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Angiogenesis: Potentials for Pharmacologic Intervention in the Treatment of Cancer, Cardiovascular Diseases, and Chronic Inflammation

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Abstract——**Angiogenesis, or the formation of new blood vessels out of pre-existing capillaries, is a sequence of events that is fundamental to many physiologic and pathologic processes such as cancer, ischemic diseases, and chronic inflammation. With the identification of several proangiogenic molecules such as the vascular endothelial cell growth factor, the fibroblast growth factors (like in FGFs), and the angiopoietins, and the recent description of specific inhibitors of angiogenesis such as platelet factor-4, angiostatin, endostatin, and vasostatin, it is recognized that therapeutic interference with vasculature formation offers a tool for clinical applications in various pathologies. Whereas inhibition of angiogenesis can prevent diseases with excessive vessel growth such as cancer, diabetes retinopathy, and arthritis, stimulation of angiogenesis would be beneficial in the treatment of diseases such as coronary artery disease and critical limb ischemia in diabetes. In this review we highlight the current knowledge on angiogenesis regulation and report on the recent findings in angiogenesis research and clinical studies. We also discuss the potentials, limitations, and challenges within this field of research, in light of the development of new therapeutic strategies for diseases in which angiogenesis plays an important role.**

I. General Aspects of Angiogenesis

A. Introduction

The formation of new blood vessels out of pre-existing capillaries, or angiogenesis, is a sequence of events that is of key importance in a broad array of physiologic and pathologic processes. Normal tissue growth, such as in embryonic development, wound healing, and the menstrual cycle, is characterized by dependence on new vessel formation for the supply of oxygen and nutrients as well as removal of waste products. Also, a large number of different and nonrelated diseases is associated with formation of new vasculature. Among these pathologies are diseases, such as tissue damage after reperfusion of ischemic tissue or cardiac failure, where angiogenesis is low and should be enhanced to improve disease conditions (Carmeliet et al., 1999; Ferrara and Alitalo, 1999). In several diseases, excessive angiogenesis is part of the pathology. These diseases include cancer (both solid and hematologic tumors), cardiovascular diseases (atherosclerosis), chronic inflammation (rheumatoid arthritis, Crohn's disease), diabetes (diabetic retinopathy), psoriasis, endometriosis, and adiposity. These diseases may benefit from therapeutic inhibition of angiogenesis (Folkman, 1995; Hanahan and Folkman, 1996).

The initial recognition of angiogenesis being a therapeutically interesting process began in the area of oncology in the early 1970s, when Drs. Folkman and Denekamp put forward the idea that tumors are highly vascularized and thereby vulnerable at the level of their blood supply. In those early years, it was already hypothesized that the process of angiogenesis might be a target for therapy. It was only after the discovery of the first compounds with specific angiostatic effects in the early 1990s (Ingber et al., 1990; O'Reilly et al., 1994) that the research field of angiogenesis rapidly expanded and provided an increasing body of evidence that inhibition of angiogenesis could attenuate tumor growth. More recently, novel angiogenesis inhibitors have shown great potential in the treatment of cancer in preclinical studies. Several of those compounds are currently being tested in clinical trials (Molema and Griffioen, 1998). With increasing insight into the role of angiogenesis in other diseases as well, modulation of vascular outgrowth is now also regarded as a therapeutic target in these diseases.

To date, antiangiogenesis therapy is considered, worldwide, a promising approach, supposedly leading to the desperately needed breakthrough in cancer therapy and other proangiogenic diseases. Nevertheless, many questions remain unanswered and many concepts unverified at present. For example, it has to be established whether the exciting effects seen in preclinical investigations using xenogeneic and syngeneic tumor transplant models and transgenic systems also prevail in the human situation. Furthermore, it has been shown that the angiogenesis inhibitors angiostatin and endostatin (O'Reilly et al., 1994, 1997) do not elicit drug-induced resistance on prolonged treatment in tumor-bearing animals, although being highly effective in tumor growth

[2] Abbreviations: BM, basement membrane; Ang-1, angiopoietin-1; BsAb, bispecific antibody; CAI, carboxyamidotriazole; ECM, extracellular matrix; HIF, hypoxia-inducible factor; HUVEC, human umbilical vein endothelial cells; FGF, fibroblast growth factor; FGF-R, FGF receptor; ICAM-1, intercellular adhesion molecule-1; IFN, interferon; IL, interleukin; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MMP, matrix metalloproteinase; NFAT, nuclear factor of activated T cells; NO, nitric oxide; PDGF, platelet-derived growth factor; t-PA/u-PA, tissue type/urokinase plasminogen activator; SPARC, secreted protein acidic and rich in cysteine; TGF, transforming growth factor; TNF, tumor necrosis factor; tTF, truncated tissue factor; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; VEGF-R, VEGF receptor; phVEGF$_{165}$, plasmid-encoding human VEGF$_{165}$ isoform; vWF, von Willebrand factor/factor VIII-related antigen.

reduction. This observation is of extreme importance, because it opens possibilities for long-term treatment or the development of treatment modalities for the prevention of disease in high-risk populations prone to develop tumors. It remains to be seen whether this scenario can be extended to other angiogenesis inhibitors as well as to other proangiogenic diseases of interest. Another important issue is the concept of cancer treatment with angiogenesis inhibitors as a single-compound strategy. Is this a feasible treatment strategy or should antiangiogenic therapy be used in combination with other treatment modalities such as immuno- or chemotherapy? Also, although antiangiogenesis therapy is considered to have low toxicity, there is as yet little information on the safety of therapeutic angiostatic strategies; there is little or no information to what extent inhibition of angiogenesis as tumor treatment will affect normal physiological processes in embryonic development or in wound healing and what the long-term side effects will be.

Although current interest in angiogenesis comes mainly from oncology researchers, also nononcological research fields have now recognized that modulation of angiogenesis may provide a tool for clinical interventions. This demonstrates that angiogenesis is a multidisciplinary theme from a pharmacologic target point of view. In addition, many disciplines of biomedical origin are contributing to basic angiogenesis research, because the processes involved are so complex. In this review, the molecular players of vessel growth, methodology of angiogenesis research, and preclinical and clinical use of angiogenesis as a target for therapy will be discussed.

B. Function of Endothelial Cells in Normal Physiology

The blood vessels in the body have long been considered to merely function as a transport compartment of the blood. Nowadays, it is appreciated that the vasculature is one of the main organs in the body, extending more than 900 m^2 and playing a major role in maintaining the body's integrity in various ways.

Blood vessels consist of endothelial cells that are in direct contact with the blood, and subendothelially located pericytes, smooth muscle cells, fibroblasts, basement membrane (BM),[1] and extracellular matrix (ECM). Depending on the location in the body, the organ microenvironment, the cellular constituents, BM, and ECM of the vasculature differ in phenotype, composition, and function (Rajotte et al., 1998).

The endothelial cells form a monolayer in every single blood vessel in the circulation and are actively involved in several regulatory processes in the body (Fig. 1). Besides being metabolically active and selectively permeable for small solutes and peptides/proteins, they regulate blood coagulation. When their integrity is maintained, endothelial cells exert anticoagulative properties via the synthesis of thrombomodulin, tissue factor (TF) pathway inhibitor and tissue-type plasminogen activator (t-PA). On activation or damage, endothelial cells quickly release proteins like multimeric von Willebrand factor (vWF), which promotes platelet adhesion and aggregation, and plasminogen activator inhibitor-1, a member of the serpin family. In addition, TF expression by endothelium leads to initiation of the extrinsic blood coagulation pathway (Verstraete, 1995). Another important feature of endothelial cells is their ability to direct cells of the immune system to specific sites in the body. Constitutively expressed or cytokine-inducible cellular adhesion molecules [e.g., E-selectin and intercellular adhesion molecule-1 (ICAM-1)] and soluble factors such as chemoattractants, cytokines, and chemokines act in concert to recruit the immune cells to lymphoid organs or inflammatory sites (Carlos and Harlan, 1994). Last, endothelial cells are actively involved in vascular remodeling during, for example, ovulation, wound healing, tumor growth, and diabetic retinopathy. Although complex in regulation and sometimes difficult to functionally analyze in vitro, as well as during disease progression, data have become available that link (parts of) these endothelial cell functions to various steps in the angiogenic cascade.

C. Molecular Control of Angiogenesis

In vasculogenesis during embryonic development, new endothelial cells differentiate from stem cells. In contrast, in angiogenesis new blood vessels mainly emerge from pre-existing ones (Risau, 1997). In adult life, physiologic stimuli during wound healing and the reproductive cycle in women lead to angiogenesis, whereas vasculogenesis is absent. Pathologic conditions such as tumor growth, rheumatoid arthritis, and diabetic retinopathy are characterized by abundant angiogenesis. The active vascular remodeling phase in tumors, e.g., is reflected by the fact that tumor endothelial cells proliferate 20 to 2000 times faster than normal tissue endothelium in the adult (Denekamp, 1984). In the last decade, several molecular players have been identified that significantly contribute to the molecular processes leading to new blood vessel formation. In the following sections, recent advances in this area of research are discussed.

1. Initiation of the Angiogenic Response. Angiogenesis is rapidly initiated in response to hypoxic or ischemic conditions. Vascular relaxation, for example, mediated by nitric oxide (NO) is a prerequisite for endothelial cells to enter the angiogenic cascade. Likely, morphologic changes of the endothelial cells lead to a decrease in confluency status to make them susceptible to mitogens (Folkman, 1997). In all types of angiogenesis, either under physiologic or pathologic conditions, endothelial cell activation is the first process to take place. Cytokines from various sources are released in response to hypoxia or ischemia. It is suggested that vascular endothelial growth factor (VEGF) is a major player in angiogenesis initiation based on its ability to induce

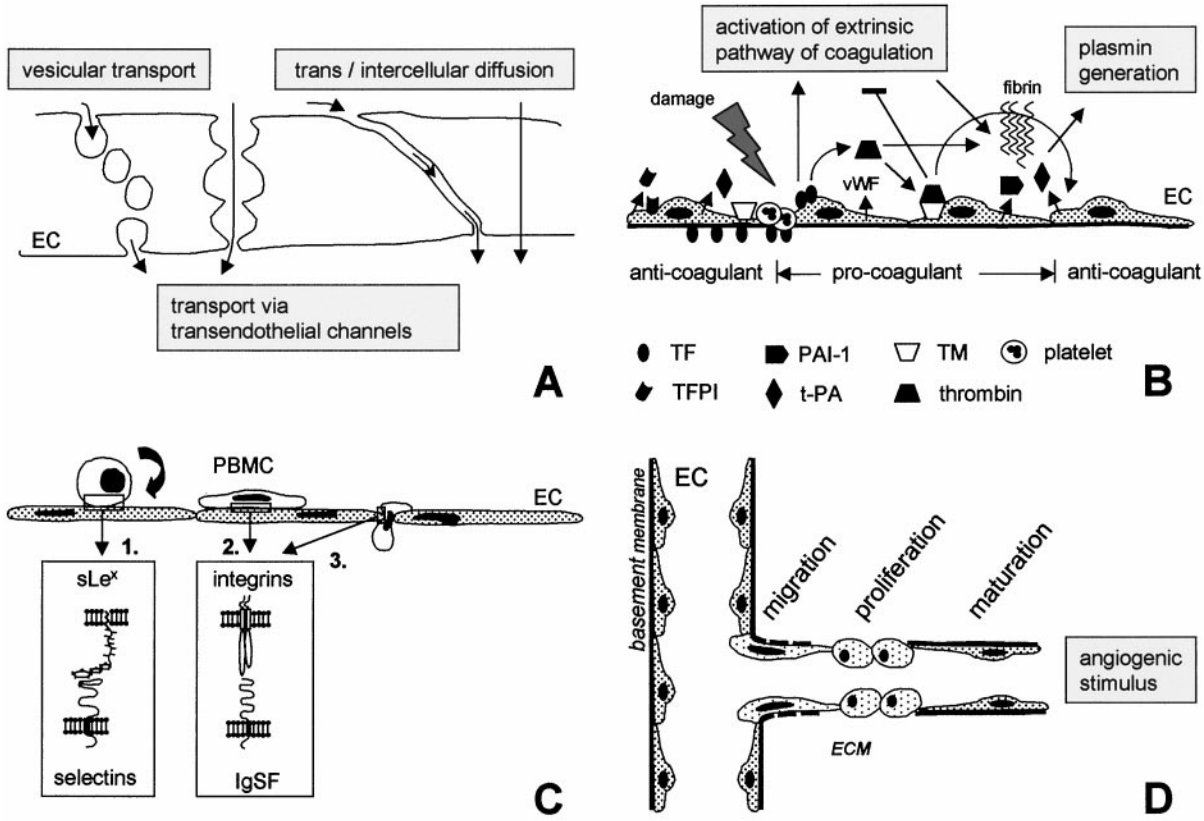

FIG. 1. Endothelial cells exert several important functions in the body. A, the endothelium forms a semipermeable barrier for the transport of blood-borne peptides, proteins, and other soluble molecules to underlying tissue; B, via the regulated expression of pro- and anticoagulative activities, endothelium actively participates in the hemostatic balance in the body; C, under the influence of proinflammatory cytokines, endothelial cells up-regulate a variety of cellular adhesion molecules to tether and activate leukocytes and facilitate leukocyte adhesion and transmigration from the blood into the tissue; D, during wound healing and tumor growth, among others, angiogenesis takes place. In this process, an active role exists for endothelial cells. EC, endothelial cells; IgSF, Ig superfamily; PAI-1, plasminogen activator inhibitor; PBMC, peripheral blood mononuclear cells; sLe^x, sialyl Lewis X; TFPI, Tissue Factor pathway inhibitor; TM, thrombomodulin.

vasodilation via endothelial NO production and its endothelial cell permeability increasing effect (Ziche et al., 1997). This allows plasma proteins to enter the tissue to form a fibrin-rich provisional network (Dvorak, 1986). The observation that VEGF production is under control of hypoxia inducible factor (HIF) strengthens the suggestion of an early involvement of VEGF in the angiogenic response. Moreover, VEGF receptor (VEGFR) expression is up-regulated under hypoxic or ischemic conditions as well (Forsythe et al., 1996).

