some high- and low-affinity receptors have been described. For example, endostatin seems to mediate its activity through binding to $\alpha5\beta1$-integrin,

explaining the inhibition of migratory activity of endothelial cells (Rehn et al. 2001; Wickstrom et al. 2002). However, binding of endostatin to several other receptors, such as $\alpha v\beta 3$-integrin, glypican-1, and E-selectin, have been described as well (Karumanchi et al. 2001; Rehn et al. 2001; Yu et al. 2004). Angiostatin binds membrane ATP-synthase, a molecule that is involved in the energy household of endothelial cells (Moser et al. 1999). TNP-470/caplostatin has been found to mediate its activity partly through binding to methionine aminopeptidase-2, a cytoplasmic metalloenzyme (Griffith et al. 1997). Thrombospondin-1 was found to bind to CD36 on activated endothelium (Dawson et al. 1997).

To identify the molecules that can serve as target molecules on tumor endothelial cells, several studies using large-scale gene expression profiling techniques report on genes and proteins that are overexpressed in the tumor vasculature (reviewed in van Beijnum and Griffioen 2005). St Croix et al. (2000) used serial analysis of gene expression technology to identify markers in colorectal carcinoma endothelial cells isolated with magnetic beads. These tumor endothelial markers showed high expression in tumor-derived endothelial cells but not in normal endothelial cells. A strong bias toward genes functioning in extracellular matrix turnover such as collagens Iα1, Iα2, IIIα1, IVαI, VIα3, and XIIα1, matrix metalloproteinases MMP-11 and MMP-2, serpinE, and SPARC were found, stressing the importance of extracellular matrix remodeling during angiogenesis in vivo (St Croix et al. 2000). Using the same technique, profiles were published representing malignant brain endothelium and invasive breast carcinoma endothelium. These studies also revealed collagens and matrix MMPs (Madden et al. 2004; Parker et al. 2004).

We have used a molecular method, suppression subtractive hybridization, to compare gene-expression profiles of isolated endothelial cells from colon carcinoma tissues, nonmalignant angiogenic placental tissues, and nonangiogenic normal tissues. We identified 17 tumor angiogenesis genes overexpressed in tumor endothelium compared with angiogenic and nonangiogenic endothelium (van Beijnum et al. 2006). Four of these genes encode surface-expressed or secreted proteins, namely vimentin, CD59, high-mobility group box-1, and insulin-like growth factor binding protein-7. Further investigation showed that antibody targeting of these proteins inhibited angiogenesis in in vitro and in vivo assays. The success of this approach was demonstrated by targeting one of these markers, vimentin, in a mouse tumor model, resulting in markedly inhibited tumor growth, accompanied by reduced microvessel density (van Beijnum et al. 2006).

To identify the receptor mediating the activity of anginex, we applied yeast 2-hybrid analysis. After building a gene encoding anginex (Brandwijk et al. 2006; Brandwijk et al. 2005) and using that as a 'bait' vector in the yeast 2-hybrid screening, most of the selected transcripts identified galectin-1 as the receptor that mediates anginex' activity (Thijssen et al. 2006).

• Resistance to Angiogenesis Inhibitors

It has been hypothesized that antiangiogenesis therapy is not subject to drug-induced resistance. This assumption is based on the fact that the cells to be treated, the endothelial cells, are genetically stable cells that are not likely to mutate into resistant variants. Several arguments against this dogmatic view can be made. Next to the resistance that neutralization or blocking of tumor-derived growth factors may induce, which, as discussed before, is inherent in the drug itself, it has been disputed whether endothelial cells in tumors are indeed genetically stable. Recent studies have demonstrated the presence of genetic abnormalities in tumor-associated endothelium (Hida and Klagsbrun 2005; Streubel et al. 2004), at least suggesting the potential to mutation. A theoretical possibility of generation of resistance is through the phenomenon of vasculogenic mimicry. This is the process of making blood-transporting vascular structures by the tumor cells themselves (Hendrix et al. 2003; van der Schaft et al. 2005; van der Schaft et al. 2004). This process exists in aggressive tumors and potentially makes tumors angiogenesis independent. Treatment with angiogenesis inhibitors will eventually give a growth advantage to the angiogenesis-independent tumor cells (van der Schaft et al. 2004), leading to ineffectiveness of angiogenesis inhibitors.

• Direction of Future Research

At this writing, the first angiogenesis inhibitor has been in the market for 2 years, several new compounds with angiostatic activity have been approved since, and several are in late stages of clinical testing. These compounds have shown proof of concept for applicability of angiogenesis inhibition as a strategy of cancer therapy. It is an attractive idea that these achievements mark the beginning of a useful new strategy of clinical management of cancer. At the same time, we need to question whether the newly approved compounds are tackling the problem at the right level. As mentioned above, blocking (the activity of) tumor-derived growth factors, either by neutralizing anti-growth-factor antibodies or by antagonizing receptor activity, may eventually lead to drug-induced resistance. It is therefore argued that more attention be paid to the development of direct inhibitors of angiogenesis. It is well imaginable that the development of such direct angiogenesis inhibitors is lagging behind because of the lack of knowledge on how these molecules work. For this reason, more research on signaling pathways and their exact working mechanisms is needed.

Although angiogenesis inhibitors are now also used in vascular eye diseases, pathologies that do not depend on systemic treatment, it is expected that clinical experience with angiogenesis inhibitors will come from studies in patients with cancer. Studies with these compounds in cardiovascular diseases, rheumatoid arthritis, and endometriosis, for example, will therefore likely to be limited in the near future.

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