VEGF is abundantly produced by hypoxic tumor cells, macrophages and other cells of the immune system (Brown et al., 1997). Besides affecting vasodilation and vascular permeability, VEGF can induce the expression of proteases and receptors important in cellular invasion and tissue remodeling and is able to prevent endothelial cell apoptosis (Ferrara and Keyt, 1997; Gupta et al., 1999). That angiogenesis is not completely dependent on VEGF production was recently shown by Hansen-Algenstaedt et al. (1999); the consequences of which will be discussed in more detail in *Section V*. For a more detailed overview on the role of VEGF in the regulation of angiogenesis, the reader is referred to a recently published review by Ferrara (1999).

After proper activation of the endothelial cells, endothelial penetration into new areas of the body is achieved by degradation of the BM by matrix metalloproteinases (MMPs). These extracellular endopeptidases are secreted as zymogens that become activated in the ECM compartment and subsequently selectively degrade components of the ECM (Stetler Stevenson, 1999). They are produced by a variety of cells, including epithelial cells, fibroblasts, inflammatory cells, and endothelial cells. MMP activity and, hence, angiogenesis is counteracted by the family of tissue inhibitors of metalloproteinase (TIMPs) (Gomez et al., 1997; Valente et al., 1998).

2. Endothelial Cell Migration and Proliferation. Plasminogen activators u-PA and t-PA convert the ubiquitous plasma protein plasminogen to plasmin. Plasmin has a broad trypsin-like specificity and degrades, e.g., fibronectin, laminin, and the protein core of proteoglycans. In addition, plasmin activates certain metalloproteinases. Plasmin is believed to be the most important protease for the mobilization of fibroblast growth factor-2 (FGF-2 or basic FGF) from the ECM pool.

FGF members are directly acting proangiogenic molecules. FGF-2 consists of, in two modifications, an 18-

kDa low-molecular weight form and a 22- to 24-kDa high-molecular weight form. During angiogenesis, low-molecular weight FGF-2 binding to endothelium induces FGF receptor (FGF-R) down-regulation, increased motility, proliferation and proteinase activity, and modulates integrin levels. High-molecular weight FGF-2 may act on endothelial cell proliferation after nuclear translocation in the endothelial cells (Gleizes et al., 1995; Klein et al., 1997). Recently, it was shown that a secreted FGF-2-binding protein could bind FGF-2 that is normally inactive due to strong adherence to heparan sulfate proteoglycans in the ECM. The displaced FGF-2 molecules were thus released to mediate biological function. Of note is the observation in this and other studies that angiogenesis seems exquisitely sensitive to small changes in factors such as VEGF and FGF-2 that drive the angiogenic process. This may have important therapeutic implications in treating angiogenesis-driven disorders (Czubayko et al., 1997). Besides its effect on angiogenesis initiation, VEGF also affects endothelial cell proliferation. This effect can be (partly) attributed to NO and cGMP-mediated activation of the mitogen-activated protein kinase (MAPK) family (see *Section II.B.* for a more detailed description on VEGF and FGF-2-mediated signal transduction and cell activation pathways).

Integrins are transmembrane proteins composed of an α and β subunit in over 20 different heterodimeric combinations. They bind to ECM proteins or cell surface ligands through short peptide sequences. Combinations of different integrins on cell surfaces allow cells to recognize and respond to a variety of different ECM proteins (Varner, 1997). They are able to transduce signals from within the cells to the outside as well as from the outside into the cell (Aplin et al., 1998). Integrin-mediated cell adhesion impacts two key aspects of growth regulation. First, it can influence the activity of the basal cell cycle machinery consisting of cyclin-dependent kinase complexes. Second, integrins play a pivotal role in anchorage-dependent cell death or anoikis (Frisch and Ruoslahti, 1997; Howe et al., 1998). Integrin $\alpha_v\beta_3$ mediates cellular adhesion to vitronectin, fibrinogen, laminin, collagen, vWF, or osteopontin through their exposed tripeptide Arg-Gly-Asp (RGD) moiety (Cheresh, 1993). $\alpha_v\beta_3$ is minimally expressed on normal resting endothelium, but significantly up-regulated on activated endothelium and is believed to play a critical role in angiogenesis. Both peptide and antibody inhibitors of $\alpha_v\beta_3$ induced endothelial cell apoptosis, suggesting a role for this integrin in endothelial cell survival during angiogenesis (Brooks et al., 1994a). Another α_v integrin associated with angiogenesis is $\alpha_v\beta_5$. Whereas FGF-2 or tumor necrosis factor-α (TNF-α) induced $\alpha_v\beta_3$-dependent angiogenesis in vivo, VEGF or transforming growth factor-β (TGF-β) initiated an angiogenesis pathway merely dependent on $\alpha_v\beta_5$ (Friedlander et al., 1995).

Components of the ECM also contribute to the regulation of endothelial cell morphology and function. Thrombospondin, for example, inhibits endothelial cell proliferation when added in soluble form. When endothelial cells on the other hand are plated on matrix bound thrombospondin, they become more permissive for proliferative signals. Furthermore, through binding to and activation of TGF-β and affecting protease activity, thrombospondin may be able to influence cell growth, migration, and differentation as well (DiPietro, 1997). In patients with invasive bladder cancer, low-thrombospondin expression in the tumor was associated with increased recurrence rates, decreased overall survival, and high-microvessel density counts. These data are suggestive of an antiangiogenic role for this ECM constituent under physiologic conditions (Grossfeld et al., 1997). Laminin is another ECM protein with functions in endothelial cell attachment, growth promotion, protease secretion, and interactions with other ECM components. Laminin can bind to cell surface-binding proteins including integrins, which leads to integrin signaling (Grant and Kleinman, 1997). SPARC (secreted protein acidic and rich in cysteine), also known as BM40 or osteonectin, is a protein of which the expression is elevated under stress conditions such as endotoxin stimulation, heat shock, and sparse cell density. SPARC overexpression has been observed in tumors such as human esophageal carcinoma and cutaneous malignant melanoma (Porte et al., 1998; Massi et al., 1999). Furthermore, transient expression of SPARC during endothelial cell injury and cellular activation indicated a role in tissue repair, remodeling and angiogenesis (Jendraschak and Sage, 1996).

3. Maturation of the Neovasculature. Endothelial cell interaction with ECM and mesenchymal cells is a prerequisite to form a stable vasculature. Therefore, after endothelial cell proliferation and maturation, and the formation of endothelial tube structures, surrounding vessel layers composed of mural cells (pericytes in small vessels and smooth muscle cells in large vessels), need to be recruited. Endothelial cells may accomplish this via the synthesis and secretion of platelet-derived growth factor (PDGF), a mitogen and chemoattractant for a variety of mesenchymal cells. Subsequent differentiation of the mural precursor cells into pericytes and smooth muscle cells is believed to be a cell-cell contact-dependent process. On endothelial cell-mural cell contact, a latent form of TGF-β, produced by both endothelium and mural cells, is activated in a plasmin-mediated process. Activated TGF-β can induce changes in myofibroblasts and pericytes, which may contribute to the formation of a quiescent vessel, ECM production, and maintenance of growth control. The coinciding investment of growing capillaries by pericytes with the deposition of BM and cessation of vessel growth during wound healing also indicates vessel growth regulation by pericytes (Hirschi and D'Amore, 1997). FGF-1 is also implicated in endothelial cell differentiation leading to vascular tube formation. Besides inducing plasminogen

activator and endothelial cell proliferation and migration, FGF-1 receptor signaling resulted in endothelial tube formation in collagen (Kanda et al., 1996).

Angiopoietins and receptor tyrosine kinase Tie1 and Tie2 play critical roles in the later stages of angiogenesis as well. They are required for communication of endothelial cells with the surrounding mesenchyme to establish stable cellular and biochemical interactions (Maisonpierre et al., 1997). Tie1 function is related to endothelial cell differentiation and the establishment of blood vessel integrity. Tie2, on the other hand, is particularly important for vascular network formation (Dumont et al., 1994; Puri et al., 1995; Sato et al., 1995). Tie2 expression is restricted to the endothelial cells. Surprisingly, Tie2 was present on quiescent as well as angiogenic endothelium in the adult rat. Moreover, the receptor tyrosine kinase was constitutively phosphorylated in both types of vasculature. These data suggest that Tie2 has a dual function involving both angiogenesis and vascular maintenance (Wong et al., 1997). Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are Tie2-specific ligands that activate or antagonize Tie2 signaling in endothelium, respectively. In postnatal neovascularization, Ang-1 is likely to promote vascular network maturation (see *Section II.B*. for a more detailed description of Ang-1 as a proangiogenic protein for therapeutic purposes). In contrast, Ang-2 rendered endothelium sensitive to angiogenic factors via induction of smooth muscle cell/pericyte loss and hence destabilized the neovasculature (Maisonpierre et al., 1997; Asahara et al., 1998). The observation that Ang-2 was able to phosphorylate Tie2 when expressed by fibroblasts, indicates that in endothelial cells other regulatory mechanism(s) prevail leading to antagonistic activity. Whereas Ang-1 is widely expressed, Ang-2 is only found at sites of vascular remodeling. Here it may block vessel stabilization, maturation, or survival signals from Tie2 (Maisonpierre et al., 1997; Korpelainen and Alitalo, 1998). In human glioblastomas, a cell-specific up-regulation of Tie2, Ang-1, and Ang-2 during tumor progression was demonstrated in a pattern compatible with a role in tumor-induced angiogenesis (Stratmann et al., 1998). Using homology based cloning, two new members of the angiopoietins, Ang-3 (mouse-specific) and Ang-4 (human-specific) were identified. They are distributed differently in the respective species, where Ang-3 acts as an antagonist and Ang-4 as an agonist of receptor tyrosine kinase signaling. Their respective roles in vascular maintenance have not been established yet (Valenzuela et al., 1999).

4. Other Mechanisms Implicated in Angiogenesis Control. Although the roles of several factors during angiogenesis have been discussed here separately, it is important to note that the activity of an angiogenesis-regulating cytokine depends on the presence and concentration of other factors or cytokines in the environment of the responding endothelium (Pepper et al., 1998). For example, exogenous factors such as hormones can affect conditions leading to angiogenesis (Schiffenbauer et al., 1997). Isoforms of VEGF that bind to ECM-associated heparan sulfate proteoglycans can release ECM-stored FGF-2 in a bioactive form (Jonca et al., 1997), and angiopoietins potentiate the effects of VEGF (Asahara et al., 1998).

Although their relative role in angiogenesis is not yet fully elucidated, it is now well appreciated that cells of the immune system such as monocytes/macrophages, lymphocytes, and mast cells can affect pro- and antiangiogenic balances (Sunderkotter et al., 1996; Blair et al., 1997). T lymphocytes were able to activate endothelial expression of various metalloproteinases via CD40/CD40-ligand interactions. As a consequence, increased tube formation in a three-dimensional gel was observed (Mach et al., 1999). Based on the effect of cells of the immune system on angiogenic parameters and the overt neovascularization in chronic inflammatory diseases, antiangiogenic strategies were put forward as treatment modalities for these diseases as well (see *Section IV*).

Recently, Keshet and coworkers identified the importance of the presence of periendothelial cells in the microenvironment as a control mechanism of angiogenesis. Loss of VEGF by androgen ablation therapy led to selective apoptosis of endothelial cells in vessels devoid of periendothelial cells. Based on this observation, it is now hypothesized that VEGF is required to maintain cell anchorage to a provisional ECM until periendothelial cells facilitate a more permanent mode of adhesion (Benjamin et al., 1999).

Besides the already mentioned proangiogenic factors, VEGF, FGF-1, and FGF-2, many others have now been identified in various settings of physiologic and pathologic angiogenesis. Among them are TGF-α and TGF-β, granulocyte macrophage-colony-stimulating factor, epidermal growth factor, interleukin-1 (IL-1), scatter factor, platelet-activating factor, IL-8, and substance P (Bouck et al., 1996; Yoshida et al., 1997). Their effects can be either directly or indirectly on the endothelium via activation of surrounding cells to produce other factors with proangiogenic activity or modulation of receptors/receptor activities (Yoshida et al., 1997; Giraudo et al., 1998).

Hypoxia is an important environmental factor that leads to neovascularization. In the case of tumor growth, however, cancer-causing genetic changes, possibly in conjunction with environmental influences, are able to induce angiogenesis as well (Rak et al., 1995; Okada et al., 1998). Many oncogenes, among which *c-myb, sis,* and *src*, were shown to stimulate the expression of a wide variety of molecules that induce angiogenesis. Furthermore, mutant *ras* oncogenes strongly up-regulated the proangiogenic factors TGF-α, TGF-β, and VEGF. Activated oncogenes can also indirectly contribute to the angiogenic phenotype by affecting the production and activation of BM and ECM-degrading enzymes (Bouck et

al., 1996; Okada et al., 1998). Tumor suppressor genes have now also been identified to play a role in angiogenic activities of cells. Inactivation of p53, for example, downregulated the antiangiogenic ECM component thrombospondin (Dameron et al., 1994; Grossfeld et al., 1997). In nonsmall cell lung cancer, loss of p53 was associated with a high-vascular maturation index (Kakolyris et al., 1999). Besides the involvement of tumor cell-associated changes in p53, this tumor suppressor gene also plays a role in endothelial cell-mediated control of angiogenesis. Adenovirus-mediated overexpression of endothelial p53 inhibited human umbilical vein endothelial cells (HUVEC) proliferation and capillary network formation in vitro (Riccioni et al., 1998). In endothelial cells existing in atherosclerotic and normal human aorta, variations in p53 expression levels could be detected. Moreover, the multinucleated variant endothelial cells expressed a mutant p53 type, which may be indicative for loss of endothelial cell growth control (Satoh et al., 1998). As with tumor cells, it is most likely that in vivo a combination of mutations in various tumor suppressor genes and oncogenes leads to a proliferative proangiogenic character of the endothelial cells.

Most of the experimental data on angiogenesis control published so far deal with angiogenesis in cancer. The advances made in this area are of prime importance for understanding molecular players involved in the regulatory pathways. It should be realized, however, that the process of angiogenesis may be differentially regulated in the various disease settings. Therefore, care should be taken in extrapolating data on, e.g., regulatory pathways and their activators and inhibitors from these tumor growth-related experiments to other diseases.

II. Angiogenesis Stimulation

Much attention has been payed to therapeutic strategies that are able to stop the angiogenic cascade in tumor growth (see *Section III*) and more recently, in chronic inflammatory situations such as rheumatoid arthritis (see *Section IV*). There are, however, various diseases affecting millions of people every year that would benefit from the induction of angiogenesis, so-called therapeutic angiogenesis (Takeshita et al., 1994). Although the number of studies reported in this area of research are not nearly as high as the number of studies on antiangiogenic therapies, the approach appeared to be quite successful in a preclinical setting as well as in the recently performed first clinical trials.

A. Target Diseases for Angiogenesis Stimulation

The disease conditions that may benefit from therapeutic angiogenesis encompass ischemic diseases such as ischemic coronary artery disease, critical limb ischemia with various etiologies, and decubitus. In these diseases, functional blood flow is partially lost in an organ or limb. For coronary artery disease, the leading cause of morbidity and mortality in Western countries, the therapeutic options (reducing the risk factors, restoration of the blood flow by angioplasty, or coronary bypass grafting), are insufficient. In critical limb ischemia, estimated to develop in 500 to 1000 individuals per million per year, the anatomic extent and the distribution of the arterial occlusions render the patients unsuitable for operative or percutaneous revascularization. At present, no pharmacologic treatment could favorably affect the ischemia. Often, loss of the limb by amputation is the recommended treatment for these patients (Baumgartner et al., 1998). A specific form of vascular occlusive disease that leads to critical limb ischemia, is thromboangiitis obliterans, or Buerger's disease. The disease afflicts arteries of young smokers and is characterized by the onset of distal extremity ischemic symptoms, leading to ulceration and gangrene (Isner et al., 1998). Gastroduodenal ulcers, also caused by local insufficient perfusion, have been subject of angiogenesis stimulation therapies (Wolfe et al., 1995). It has recently been suggested that for congestive heart failure, possibly a result of myocardial ischemia, stimulation of angiogenesis may also become a therapeutic option (Carmeliet et al., 1999; Isner and Losordo, 1999).

The treatment of arterial occlusions by balloon angioplasty is frequently associated with delinquent re-endothelialization and smooth muscle cell proliferation. One therapeutic option to reduce subsequent intimal thickening is the induction of apoptosis in infiltrating immune cells (Sata et al., 1998). Therapeutic angiogenesis to facilitate endothelial cell regeneration in this specific pathology has been proposed as well (Callow et al., 1994; Asahara et al., 1996).

In the case of organ transplantation, surgical procedures dictate loss of vessel integrity and function of the transplanted organ (Taub et al., 1998). Transplantation of encapsulated pancreatic islets as a treatment modality for type I diabetes, for example, may be more successful when prevascularized solid supports are used or solid supports are pretreated with proangiogenic factors (de Vos et al., 1997).

B. Proangiogenic Compounds

Ischemic diseases from different etiologies may improve when treated with agents that induce neovascularization. Although a vast number of proangiogenic factors are available (see *Section I.C*), to date mostly VEGF and FGF-2 have been explored for this purpose. More recently, the proangiogenic protein angiopoietin-1 (Ang-1), ligand for the Tie2 receptor on endothelium, has been applied in therapeutic angiogenesis strategies as well.

1. Vascular Endothelial Growth Factor. Angiogenesis is driven by numerous mediators produced by numerous cells under a variety of conditions. These mediators are either soluble, ECM or membrane bound growth factors, or components of the ECM itself. Of the soluble factors, one of the best studied and the most

potent proangiogenic factor is VEGF, discovered in the early eighties by Dvorak and colleagues (Senger et al., 1983). VEGF (also known as VEGF-A) isoforms VEGF-121, -145, -165, -183, -189, and -205 are a result of alternative splicing from a single VEGF gene located on chromosome 6 (Mattei et al., 1996). Together with VEGF-B, -C, and -D, they belong to the VEGF/PDGF super family. Recently, a viral VEGF family member, designated VEGF-E, was described (Meyer et al., 1999). The two VEGF-specific tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), are expressed on vascular endothelium, and to a lesser extent on monocytes/macrophages and certain tumor cell types. VEGFR-3 (Flt-4), which binds VEGF-C and VEGF-D, is mainly expressed on lymphatic endothelium (Kaipainen et al., 1995). Interaction of VEGF with VEGFR-2 is a critical requirement to induce the full spectrum of VEGF biologic responses. Intracellular signal transduction pathways in endothelial cells through VEGFR-2 dimerization lead to permeability enhancement, cellular proliferation, and migration, as schematically shown in Fig. 2 (Abedi and Zachary, 1997; Kroll and Waltenberger, 1997; Wheeler Jones et al., 1997; Ziche et al., 1997; Gerber et al., 1998; Hood and Granger, 1998; Wellner et al., 1999; Doanes et al., 1999; Shen et al., 1999; Yu and Sato, 1999). All these studies were performed in vitro, exploiting either HUVEC or vascular endothelium from bovine or porcine origin. Evidence for at least partly similar VEGFR-mediated signal transduction pathways in vivo was recently provided using intact microvessels of mouse mesentery (Mukhopadhyay et al., 1998).

Under conditions of serum starvation, the sustained activation of c-Jun NH_2 terminal kinase (JNK) or stress-activated protein kinase (SAPK) could be counteracted by VEGF-mediated activation of MAPK, leading to the prevention of apoptosis in microvascular endothelial cells (Gupta et al., 1999). VEGF-mediated signaling was also able to confer a proliferation inhibitory signal in endothelium through regulating cell cycle progression by p38 MAPK activation (Yu and Sato, 1999) to induce endothelial expression of ECM-degrading enzymes and to recruit pericytes (Puri et al., 1995; Lamoreaux et al., 1998). VEGFR-2 and endothelial NO synthase colocalized with caveolin in plasma membrane caveolae, suggestive of VEGF signaling events within the caveolar compartment of endothelium (Feng et al., 1999).

JNK kinases participate in cellular processes via the modulation of transcriptional activation factors such as AP-1. Other transcriptional activation factors downstream of the VEGF signal transduction pathways shown to be functional in endothelial cells are nuclear factor of activated T cells (NFAT) and nuclear transcription factor ETS (Chen et al., 1997). Whereas AP-1 and NFAT switch on genes regulating tissue factor expres-

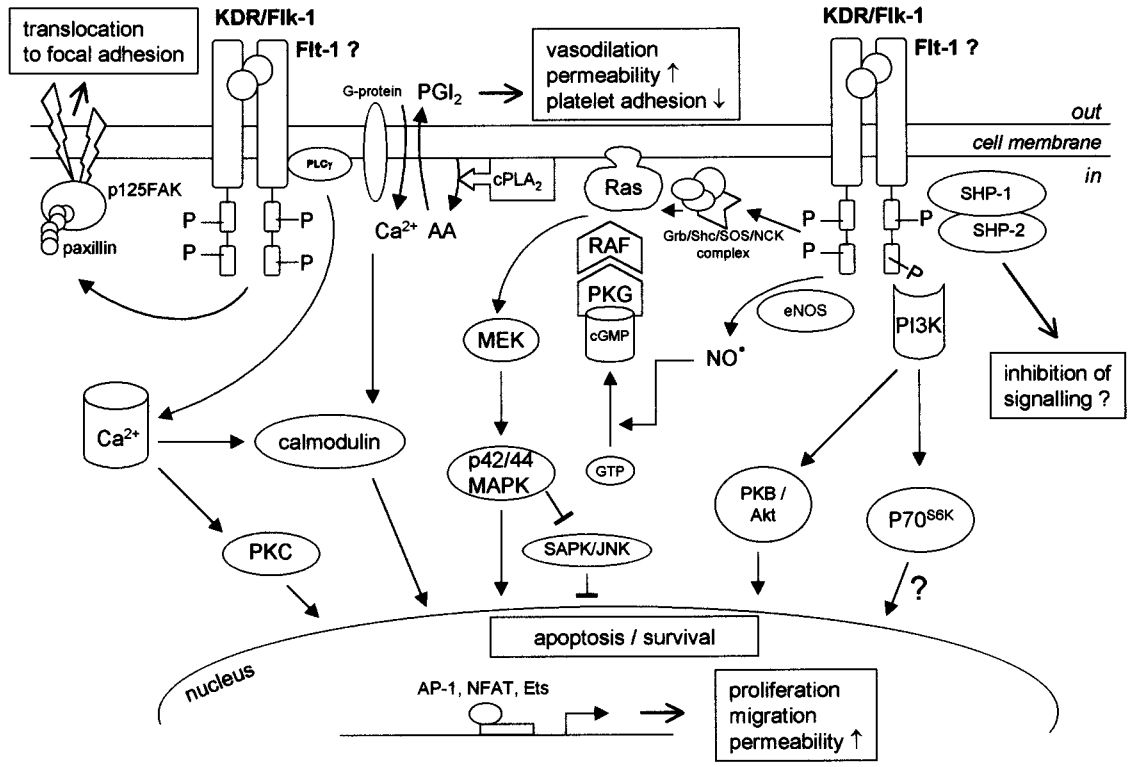

FIG. 2. Dimerization of VEGFR-2 (KDR/flt-1) or VEGFR-1 (Flt-1) leads to a series of events in endothelial cells in vitro. Via a sequence of intracellular signal transduction steps, VEGFR signaling induces permeability increases, endothelial cell proliferation and migration, and cell survival. AA, arachidonic acid; AP-1, activator protein-1; $cPLA_2$, cytoplasmic phospholipase A_2; eNOS, endothelial NO synthase; FAK, focal adhesion kinase; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PKG, protein kinase G; PLCγ, phospholipase C-γ; SAPK/JNK, stress-activated protein kinase/c-Jun NH_2 terminal kinase.

sion upon VEGF activation, ETS regulates the expression of u-PA, MMPs, and integrin 3 (Iwasaka et al., 1996; Oda et al., 1999).

The pleiotropic effects of VEGF in vitro on endothelial cell growth, proliferation and migration, among others, have now been extensively documented by many groups. However, plasma leakage and subsequent fibrin formation is one of the hallmarks of angiogenesis initiation in solid tumor growth and initiation of wound healing. The fibrin provides a functional matrix for endothelial cells to become activated, to proliferate, and to migrate (Dvorak, 1986). Therefore, it may well be that in vivo the permeability inducing capacity of VEGF is its most important function in regulating physiologic and pathologic angiogenesis.

2. Fibroblast Growth Factors. Members of the FGF family are also potent inducers of angiogenesis. Cellular responses mediated by FGFs include cell migration, proliferation, and differentiation (Kanda et al., 1997). The FGF family consists of nine structurally related polypeptides, of which FGF-1 (acidic FGF) and FGF-2 (basic FGF) are the most extensively studied. Both FGF-1 and FGF-2 are devoid of a signal sequence for secretion. Export from cells without compromising cell integrity or requiring cell death possibly follows a nonclassical, synaptotagmin-1-dependent exocytotic pathway (LaVallee et al., 1998).

The cellular effects of FGFs are mediated via specific binding to high-affinity tyrosine kinase receptors (Klein et al., 1997). In addition, low-affinity FGF receptors exist, that consist of polysaccharide components of heparan sulfate proteoglycans on cell surfaces and in ECM. Binding to the latter receptors has been proposed as a mechanism to stabilize and protect FGF from inactivation. Heparan sulfate on cell surfaces, on the other hand, plays a more active role in displacing ECM-bound FGF-2 and subsequent presentation to the high-affinity signal transducing receptors (Miao et al., 1996).

Receptor dimerization by FGF is facilitated by heparin. It results in protein kinase activity and receptor autophosphorylation. As with VEGFR signaling, this autophosphorylation enables adaptor proteins such as Grb2, Shc, and Nck to bind and subsequently activate the Ras/Raf-MAPK pathway of endothelial cell proliferation activation (Klein et al., 1997). p42 MAPK activation was also implicated in endothelial cell motility regulatory responses to FGF. p42 MAPK driven phosphorylation of cytoplasmic phospholipase-A_2 enabled arachidonic acid release upon FGF-2 activation of bovine aortic cells (Sa et al., 1995). Besides activating MAPK-mediated cell proliferation, FGF-2 induced murine brain endothelial cell proliferation via a serine/threonine kinase that phosphorylates ribosomal protein S6 ($p70^{S6K}$). This proliferation activation route was restricted to endothelial cells cultured on fibronectin. When allowed to differentiate to form tube-like structures in collagen gels, $p70^{S6K}$ was not activated (Kanda et al., 1997).

In addition to initiating receptor signaling, FGF-1 can be endocytosed and transported to the cell nucleus. This transport affects the cell cycle in the late G_1 stage, promoting transition to the S stage (Imamura et al., 1994). Entrance of FGF-2 into the nucleus correlated with phosphorylation of nucleolin and subsequent increases of rDNA transcription, likely to be mediated by the protein kinase CKII (Bouche et al., 1994). FGF-2 uptake by endothelial cell was furthermore shown to be a route of growth factor degradation and can therefore act to regulate FGF-2 activity (Gleizes et al., 1995).

3. Angiopoietin-1. Ang-1 is an endogenously secreted glycoprotein of approximately 75 kDa. Its receptor, Tie2, is generally restricted to the endothelium and of importance in angiogenesis during development, tumor growth, and wound healing (Sato et al., 1995; Lin et al., 1997; Wong et al., 1997; Stratmann et al., 1998). In vitro, Ang-1 stimulated tyrosine phosphorylation of Tie2 in endothelial cells, inhibited serum starvation-induced endothelial apoptosis, induced sprouting angiogenesis, and stabilized HUVEC network organization (Koblizek et al., 1998; Korpelainen and Alitalo, 1998; Kwak et al., 1999). When combined with other angiogenic factors such as VEGF or growth factor supplements containing FGF-1, the survival of both endothelial cells and vascular networks increased even more (Kwak et al., 1999; Papapetropoulos et al., 1999). Although being chemotactic for endothelial cells and Tie2-transfected fibroblasts, no mitogenic responses of endothelial cells to Ang-1 could be observed (Koblizek et al., 1998; Witzenbichler et al., 1998).

C. Effects of Angiogenesis Stimulation in Preclinical Studies

In various animal models, the effects of either plasmid DNA encoding angiogenic factors or their respective protein have been studied. In pigs, gradual narrowing of the coronary artery leading to complete blood vessel occlusion was induced by use of an ameroid constructor. In this model, the continuous perivascular administration of VEGF via an osmotic pump, or FGF-2 containing heparin-alginate microspheres, led to improved resting and stress-induced collateral blood flow values. Although the number of (vWF positive) blood vessels in nonischemic heart tissue was unchanged, the vessel density significantly increased in the ischemic areas (Lopez and Simons, 1996).

In a mouse model of hindlimb ischemia, created by femoral artery ligation, the question of whether diabetes leads to impaired neovascularization was addressed, because diabetes is a major risk factor for artery diseases. It was shown, that nonobese diabetic mice exerted a lower angiogenic response in ischemic tissue compared with normal mice. This impaired response could be reduced by intramuscular (i.m.) gene therapy with recombinant adenovirus expressing murine VEGF cDNA (Rivard et al., 1999). Local administration of a plasmid

encoding the 165-amino acid isoform of human $VEGF_{165}$ ($phVEGF_{165}$) during balloon cathetherization of the femoral arteries of rabbits resulted in an increased rate of re-endothelialization. This effect was also observed in the contralateral femoral artery that underwent simultaneous balloon injury but was not transfected. As a result of the increased re-endothelialization, the intimal thickening was diminished in both limbs, thrombotic occlusions were less frequent, and recovery of the endothelial cell-dependent vasomotor reactivity was accelerated (Asahara et al., 1996).

For reconstructive surgery and organ transplantation procedures, hypoxia or ischemia of the organs will negatively influence organ viability and function. The local administration of VEGF cDNA or FGF-2 protein into ischemic experimental skin flaps in rats and rabbits, respectively, significantly enhanced survival time of isolated skin flaps after 1 week (Hickey et al., 1998; Taub et al., 1998). In the case of a solid support system exploited for the grafting of, e.g., pancreatic islets as a means of bioartificial organ development, incorporation of FGF-1 led to ingrowth of blood vessels 4 weeks after implantation under the liver. Engrafting of pancreatic islets into these FGF-1 prevascularized solid support systems resulted in a better survival of the graft compared with islets engrafted without a solid support, although islet function was somewhat less than in normal rats (de Vos et al., 1997). This study indicates that therapeutic angiogenesis may also have a potential in organ transplantation and bioartificial organ development. It should be realized, however, that in the case of allotransplantation, immune cell activation will occur. Increased neovascularization may facilitate immune cell infiltration by virtue of the fact that more blood vessels allow better access to the graft. On the other hand, endothelium under the influence of proangiogenic factors may exhibit impaired leukocyte recruitment functions (see *Section IV.A*).

After i.m. administration of a plasmid encoding the human Ang-1 gene in a rabbit ischemic hindlimb model, human Ang-1 mRNA could be detected 3 to 14 days after gene transfer. No mRNA was found in sites distant from the ischemic hindlimb. Both the angiographic score and the capillary density were increased in the hindlimb 40 days after Ang-1 encoding plasmid administration (Shyu et al., 1998).

The increase in re-endothelialization of balloon injured vessels in the femoral artery that was not treated with the $phVEGF_{165}$ cDNA (Asahara et al., 1996), poses the question whether angiogenic therapy for a specific purpose may affect other sites in the body as well. For example, the question comes to mind whether therapeutic angiogenesis in a patient with myocardial ischemia is able to induce angiogenesis in an otherwise dormant, undetected, tumor nodule. Until now, however, laboratory studies did not demonstrate that stimulation of angiogenesis alone was sufficient for malignant growth (Isner et al., 1996).

D. First Clinical Studies on Angiogenesis Stimulation

So far, results are available from several pilot studies on the clinical application of therapeutic angiogenesis. In all three studies described, naked plasmid DNA encoding human $phVEGF_{165}$ under the cytomegalovirus promoter/enhancer was administered. In the case of ischemic limbs in patients with critical limb ischemia or thromboangiitis obliterans, the DNA was i.m. injected in the ischemic limb (Baumgartner et al., 1998; Isner et al., 1998). The DNA was administered directly in the myocardium of patients suffering from myocardial ischemia. In patients suffering from myocardial ischemia, the DNA was administered directly in the myocardium (Losordo et al., 1998).

Using contrast angiography, newly formed collateral blood vessels could be visualized in critical limb ischemia and thromboangiitis obliterans patients treated with $phVEGF_{165}$. Ischemic ulcers markedly improved or healed, resulting in successful limb salvage in several patients. Documented adverse effects were transient ankle or calf edema in some limbs. Patients suffering from myocardial ischemia had significant reduction in angina and reduced ischemia after $phVEGF_{165}$ treatment.

VEGF was also administered as a protein to patients with angina. Although the 120-day follow-up showed promising results, the 60-day follow-up showed no difference in exercise time or improvement of angina compared with placebo. An unexpected improvement in the placebo group may be the reason for this result. Furthermore, a clinical study on the applicability of FGF-2 in a similar patient group has started. Results are expected to be presented early 2000 (anonymous, 1999a).

It was concluded from these preliminary studies that therapeutic angiogenesis was able to induce neovascularization, and if instituted at the proper time, it may improve ischemic disease conditions in humans. The finding that endothelial progenitors can be isolated from human peripheral blood opens another possibility to augment collateral vessel growth to ischemic tissue (Asahara et al., 1997). The homing potential of these progenitors to foci of angiogenesis may be exploited for their application as autologous vectors for gene therapy with, e.g., cDNA encoding VEGF after angiogenesis induction with nontargeted plasmid DNA.

III. Angiogenesis Inhibition

A. Angiogenesis and Cancer

Virchow was among the first to demonstrate the high vascularization in tumors in his publication *Die Krankhaften Geschwülste* published in 1863. He suggested that this was associated with the disorganized nature of tumor cells. The origin of the observed blood vessels was uncertain by then, it either developed from

the transformed tumor cells or, alternatively, from normal cells that had been derived from the neighboring benign tissues. In a later period it was suggested that the trigger for enhanced blood vessel growth in tumors emanated from the invading malignant cells. It was proposed that the ability to attract new vasculature from the host was a characteristic feature of tumor cells. Recently, two paradigms were added to the vascular processes thought to prevail in tumor-induced neovascularization. During vessel co-option, tumors will initially exploit the host vasculature for survival, which coincides with host vasculature regression. Ongoing tumor cell growth will subsequently lead to initiation of angiogenesis (Holash et al., 1999). Furthermore, circulating endothelial progenitor cells can form an additional source for postnatal vasculogenesis in tumor growth (Asahara et al., 1999).

Since then many researchers have studied angiogenesis in a variety of test systems. Only after it was recognized that new vessels at the tumor site were absolutely required for solid tumor expansion beyond the size of approximately 1 to 2 mm in diameter, it was suggested that the process of angiogenesis might be a target for therapy. Recent studies have demonstrated that lymphoproliferative diseases, such as leukemia and lymphoma, are dependent on angiogenesis as well. Elevated expression of FGF and VEGF has been observed in acute myeloid leukemia, acute lymphoblastoid leukemia, and lymphomas (Fiedler et al., 1997; Foss et al., 1997; PerezAtayde et al., 1997). These studies indicate, therefore, that angiogenesis might also be a therapeutical target for hematologic tumors.

The first molecule identified as an angiogenic factor was described in 1984 (Shing et al., 1984) after which a large number of angiogenic factors followed. These factors can be produced by the tumor cells themselves, cells present in the tumor stroma such as fibroblasts, smooth muscle cells, or by infiltrating immune cells. To complicate matters, these cells are all able to produce angiogenesis inhibitors as well. More recent attention has been paid to the isolation and characterization of these angiogenesis inhibitors because they may have potential as therapeutic agents. Most of them have been studied for their applicability in cancer therapy but may also be suitable for the therapy of chronic inflammatory conditions (see *Section IV.C*).

B. In Vitro and in Vivo Models to Study Angiogenesis

Angiogenesis can be qualitatively and quantitatively measured in a large variety of in vitro and in vivo model systems. As discussed in *Section I.C*, the angiogenic cascade can be dissected in different sequential steps so that can each be studied separately in vitro. Research has mainly focused on proliferation and migration of endothelial cells. For this research, different endothelial cell sources can be applied. For human research most laboratories make use of HUVECs. This is the best available source of human endothelium, but the major drawback of these cells is their macrovascular origin, which makes them less suitable for studies on angiogenesis, a microvascular process. Although more laborious, human microvascular endothelial cells can be isolated from other organs such as foreskin or adipose tissue. For all primary isolates, the number of in vitro passages (4–5, and in the presence of growth factors, approximately 10) is, however, limited, which poses a major problem for their application. The required regular isolations furthermore introduce significant donor variation. To circumvent these drawbacks, one can use immortalized endothelial cell lines, such as HMEC-1 (Ades et al., 1992), EA.hy926 (Edgell et al., 1983), or ECL4n (Griffioen et al., 1996b). ECV304, a spontaneous immortalized endothelial cell line, has been applied for in vitro angiogenesis studies, although recently it has become apparent that this cell line contains a nonendothelial background as well. Endothelial cells from other species are also available, e.g., bovine capillary endothelial cells or cell lines from mouse and rat origin. It should be kept in mind that the effects of angiogenesis-inducing or -inhibiting factors can be different in the different species.

Assays to study proliferation of endothelial cells are based on cell counting or radiolabeled thymidine incorporation, or on colorimetric systems for measurement of mitochondrial activity [cell counting kit-8 (CCK-8) or dimethylthiazol diphenyl tetrazolium bromide (MTT)]. Alternatively, proliferation of endothelial cells can be analyzed by DNA profiling or determination of cell cycle-dependent expression of molecules such as proliferating cell nuclear antigen or Ki-67. Also, detection of cell death is a commonly used approach to average cell growth; e.g., apoptosis induction can be studied by detection of subdiploid cells or analysis of DNA degradation profiles, cell morphology or nick-end labeling by terminal dUTP nick-end labeling analysis.

For analysis of migration of endothelial cells, Boyden chambers are primarily used. An easier method is the wound assay. This assay system is based on wounding of a confluent monolayer of endothelial cells and measurement of the wound width in time.

Although the advantage of these in vitro assays is clearly the control over the few parameters present, the angiogenic cascade consists of multiple steps. This as a more extended process, can be studied in vitro, too. Most of these assays studying more complex processes of angiogenesis are based on tube formation of long-term cultured endothelial cells in a 3-dimensional seminatural matrix microenvironment. The most commonly used assay system to measure tube formation is the growth factor-induced sprouting of capillary-like structures from a confluent monolayer of endothelial cells grown on a thick gel. These gels can either be composed of a seminatural matrix with or without growth factors (e.g., Matrigel), or be based on collagen (Barendsz-Janson et al., 1998) or fibrin (Koolwijk et al., 1996). The demon-

stration of lumina in these endothelial cell sprouts is regarded as a criterion for vessel growth, in contrast to just migration of endothelial cells in the matrix or just rearrangement of endothelial cells *on* the gel. An elegant method to measure capillary formation has been described where endothelial cells grown on gelatin-coated cytodex-3 microcarrier beads were cultured in a fibrin gel (Nehls and Drenckhahn, 1995; Trochon et al., 1998). Quantification of sprouting can subsequently be performed by either measurement of maximal sprouting distance or by computer-based determination of total vessel length. Assays based on the sprouting of capillaries out of fresh tissue embedded in matrix gels more closely reflect the in vivo situation. This has been described for rat aortic rings (Nicosia and Ottinetti, 1990; Malinda et al., 1999) and human placenta tissue (Brown et al., 1996). This procedure is not applicable for all tissues because it has been described that, e.g., tumor biopsies often produce too much proteases digesting the matrix and thereby prevent endothelial cell sprouting (Barendsz-Janson et al., 1998). Figure 3 shows some examples of in vitro angiogenesis assays. A recently published novel way of measurement of angiogenesis in vitro, which is even more close to the in vivo situation, is the use of embryoid bodies (Wartenberg et al., 1998). In vitro cultured mouse blastocyst cells (Evans and Kaufman, 1981) recapitulate several steps of murine embryogenesis, including vasculogenesis and angiogenesis (Risau et al., 1988). There is a complete blood vessel development in these embryoid bodies (Vittet et al., 1996) making this system suitable for the study of angiogenesis modulators.

Besides the advantages that in vitro angiogenesis assays clearly have, the major drawback of all these assays is that they require the endothelial cells to be removed from their natural microenvironment, which may alter their physiologic properties. To study angiogenesis in vivo, the most frequently used assay systems are the chicken chorioallantoic membrane assay (Nguyen et al., 1994), the corneal pocket (Conrad et al., 1994), transparent chamber preparations such as the dorsal skin-fold chamber (Algire, 1943; Lichtenbeld et al., 1998), the cheek pouch window (Shubik et al., 1976), and the polymer matrix implants (Mahadevan et al., 1989; Plunkett and Hailey, 1990). However, in vivo assays also have several disadvantages: the pharmacokinetic properties of the compounds tested, necessary for proper interpretation of results, are often not known and the host will respond nonspecifically to the implantation. In this review, these assays will not be discussed in more detail because recently an elegant review on this issue appeared, discussing the pro's and con's of in vivo quantitative angiogenesis assays (Jain et al., 1997).

C. Ways to Interfere with Angiogenesis

A broad spectrum of strategies for modulation of angiogenesis has been described. As discussed in *Section I.C*, angiogenesis mainly depends on proper activation, proliferation, adhesion, migration, and maturation of endothelial cells. Most approaches to modulate angiogenesis are therefore focused on these endothelial functions during blood vessel formation.

1. Intervention with Endothelial Cell Growth. The most successful approach to modulate angiogenesis, to date, is the use of agents that specifically inhibit the growth of the endothelial cells. One of the first compounds identified to exhibit inhibitory effects on cell growth with specificity for endothelial cells was *O*-chloroacetylcarbamoyl fumagillol or AGM-1470/TNP-470, an analog of the fungus-derived antibiotic fumagillin (Ingber et al., 1990;

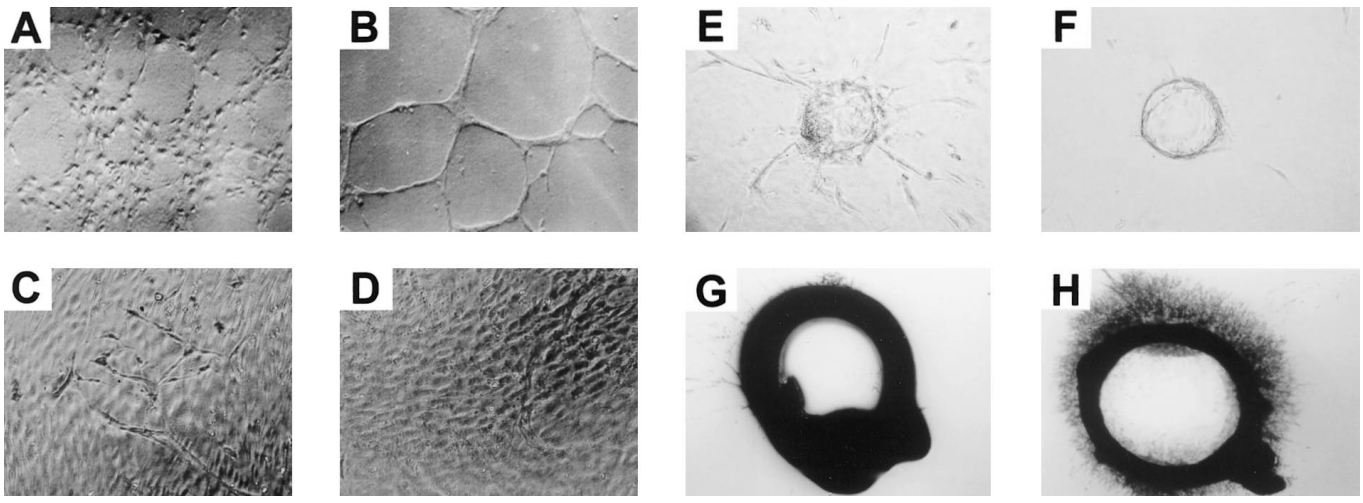

FIG. 3. In vitro angiogenesis assays. Tube formation of human umbilical vein endothelial cells on the seminatural matrix, Matrigel, 2 h (panel A) and 16 h (panel B) after seeding. Growth factor induced (20 ng/ml bFGF) sprouting of bovine microvascular endothelial cells grown as a monolayer on a collagen-based gel (panel C) can be inhibited by the angiogenesis inhibitor platelet factor-4 (panel D). Similar regulation is shown in panels E and F in a tube formation/migration assay of bovine endothelial cells grown on cytodex-3 beads and embedded in a fibrin gel. Panel G represents the rat aortic ring assay under control conditions and after exposure for 5 days with 200 μg/ml endothelial cell growth supplement (H). Panels A, B,G, and H were kindly provided by Dr. H. Kleinman (Bethesda, MD).

Kusaka et al., 1991). The mechanism of action of this compound was found to be prevention of endothelial cells to enter G_1 phase of the cell cycle, resulting in a decrease in proliferation (reviewed in Castronovo and Belotti, 1996). In later years, several endogenous molecules with angiostatic activity were described. Among these molecules are thrombospondin-1 (Rastinejad et al., 1989; Good et al., 1990; Grossfeld et al., 1997), platelet factor-4 (Gupta et al., 1995; Kolber et al., 1995), and interferon-inducible protein-10 (Luster et al., 1995). Two other members of this class of endogenously produced antiangiogenic proteins are angiostatin (O'Reilly et al., 1994) and endostatin (O'Reilly et al., 1997). Angiostatin is an internal fragment of plasminogen with multiple antiangiogenic activities in vitro and in vivo. Endostatin is a proteolytic fragment of collagen XVIII that affects endothelial cell survival via the induction of an imbalance between the antiapoptotic proteins Bcl-2 and Bcl-X_L and the proapoptotic protein Bax (Dhanabal et al., 1999). Both induced tumor regression, not only growth inhibition, in tumor-bearing mice, an effect that was most pronounced with endostatin and demonstrates the potential of these proteins. Direct inhibition of endothelial cell growth was also obtained with two other recently described endogenously produced angiostatic proteins, namely vasostatin (Pike et al., 1998) and restin (Ramchandran et al., 1999). Detailed mechanisms of action have not been described yet for these angiogenesis inhibitors.

A separate method for modulation of angiogenesis is the interference with angiogenic factors such as VEGF or FGF and their receptors. VEGF is also a major ruler during the development of tumors. Angiogenesis and subsequent tumor growth can be inhibited by blocking these factors (Kim et al., 1993). This can be performed by treatment with humanized blocking antibodies to these factors, antibodies to their receptors, with soluble receptors functioning as antagonists, dominant negative growth factor variants, or VEGF antisense constructs. Functional interference with growth factor signaling can also be performed by specific inhibitors of growth factor receptor signaling, as has been described for SU5416, a specific inhibitor of VEGFR-2 phosphorylation (Fong et al., 1999).

Recently a new nonendothelial cell-specific inhibitor of angiogenesis was described. Carboxyamidotriazole (CAI) is an inhibitor of tumor cell motility; the mechanism of action is the inhibition of transmembrane calcium influx. The inhibition of calcium influx prevents the activation of the focal adhesion kinase and RhoA pathways. CAI inhibited invasion by its ability to decrease the production of MMPs, blocked migration of cells, and caused cytostasis in tumor cells and endothelial cells. By interference in the biochemical pathways involved in endothelial spreading on extracellular matrix, the integrity of the vascular tube as well as stabilization of newly formed vessels were affected. Local administration of CAI inhibited capillary expansion in the chick chorioallantoic membrane assay. In vivo studies confirmed the antiangiogenic and anticancer effect of CAI for, e.g., ovarian cancer (Kohn and Liotta, 1995; Kohn et al., 1995).

2. Intervention with Endothelial Cell Adhesion and Migration. Because the process of angiogenesis also depends on endothelial cell adhesion events to, and migration of cells through, the extracellular matrix, effort is put in the search for modulators of these interactions. The first identified member of this group of compounds is the endogenously produced cytokine interferon. Antiendothelial activity was recognized by the observation that interferon could inhibit the migration of capillary endothelial cells (Brouty and Zetter, 1980). Subsequently, both interferons α and β were shown to have in vivo antiangiogenic activity (Sidky and Borden, 1987). Although interferons are probably not sufficiently active for treatment of all tumors, benign tumors predominantly comprised of endothelial cells are particularly sensitive to treatment with interferon (Ezekowitz et al., 1992). When it was found that activated endothelial cells up-regulate receptors for extracellular matrix components (Re et al., 1994; Frisch et al., 1996; Griffioen et al., 1997), interaction of endothelial cells with the matrix was chosen as a target for inhibition of angiogenesis. This proved to be a relevant approach by the demonstration that $\alpha_v\beta_3$ integrin molecules, the biological function of which is binding of vitronectin and other RGD-containing matrix components, are overexpressed in angiogenically stimulated blood vessels. Ligation of these receptors with an antibody called LM609 interferes with endothelial cell growth leading to inhibition of subsequent tumor growth (Brooks et al., 1994a). In addition, the exposure of endothelial cells to anti-$\alpha_v\beta_3$ antibodies resulted in the induction of apoptosis in these cells via loss of cell anchorage to the extracellular matrix (Brooks et al., 1994b). This is most likely the mechanism by which proliferation of endothelial cells and angiogenesis in vivo is blocked by $\alpha_v\beta_3$-directed antibodies.

3. Intervention with Metalloproteinases. Another mechanism of angiogenesis inhibition, related to the inhibition of endothelial cell adhesion and migration, is the use of specific inhibitors of proteinases that dissolve the connective tissue, thereby facilitating endothelial cell migration and subsequent vessel formation. Matrix metalloproteinases are a homologous family of enzymes that are involved in tissue remodeling and morphogenesis. Collectively, these enzymes are capable of degrading all components of the extracellular matrix (Rasmussen and McCann, 1997). Increased activity of these enzymes has been observed in tumor formation, and therefore inhibitors of MMPs represent an attractive approach to treat cancer. MMP inhibitors can be divided in synthetic protease inhibitors and naturally occurring MMP inhibitors, the tissue inhibitors of metalloproteinase. Belonging to the former group, batimastat, marimastat, and prinomastat/AG3340 are potent broad-spectrum inhibitors of the major MMPs and can prevent or

reduce spread and growth of several different malignant tumors in numerous animal models (Brown and Giavazzi, 1995; Shalinsky et al., 1999). Cell adhesion and proteolytic mechanisms are functionally associated, as recently demonstrated by the observation that the collagenase MMP-2 can bind to integrin $\alpha_v\beta_3$ on angiogenic blood vessels. Most interestingly, it was found that a naturally occurring MMP-2 breakdown product, called PEX, can inhibit cell-associated collagenolytic activity. It is suggested that this breakdown product is an important regulator of protease activity during angiogenesis and vasculogenesis. A recombinant form of PEX was useful in blocking angiogenesis and tumor growth in vivo, providing a novel therapeutic approach for angiogenesis inhibition at this level (Brooks et al., 1998).

D. Preclinical Use of Angiogenesis Inhibitors in Cancer

The pivotal role of angiogenesis in tumor progression and metastasis has urged researchers to test newly developed angiogenesis inhibitors in a broad variety of animal tumor growth models. Studies with one of the first angiogenesis inhibitors, AGM-1470/TNP-470, were performed in the early 1990s. Although in vitro the sensitivity for TNP-470 was not completely restricted to endothelial cells, doses of the compound had to be 10 to 100 times higher for comparable inhibition of tumor cell lines. Treatment of tumor-bearing mice resulted in a significantly increased survival time of 260% over untreated control animals. Daily treatment was not necessary; optimal treatment regimens were s.c. or i.v. administration once every three days. Oral administration had weaker effects on tumor growth, likely a result of lower bioavailability. The fact that sensitivity of tumor cells in vitro and effect on tumor growth in vivo did not correlate is seen as evidence for antitumor effects through the tumor vasculature (Ingber et al., 1990; Yamaoka et al., 1993). Angiogenesis inhibitors have also been expected to be efficient metastasis inhibitors based on the concept that tumors require new vasculature for spreading and outgrowth at a secondary site. TNP-470 reduced both the number and size of metastases of the B16BL6 melanoma and the M5076 reticulum cell sarcoma cell line in mice by 80 to 90% (Yamaoka et al., 1993). Also in rats, inhibition of tumor growth and metastasis was observed (Futami et al., 1996).

The next breakthrough in the search for novel antiangiogenic compounds occurred when the hypothesis that a primary tumor, whereas capable of stimulating angiogenesis for its own blood supply, can produce angiogenesis inhibitors that suppress the outgrowth of distant metastases, was proven to hold true. This hypothesis came from the observation that the removal of primary tumors could lead to the accelerated growth of metastases (Sugarbaker et al., 1977). To test this hypothesis, the Lewis lung carcinoma mouse model was used in which the primary tumor completely suppresses the growth of its metastases. From the urine of these mice a cleavage fragment of plasminogen called angiostatin was purified, which completely replaced the inhibitory activity of the primary tumor (O'Reilly et al., 1994). Treatment of tumor-bearing mice with angiostatin almost completely prevented metastasis formation in the lung. Using a similar strategy, endostatin was discovered (O'Reilly et al., 1997). Treatment of mice carrying different syngeneic malignant tumors with endostatin led to a rapid regression of tumors. As with angiostatin, there was no sign of toxicity, and continued endostatin therapy maintained the tumors in a state of dormancy. Discontinuation of treatment led to renewed growth at the primary site, which would eventually lead to death of the animals. However, when therapy was restarted tumors regressed again for nearly 100%. Subsequent intermittent cycles of treatment were able to maintain the tumors in a state of dormancy and showed no sign of drug-induced resistance (Boehm et al., 1997). When angiostatin and endostatin therapy were combined for 25 days, both at relatively high doses (20 mg/kg/day), tumors regressed completely yielding tumor-free survival of up to 11 months after start of therapy.

The nature of this dormancy is still obscure since these tumors have the capacity of renewed outgrowth when transplanted to the other flank or to another animal. This makes the involvement of the immune system, i.e., the development of a specific antitumor immune response in these mice, unlikely. It has been suggested that the sequential accumulation of endostatin during these cycles of growth and regression can locally lead to a high concentration, and hence keep the tumor dormant (Black and Agner, 1998). The data on endostatin suggest that the endothelial cell compartment is directly involved in the dormancy of the tumor cells, demonstrating the powerful control exerted by the vascular endothelial cell population over the tumor cell population.

The discovery of overexpression of $\alpha_v\beta_3$ integrin in tumor vessels and the dependence of angiogenesis on this matrix receptor (Brooks et al., 1994a) introduced the first strategy for angiogenesis inhibition by targeting specific tumor endothelial cell determinants. Antibody and specific RGD motif containing peptide ligands both inhibited human tumor growth in animal models (Brooks et al., 1994b; Friedlander et al., 1995). The prevention of the formation of a suitable matrix to migrate through by the inhibition of MMPs is related to this inhibition of cell adhesion to suppress angiogenesis. Treatment of mice carrying the highly metastatic tumor B16BL6 with batimastat resulted in reduction of lung colony number. It was found that not cell arrest in the lungs but actually extravasation of the cells was inhibited (Chirivi et al., 1994). Batimastat also inhibited primary tumor cell outgrowth. A 58% inhibition was observed when treatment was started from the day of tumor cell inoculation. In numerous other models, such as ovarian cancer and an orthotopic colon cancer model, similar results were obtained (Davies et al., 1993; Wang

et al., 1994). Several other low-molecular weight synthetic MMP inhibitors is currently under development. One of them is prinomastat/AG3340, which has one of the lowest K_i values (K_i is the concentration that inhibits enzyme activity by 50%) for gelatinase A (MMP-2) and gelatinase B (MMP-9). In a chemoresistant human non-small cell lung carcinoma mouse model, AG3340 was well tolerated at 400 mg/kg/day (administered twice daily, 7 days a week) and exhibited significant inhibition of angiogenesis (up to 77%) and tumor growth (up to 65%). A suboptimal dose of AG3340 in combination with carboplatin or paclitaxel resulted in greater inhibition than was observed with either agent alone. In colon and prostatic tumors, angiogenesis was inhibited by approximately 50% (Shalinsky et al., 1998).

The potential of the use of inhibitors of specific signal transduction pathways is nicely exemplified by the use of CAI. This inhibitor of calcium mobilization, arachidonic acid release, and generation of inositol phosphates, inhibits growth of tumor cell lines. In the same way that it inhibits tumor cells, endothelial cells are affected, resulting in inhibition of angiogenesis. Oral administration of CAI to mice carrying human xenografts resulted in a >80% inhibition of tumor outgrowth (Kohn et al., 1992). CAI was not toxic as determined by macroscopic and histological evaluation. These studies are highly interesting because one might expect that pharmacologic interference with intracellular signaling pathways, which are so fundamental to cellular function in both pathologic and normal tissues, will lead to massive toxicity. Because this is not the case, the conclusion seems justified that tumors display an enhanced dependence and sensitivity to these second messenger pathways or are exposed to higher drug concentrations by increased uptake or accumulation at the tumor site.

E. Clinical Trials with Inhibitors of Angiogenesis for Cancer Treatment

Currently some 30 antiangiogenesis compounds are being tested in human clinical anticancer trials. Most of them are in phase I or II testing, to determine optimal dosage, nature and severeness of side effects, safety and preliminary effectivity. Only a few compounds, as discussed in this chapter, are further down the clinical road and currently being tested in phase III trials. Usually the angiogenesis targeted studies are designed in a way that patients receive either standard (chemo- or immuno-) therapy and a placebo, or standard therapy combined with a new antiangiogenic drug. The National Cancer Institute, which is involved in some of these clinical trials, has classified these drugs in different categories. Without trying to be complete, a few examples of clinical studies with these compounds will be discussed here.

i. *Drugs that inhibit endothelial cell proliferation directly.* As a member of the "first generation" of angiogenesis inhibitors, TNP-470 was used in the first formal clinical trial of antiangiogenic therapy. In cooperation with the National Cancer Institute, currently three clinical studies are operative: 1) a phase I study of TNP-470 in patients with recurrent or refractory pediatric solid tumors, lymphomas, and acute leukemias; 2) a phase II study for advanced or recurrent squamous cell carcinoma of the cervix; and 3) a phase III randomized study of TNP-470 versus synchronous radiotherapy and chemotherapy for locally advanced nonresectable nonmetastatic pancreatic cancer. Recently, one phase II multi-institutional clinical study in metastatic renal cell carcinoma patients was reported. In the first 20 patients with an adequate follow-up, there was one partial response and one minor (40% shrinkage) response. Five of these 20 patients remained progression-free for 16 weeks or more. The mild toxicity reported and the high incidence of apparent prolonged progression-free survival in this heavily pretreated cohort is encouraging (Stadler et al., 1998).

ii. *Drugs that block matrix breakdown.* Among the clinical trials with angiogenesis inhibitors, the metalloproteinase inhibitors have probably been the most extensively studied in clinical trials to date. The first trial in patients with a MMP inhibitor, batimastat, started in 1991 in patients with ovarian carcinoma. Because the drug showed low bioavailability, other administration routes were used. A phase I study using an i.p. formulation yielded high and sustained plasma concentrations of the drug, low toxicity, and early signs of efficacy. A randomized controlled trial of marimastat in 400 patients with advanced pancreatic cancer has now been completed. The study was designed to compare the effect of orally and twice daily administered marimastat with the conventional treatment with gemcitabine. The study did not meet its primary endpoint, designated as a 16% or greater reduction in mortality versus gemcitabine treatment, i.e., there was no significant difference between the survival curves for gemcitabine and the highest marimastat dose. Safety data revealed no difference between the treatment groups other than the expected musculoskeletal events (stiffness and pain) caused by the MMP inhibitor that have been reported in most studies. Although this study has not shown any benefit of treatment with marimastat over the conventional treatment, there is evidence that marimastat is as effective as the "drug of first choice" treatment. This holds promise for the use of marimastat in future combination regimens, studies which are currently being enrolled. Oral administration of prinomastat/AG3340 was tested in a single dose phase I study in healthy volunteers. Favorable absoption, pharmacokinetics, and toxicity profiles were observed. In a phase I safety and tolerability study of AG3340 administered orally and twice daily in patients with advanced cancer, including lung, prostate, kidney, and colorectal cancers as well as sarcoma and melanoma, disease was stabilized in more than 25% of 47 evaluable patients who were treated for periods of

16 to 40 weeks. Three patients (one each with nonsmall cell lung cancer, renal carcinoma, and melanoma) were found to have minor reductions in tumor volume. AG3340 was found to be generally well tolerated.

A phase III clinical trial of AG3340 has been initiated in combination with chemotherapy in patients with advanced nonsmall cell lung cancer. This trial is designed to evaluate the safety and efficacy of AG3340 as part of first-line therapy in combination with chemotherapy. In a study being conducted at sites in North America, Europe, and Australia, patients with advanced nonsmall cell lung cancer will be randomized to receive either AG3340 (tablet form) in combination with gemcitabine and cisplatin or placebo in combination with gemcitabine and cisplatin. The primary objective of this study is to compare time of overall survival between patients receiving AG3340 or placebo in combination with gemcitabine and cisplatin.

iii. *Drugs that inhibit endothelial-specific integrin/survival signaling.* The results of a completed phase I clinical trial with Vitaxin, the humanized $\alpha_v\beta_3$-directed LM609 monoclonal antibody have been presented (Gutheil et al., 1998). This trial of 14 patients revealed no significant toxicities, and in addition, showed that two-thirds of the patients experienced an objective clinical response to the Vitaxin treatment. Most recently, a phase I/II study was initiated to determine the optimal dosage, whereas a phase II clinical trial to determine efficacy is planned to start early 2000.

iv. *Drugs with nonendothelial cell-specific mechanism(s) of action.* Phase I clinical trials with CAI, the inhibitor of transmembrane calcium influx, have been completed, demonstrating that oral treatment with a single dose daily was relatively well tolerated (Kohn et al., 1997). Cytostasis in the form of disease stabilization was observed in almost half the solid tumor patients treated. Several refractory ovarian cancer patients were found to have disease stabilization lasting up to 10 months. This led to the initiation of a phase II clinical trial for women with relapsed epithelial ovarian cancer and subjected to no more than three prior therapeutic regimens. This single agent trial is aimed at an outcome of 6 or more months of disease stabilization.

v. *Drugs that block activators of angiogenesis.* Angiogenesis seems exquisitely sensitive to small changes in factors such as VEGF and FGF-2 that drive the angiogenic process. This has important therapeutic implications in treating angiogenesis-driven disorders (Rak and Kerbel, 1997). Next to anti-VEGF antibodies used to neutralize the cytokine in serum/extracellular fluids, another approach is the blockade of VEGF receptor signaling. SU5416 is a specific inhibitor of the signaling pathway of the VEGFR-2 found on endothelial cells. This compound entered phase I clinical testing in the fall of 1997 and has shown good safety profiles in approximately 100 patients with solid tumors. The phase III clinical study in colorectal and lung carcinoma patients will compare the efficacy of standard chemotherapy regimens with the efficacy when combined with SU5416. SU6668 is a broad-spectrum inhibitor of angiogenesis and tumor growth, acting by interference with VEGF, FGF, and PDGF receptors. This drug is currently entering phase I studies in both i.v. and oral formulations.

A listing of angiogenesis inhibitors that are currently in clinical trials, can be found on an internet website of the National Cancer Institute (http://cancertrials.nci.nih.gov). Moreover, most of the data available so far can only be retrieved from the internet. Inhibition of angiogenesis is a new strategy of cancer therapy, in which the concept of treatment is fundamentally different from conventional therapies. In standard chemotherapy the desired endpoint is shrinkage of the tumor and complete remission, whereas in antiangiogenic therapy the endpoint aimed at is achievement of stable disease and prolonged progression-free periods leading to a relatively long survival. This underscores the novelty of antiangiogenesis therapy because until now, to our knowledge, there has never been a drug for oncological use that has been approved by the FDA on the basis of stabilization of the disease. As a consequence, the surrogate endpoints, such as stabilization or decrease of circulating tumor markers [e.g., prostate specific antigen (PSA) in prostate cancer, carcino-embryonic antigen (CEA) in gastrointestinal cancer, and CA15 in breast cancer], do not necessarily need to be the same. An important issue in antiangiogenesis treatment that concerns most clinicians, is the question whether there are surrogate endpoints for angiostatic treatment. The most commonly used parameter for ongoing angiogenesis is the circulating (plasma) level of VEGF. However, this is an indirect method and in some instances, e.g., during treatment with VEGF-blocking antibody, this parameter cannot be used. More useful surrogate endpoints have not been described yet, although circulating levels of E-selectin and vascular cell adhesion molecule-1 (VCAM-1) have been put forward for this purpose (Ferrara, 1995; Koch et al., 1995). Considering the fact that their counter ligands are abundantly present in the serum, it is however likely that these soluble adhesion molecules are rapidly cleared from the circulation, and thereby mask ongoing angiogenesis.

Although stabilization of tumor size in antiangiogenesis therapy may be an endpoint in the clinic, in preclinical animal studies it is obvious that the most promising angiogenesis inhibitors do not stabilize the tumor. Instead, they induce partial or sometimes even complete regression. This may partly be explained by the rapid turnover of cells in animal tumors, even in a stabilized tumor, in which case inhibition of angiogenesis would lead to a disturbed balance between cell growth and cell death leading to an overall enhanced cell death.

Animal experiments also may provide a clue to whether benefit from antiangiogenesis therapy should be expected in patients with large tumor burdens. Some

unpublished studies indicate that the largest benefit from angiogenesis inhibition is expected when the therapies are started as early as possible, with the largest effects seen on starting the treatment even before the angiogenic switch occurred. Because this does not meet the situation in the clinic, where cancer is normally diagnosed at a much later stage, combination therapies are most likely to give the best results.

F. Novel Approaches to Interfere with Tumor Blood Flow

Angiogenesis inhibitors are compounds that specifically interfere with regulatory processes of the various steps of angiogenesis, as described above. Tumor vasculature targeting aims at specifically delivering pharmacologic agents to the site of the blood vessels of a tumor. The pharmacologically active moieties can exert a variety of activities, but in general all strategies aim to block tumor blood flow and hence tumor growth. Moreover, as a consequence, site-specific immunologic responses may be brought about and potentiate anti-tumor effects. Besides the choice of the effector modality (e.g., coagulation factors or toxins), the choice of the target epitope is of great importance for feasibility and effectiveness of these approaches, as will be discussed below.

1. Targeted Strategies to Induce Tumor Blood Coagulation. At present, two studies on targeting blood coagulation-inducing activity to tumor endothelium as a therapy for cancer have been reported. In the first study, an animal model with artificially induced target epitopes on the tumor vasculature was exploited. In this model, transfected tumor cells s.c. inoculated locally produced high amounts of interferon (IFN), leading to tumor endothelium-specific expression of major histocompatibility complex (MHC) class II. Bispecific antibodies (BsAb) against MHC class II and a truncated form of the activator of the extrinsic coagulation pathway, tTF, were subsequently developed. Intravenous administration of a mixture consisting of BsAb and tTF (BsAb·tTF), the so called "coaguligand" formulation, to mice with clinically relevant tumor burden resulted in dramatic tumor reduction without concurrent toxicity in other organs. Site-specific blood coagulation in the tumor blood vessels caused an almost instantaneous and persisting blockade of tumor blood flow. Treatment of mice bearing s.c. tumors twice with BsAb·tTF coaguligand lead to 38% complete tumor regressions and 24% partial responses (Huang et al., 1997). The attractiveness of the coaguligand approach is the use of a truncated form of TF that is completely devoid of coagulation-inducing activity as long as it is kept from complexation with the lipophilic factor X at cell membranes. On cross-linking of the hydrophilic tTF with the target cell membranes by the BsAb, tTF becomes complexed with factor X in the presence of factor VII/VIIa, leading to the induction of blood coagulation (Fig. 4). It is thought that a threshold in the number of tTF cross-linked to factor X-containing cell membranes exists, above which the coagulation cascade is initiated. This allows the use of target epitopes

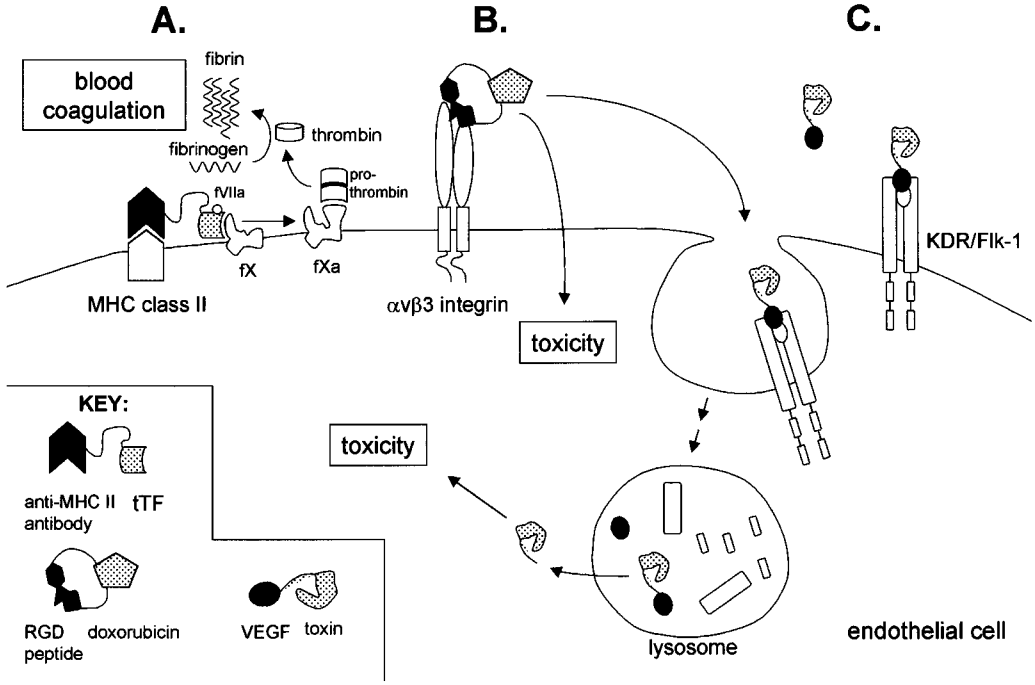

FIG. 4. Schematic representation of drug-targeting approaches aimed at selectively blocking tumor blood flow. A, coaguligand approach: by delivering the coagulation factor truncated tissue factor to the tumor endothelium, blood coagulation is initiated and the tumor blood flow blocked; B, intracellular delivery of doxorubicin by RGD peptide, leading to endothelial cell death via doxorubicin-related cytotoxic effects such as DNA topoisomerase inhibition and the generation of free radicals; C, immunotoxins or "angiotoxins" deliver deglycosylated ricin A chain or diphtheria toxin in the target cells, leading to endothelial cell killing. fVIIa, factor VIIa; fX(a), factor X(a).

that are highly but not exclusively expressed on tumor endothelium.

Using a similar approach of tumor infarction, mouse solid Hodgkin's tumors spontaneously expressing endothelial VCAM-1 were significantly retarded in outgrowth (Ran et al., 1998). The antitumor effect was not as dramatic as that seen in the MHC class II model. Possibly, the number of tTF molecules delivered at the site of the tumor endothelium was not sufficient to create a rapid and more or less generalized blood coagulation throughout the tumor vasculature. Only if the blood in the majority of vessels becomes coagulated by the coaguligand, the number of tumor cells that will be deprived of nutrients will be sufficient enough to result in strong antitumor effects. Furthermore, anticoagulative activities may be strong enough to counteract the effects when the kinetics of coagulation induction by the coaguligand are insufficient to perturb local pro- and anticoagulative activities.

The coagulation induction potency of coaguligand formulations are mainly determined by the following factors: i) the number of target epitopes on the tumor endothelium that allow BsAb-mediated interaction between tTF and factor X on the target cell membrane; ii) locally present counteracting anti-coagulative activity; and iii) kinetics of cross-linking of the BsAb and the target epitopes in relation to the kinetics of coagulation induction capacity. No detailed information is available on species differences in coagulation induction activity of the tTF coaguligand formulation, although we are aware of differences in pro- and anticoagulative properties between species. Furthermore, the number of MHC class II and VCAM-1 molecules expressed on the tumor vasculature of the animal models discussed, were high, as were the antibody affinities. This enabled a significant number of tTF to be rapidly cross-linked to the target cell membrane. For clinically relevant target epitopes and targeting devices, it needs to be established whether these parameters are as important with respect to therapy efficacy.

The research in the area of coaguligand therapy for cancer is still in its infancy. Important for its future is to define appropriate target epitopes on human tumor endothelium (*Section III.F.3*), qualitatively as well as quantitatively. Furthermore, the issue of the occurrence of distant embolism following local thrombus formation in the tumor vasculature, needs to be addressed in the appropriate settings. By obtaining more insight in these and other above-discussed processes in the coming years, we will be able to better understand the critical players in this type of therapy, and hence the value of its potential for future clinical application.

2. Targeted Strategies to Kill Tumor Endothelial Cells. In the development of drug-targeting approaches for therapy of cancer, toxins, chemotherapeutics, and ionizing radiation have been extensively studied as effector molecules (Meijer and Molema, 1995). The majority of approaches aim at targeting the effector molecules to the tumor cells, thereby increasing therapeutic efficacy and decreasing toxicity elsewhere in the body. Treatment of solid tumors, however, has not been successful, which is believed to be due to poor penetration of the drug-targeting conjugates in the solid tumor mass (Jain, 1996; Molema et al., 1997). In this respect, tumor endothelial cells are now considered a better target candidate for cancer therapy, because they are easily accessible for blood-borne therapeutics. Furthermore, hundreds of tumor cells rely on the functionality of only one blood vessel formed by relatively few endothelial cells. The number of target cells to be destroyed is therefore significantly less than when tumor cells themselves are the target for killing.

The first study on a strategy to directly kill tumor endothelium reported on the targeting of deglycosylated ricin A chain toxin to MHC class II on tumor endothelium in the IFN mouse model (Burrows and Thorpe, 1993). Treatment of tumor-bearing mice with the immunotoxin caused complete occlusion of the tumor vasculature and strong regression of large solid tumors. Another tumor endothelial cell-targeted immunotoxin recently reported consisted of deglycosylated ricin A chain conjugated with antibody against endoglin, a TGF-binding protein specifically expressed on proliferating cells (Matsuno et al., 1999). In addition, "angiotoxins" consisting of diphtheria toxin domains fused or chemically conjugated to VEGF isoforms, VEGF-121 and -165, have been investigated for this purpose. These approaches were potent in inhibiting angiogenesis and tumor outgrowth in in vitro and in vivo angiogenesis models (Ramakrishnan et al., 1996; Olson et al., 1997; Arora et al., 1999).

Targeted radioimmunotherapy of pulmonary micrometastases was feasible in mice with an antibody directed against thrombomodulin, expressed selectively and in large amounts on the luminal surfaces of capillaries and small blood vessels in the lungs. The short-lived ($t_{1/2}$ = 45 min) α-particle emitter ^{213}Bi conjugated to the antibody was delivered to healthy lung and tumor capillaries, resulting in significant tumor growth reduction and extended life span of animals treated at low doses. At higher doses, tumors almost completely regressed, but animals died of lung fibrosis induced as a result of concurrent damage to healthy tissue (Kennel et al., 1999).

By covalently conjugating the chemotherapeutic agent doxorubicin to a peptide containing an RGD motif, Arap et al. (1998) constructed drug-targeting conjugates specific for integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on endothelial cell of tumor vasculature. Treatment of human breast carcinoma-bearing mice with the conjugate at a doxorubicin equivalent 10 to 40 times lower than that of free doxorubicin, caused vascular damage in the tumors and a strong antitumor effect, whereas liver and heart toxicity was less compared with free doxorubicin (Arap et al., 1998). Whether this effect was caused by the selective delivery of the chemotherapeutic drug to the tumor en-

dothelial cells and/or tumor cells, direct caspase-3 activation (Buckley et al., 1999), or a combination has not been studied yet. More recently, the applicability of this RGD peptide-mediated tumor endothelium targeting was extended to dipeptides consisting of the RGD-targeting peptide and an apoptosis-inducing peptide. The dipeptides were selectively toxic to angiogenic endothelium and showed anticancer activity in mice (Ellerby et al., 1999).

Some major steps forward have been made in the development of novel drug-targeting approaches aimed at selectively killing tumor endothelial cells. The extensive "from the bench to the bed" experience with tumor cell targeted immunotoxins (Frankel et al., 1996) will be used for the further development of these tumor endothelial cell-targeted strategies.

3. The Quest for New Targets on Tumor Endothelium. The angiogenic phenotype of the tumor vasculature provides the most prominent markers for differentiation between tumor and normal endothelial cells. One of the first studies on the development of molecules specifically recognizing tumor endothelium was published by Hagemeier et al. (1986). They developed an antibody recognizing a 30.5-kDa antigen present at the tip of budding capillaries in proliferating tissue (placenta, umbilical vein, intestine) and in acute inflammatory reactions and tumors. This study was followed by several others, all describing molecular markers more or less selectively expressed on endothelial cells in tumor tissue (Table 1).

Most of the targets identified in the 1980s were either not evaluated for drug-targeting strategies or failed to be selective for tumor endothelium once tested in vivo. However, in recent years, several targets evolved that have now shown their potential for future development for clinical application. These targets include $\alpha_v\beta_3$ integrin and VEGFR. Not only was the $\alpha_v\beta_3$ targeted doxorubicin a potent inhibitor of tumor endothelial cell proliferation in mice (Arap et al., 1998), $\alpha_v\beta_3$ was also a suitable target for the detection of tumor angiogenesis by magnetic resonance imaging in rabbits (Sipkins et al., 1998). The conjugation of diptheria toxin to VEGF resulted in an angiotoxin with endothelial cell killing activity in vivo (see *Section III.F.2*). One may consider modifying these small peptide conjugates, because clearance from the systemic circulation can be rapid and unwanted. Modifications can be brought about by increasing molecular weight, for example, an approach also followed in the development of antibody fragments with unfavorable pharmacokinetics. A longer circulation time of the conjugates allows longer exposure of the target endothelium to the conjugates, although unwanted toxicity elsewhere may simultaneously increase.

Induction of target epitopes by irradiation of the tumor or cytokine administration may be considered an option. Using X-ray radiation, P-selectin was rapidly translocated to the vascular lumen of tumors in rats and mice (Hallahan et al., 1998). It has been known for quite some time, that TNF, combined with IFN, exerts selective cytotoxic effect on tumor macro- and microvasculature. In vitro studies demonstrated a decrease in $\alpha_v\beta_3$ integrin expression on exposure to these cytokines (Ruegg et al., 1998). One can hypothesize that this combination of cytokines is likely to affect the differential expression of other molecules as well. Up-regulated molecules may then be identified as potential targets. Especially in the context of multiple metastases in an organ, which can be subjected to isolated organ perfusion (e.g., limb, liver, kidney), cytokine-induced target epitopes may be worth considering.

CM101 is a polysaccharide exotoxin of group B streptococcus. In tumor-bearing mice, i.v. injection of CM101 led to a rapid binding of the compound to tumor neovasculature. As a result, complement was activated and leukocytes started to infiltrate, followed by the production of proinflammatory cytokines (Yan et al., 1998). Besides having a quite potent effect on tumor growth itself, CM101 could be exploited as a carrier to deliver pharmacologically active compounds at the site of the tumor vasculature. By incorporating two entities with a different mechanism of action, an approach called "dual targeting" (i.e., the carrier itself and the attached drug both exert an effect), synergistic effects can occur (Meijer et al., 1996). Similarly endostatin and TNF may be considered carrier molecules for dual targeting purposes, because tumor endothelium is more prone to endostatin- and TNF-induced cytotoxicity. The recent demonstration that the putative binding sites for endostatin are present on endothelial cells in normal tissue as well (Chang et al., 1999) challenges the use of endostatin for this purpose. As a result of its high toxicity, application of TNF for such an approach seems only feasible in isolated organ perfusion systems (Eggermont et al., 1996).

TABLE 1

Epitopes on tumor/angiogenic vascular endothelium which differentiate between normal and disease vasculature may be suitable for drug targeting or diagnostic purposes. Targets presented by molecules specific for tumor-associated basement membrane, extracellular matrix, or nonendothelial cell components have not been included.

Target Epitope	Reference
30.5-kDa. antigen	Hagemeier et al., 1986
CD34	Schlingemann et al., 1990
VEGF/VEGF receptor complex	Ramakrishnan et al., 1996
VEGF receptor	Dvorak et al., 1991
Endosialin	Rettig et al., 1992
E-selectin	Nguyen et al., 1993
α_v integrins	Brooks et al., 1994a
Endoglin	Burrows et al., 1995
Tie2	Sato et al., 1995
TNFα receptor	Eggermont et al., 1996
CD44	Griffioen et al., 1997
Angiostatin receptor	Moser et al., 1999
Endostatin receptor	not identified at present
CM101-binding protein	not identified at present
MMP-2/MMP-9	Koivunen et al., 1999

The observation in the MHC class II directed coaguligand studies that tumor endothelium of those animals not responding to treatment were devoid of the target epitope is important in light of intra- and intertumoral heterogeneity in human. In human tumors, the vasculature exists in different stages of activation and angiogenesis. In some areas the endothelium is quiescent, whereas at the same time in other areas neovessel maturation takes place. Therefore, targeting effector molecules to one target epitope only will not be successful in blocking the majority of the tumor blood flow. Conjugates targeted to a combination of epitopes should be applied for effective solid tumor debulking. In this respect, one can think of targeting to VEGFR in the early angiogenic cascade, $\alpha_v\beta_3$ integrin expressed in later stages and Tie2 during the maturation phase.

IV. The Interplay between Angiogenesis and Cells of the Immune System

A. Angiogenesis Regulates Leukocyte Recruitment

It is well established that the immune system plays an important role in the regulation of angiogenesis. Multiple studies indicate that leukocytes can induce vascular proliferation (Sidky and Auerbach, 1975; Polverini et al., 1977; Camussi et al., 1997), and specific leukocyte-derived cytokines have been identified to induce angiogenesis (Koch et al., 1992; Hashimoto et al., 1994; Richardson et al., 1994; Vanhee et al., 1994; Freeman et al., 1995). Next to the regulation of angiogenesis by leukocytes, angiogenic processes can have a major impact on cells of the immune system and on the development of an immune response as well. Normal endothelial cells contribute to the recruitment of immune cells to the site of inflammation by the expression of adhesion molecules (depicted in Fig. 1). Different families of adhesion molecules play a role in this process. The most important families identified at present are 1) the Ig superfamily of Ig related molecules, such as ICAM-1, VCAM-1, and CD31; 2) the selectins, molecules that initiate the adhesion cascade by mediating leukocyte rolling through recognition of carbohydrate epitopes; and 3) a group consisting of among others, CD34 an L-selectin binding glycoprotein that is expressed on hematopoietic progenitor cells and on the luminal side of vascular endothelial cells (Kuzu et al., 1992) and CD44, the lymphocyte homing receptor that is expressed on activated endothelial cells (Griffioen et al., 1997). Expression of endothelial adhesion molecules is controlled by cytokines such as TNF, IL-1, and IFN. These cytokines facilitate leukocyte adhesion to endothelial cells and extravasation into tissues by inducing an enhanced expression of ICAM-1, VCAM-1, and E-selectin among others (Carlos and Harlan, 1994).

In angiogenically stimulated tissues angiogenesis mediates the formation of new blood vessels (Hanahan and Folkman, 1996). The exposure of endothelial cells to angiogenic factors down-regulates adhesion molecule expression (Kitayama et al., 1994; Griffioen et al., 1996b, 1999b). Also, the induction of adhesion molecule expression by the proinflammatory cytokines TNF, IL-1, or IFN, is severely hampered (Fig. 5), a phenomenon called endothelial cell anergy (Griffioen et al., 1996a). These observations are in line with and provided a mechanistic background for earlier observations of reduced leukocyte-vessel wall interactions in tumors (Wu et al., 1994; Dellian et al., 1995; Fukumura et al., 1995; Borgstrom et al., 1997). In one study (Melder et al., 1996), it was found that during angiogenesis FGF-2 and VEGF have opposite functions with regard to natural killer cell adhesion to endothelium. Whereas FGF-2 inhibited adhesion both in vivo and in vitro, the effect of VEGF, which was only studied in vitro, consisted of stimulation of adhesion. The above results may partly explain the findings that some adhesion molecules that are absent on resting endothelial cells become overexpressed on tumor endothelial cells (Kraling et al., 1996).

A link between leukocyte-endothelium adhesion and angiogenesis seems to be present as suggested by the observation that endothelial markers originally identified to play a role in leukocyte recruitment appear to be involved in neovascularization as well (Ferrara, 1995). E-selectin has a function in the formation of capillaries (Nguyen et al., 1993), whereas platelet endothelial cell adhesion molecule-1 or CD31 has been reported to play a role in tube formation of endothelial cells (Berger et al., 1993; Fox et al., 1995; Delisser et al., 1997). These observations were extended for VCAM-1 being expressed in medullo- and neuroblastoma, lung and renal cell carcinoma (Kuzu et al., 1993; Patey et al., 1996). The description of soluble VCAM-1 and E-selectin as activators and attractors of endothelial cells (Koch et al., 1995), added to this leukocyte endothelial cell adhesion-angiogenesis link.

At the molecular level, expression of endothelial cell adhesion molecules required to facilitate leukocyte recruitment is differentially regulated from the expression of adhesion molecules in angiogenesis. Although inflammatory signals increase expression, angiogenic stimuli often down-regulate adhesion molecules involved in leukocyte-endothelial cell interactions. The net adhesion molecule receptor make-up on the endothelium is hence determined by both signals.

Recently, additional studies addressing the role of VEGF and FGF in leukocyte vessel wall interactions showed that in general a strong induction of angiogenesis inhibits leukocyte adhesion (Booth et al., 1999). These studies are especially important in the light of tumor biology because these phenomena contribute to the escape of tumors from immune surveillance. It has been suggested that this "aberrant" responsiveness in adhesion molecule expression is not specific for tumors but has its origin in embryonic development where

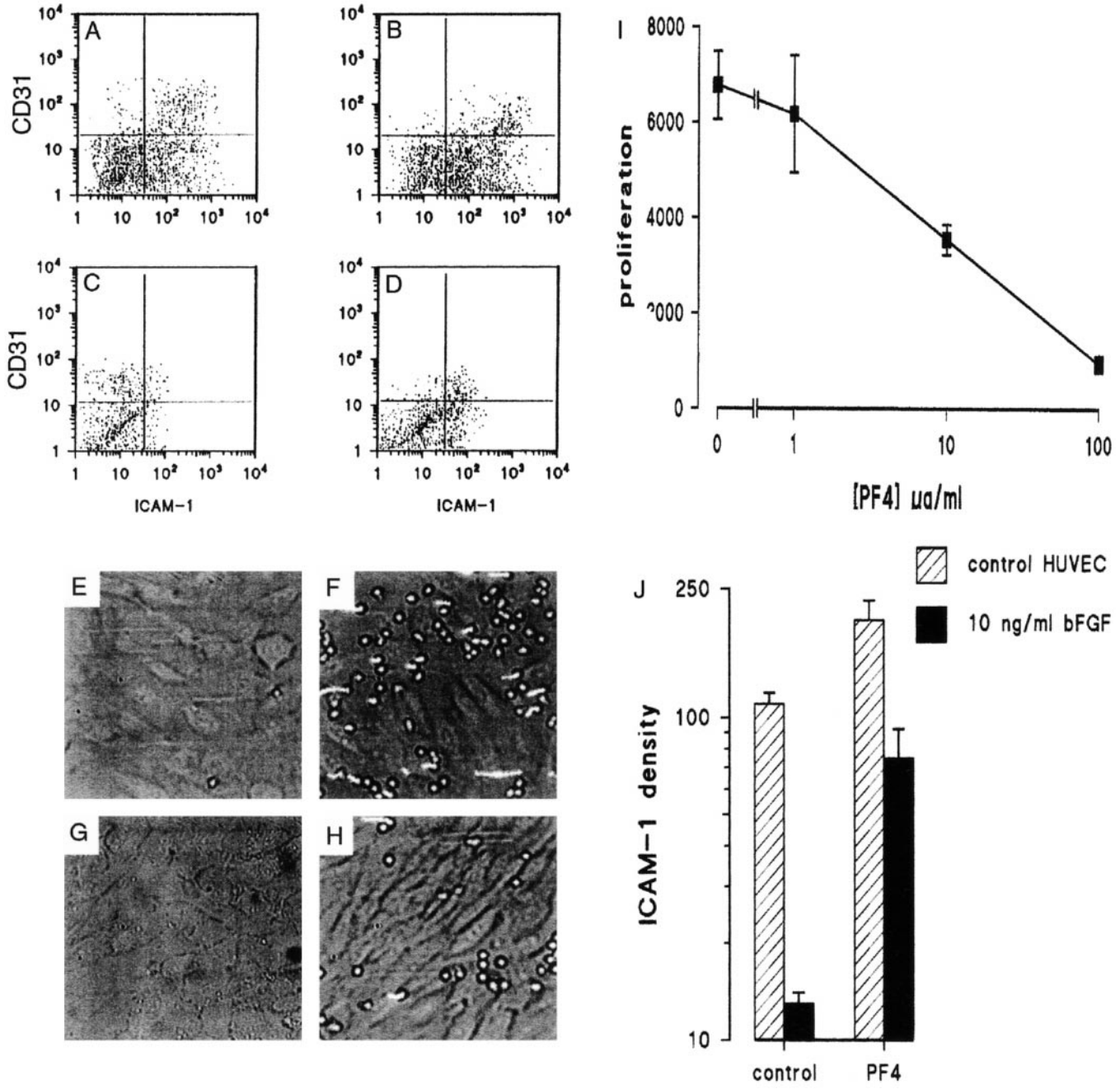

FIG. 5. Angiogenesis down-regulates endothelial cell adhesion molecules, an effect that can be overcome by angiogenesis inhibitors. Tumor endothelial cells (C, D), identified with CD31 antibodies display a suppressed ICAM-1 expression as compared with endothelial cells obtained from normal tissue (A, B). Up-regulation of ICAM-1 with TNF-α (B) is less efficient in tumor endothelial cells (D). Video images of PMN perfusion experiments with control (E), FGF-2 (bFGF) treated (10 ng/ml; G), TNF-α treated (4 ng/ml; F), and TNF-α plus FGF-2 treated HUVEC (H) demonstrate the suppression of rolling and adhesion by the proangiogenic factor FGF-2. Rolling of PMN is visualized by showing the track of rolling during a 2.5-s period. PMN without a track represents the firmly adhered population. The angiogenesis inhibitor platelet factor-4 down-regulates endothelial cell proliferation (I) and overrules ICAM-1 downregulation (J).

growing tissue should not be infiltrated by excessive numbers of leukocytes.

Inhibition of angiogenesis may counteract the down-regulation of adhesion molecules and endothelial cell anergy in tumors, and thereby restore the inflammatory response of endothelial cells and enhance the infiltration of leukocytes (Griffioen et al., 1999a) (Fig. 5). This would, next to the direct inhibition of tumor growth by prevention of blood vessel formation, be beneficial in the treatment of cancer and could be enhanced even further by combination of the therapy with, e.g., bispecific antibodies (Kroesen et al., 1998).

B. The Role of Angiogenesis in Chronic Inflammation

The relation between angiogenesis and leukocyte infiltration in cardiovascular diseases and chronic inflam-

mation has attracted a lot of attention in the last years. In these diseases, the leukocyte involvement, e.g., macrophage infiltration in atherosclerotic plaques and the immune response against cartilage in rheumatoid arthritis, is expected to mediate the pathology.

In the first acute phase of inflammation, functional changes in the vasculature such as dilatation, increase in permeability, and endothelial activation occur. In the second subacute phase, capillaries and venules remodel with extensive endothelial mitotic activity (Majno, 1998). Upon chronic stimulation, both increases in capillary density and vascular dilatation can be observed, although these responses can differ significantly between strains of mice and possibly between species (Thurston et al., 1998). In many chronic inflammatory diseases in human, neovascularization can be identified in the inflamed lesions. In rheumatoid arthritis, neovascularization, which is one of the earliest histopathological findings, is thought to be required for pannus development. Rheumatoid synovial endothelium is constantly subjected to remodeling. Besides nurturing the pannus, the blood vessels also play an active role in the inflammation by being a source of cytokines, chemokines, and proteases (Storgard et al., 1999). Synoviocytes in the rheumatoid lesions exhibit characteristics of tumor cells, including somatic mutations in regulatory genes such as Ha-*ras* and p53. Therefore, the rheumatoid synovium can be envisioned as a tumor-like mass that invades and destroys its local environment and is enriched in angiogenesis-promoting cytokines, such as FGF-2, VEGF, and IL-8, and soluble adhesion molecules VCAM-1 and E-selectin (Koch, 1998; Firestein, 1999). Also in psoriasis, expansion of the dermal microvasculature is a prominent feature (Creamer et al., 1997). Capillary leakiness and vascular anomalies develop in psoriatic skin after the occurrence of epidermal alterations. TGF, overexpressed in psoriatic epidermis, induced VEGF production by keratinocytes in vitro. Furthermore, extensive VEGF production in psoriatic skin lesions coincided with VEGFR-2 overexpression in lesional endothelium (Detmar et al., 1994). Interleukin-8 may play a key role in psoriasis because it is expressed in the stratum granulosum, attracts polymorphonuclear cells, and stimulates angiogenesis and keratinocyte mitogenesis (Konstantinova et al., 1996).

As is demonstrated for psoriasis, obesity is also dependent on angiogenesis (Crandall et al., 1997). Rats that are exposed to cold perform adaptive hyperplasia of adipose tissue. This coincides with abundant overexpression of VEGF in this tissue. This reaction was regulated by β-adrenergic stimulation of the adipose tissue and was mediated by $VEGF_{120}$, the short heparin binding site lacking isoform of VEGF (Asano et al., 1997).

Interestingly, the endothelial adhesion molecule E-selectin, which was identified many years ago as a prime mediator of leukocyte tethering from the blood stream, was more recently identified as a marker of angiogenic endothelium (Luscinskas et al., 1989; Nguyen et al., 1993). It may well be that specific isoforms of E-selectin prevail during the angiogenic process, although at present, the mechanism(s) leading to this and the functional consequences of such molecular modulations have not been extensively investigated (Verkarre et al., 1999).

In vitro, activated T cells were able to induce endothelial expression of MMPs and vascular tube formation in a three-dimensional gel assay by CD40-CD40 ligand interactions. In sites of chronic inflammation such as atherosclerotic plaques, vascular expression of CD40 and its ligand has been reported. These data suggest that ligation of CD40 on endothelium can mediate several aspects of vascular remodeling and neovascularization during atherogenesis and other chronic inflammatory diseases (Mach et al., 1999). In addition, VEGF was shown to be moderately to strongly expressed in atherosclerotic human arteries. Smooth muscle cells as well as macrophage-derived foam cells and ECM near macrophages contained extensive VEGF levels. In regions rich in macrophages, the prominent T cell infiltrate was a major source of VEGF (Couffinhal et al., 1997; Inoue et al., 1998; Chen et al., 1999a). The more the atherosclerotic lesions advanced, the more often the lesions contained intimal blood vessels. VEGFR-1 and -2 were both distinctly upregulated in macrophages and endothelial cells in the lesions (Inoue et al., 1998).

The increased serum levels of VEGF that correlated with disease activity in patients with inflammatory bowel diseases, Crohn's disease and ulcerative colitis, indicate a role for this cytokine in promoting inflammation in these chronic inflammatory diseases as well. The mechanism of action may be through increasing the vascular permeability and/or wound healing via its proangiogenic effects (Bousvaros et al., 1999).

In endometriosis, excessive endometrial angiogenesis is proposed as a mechanism in the pathogenesis of this disease. The endometrium of women with endometriosis has an increased capacity to proliferate, implant, and grow in the peritoneal cavity. There is enhanced endothelial cell proliferation in the endometrium of women with this disease, and these vessels express the cell adhesion molecule $\alpha_v\beta_3$ integrin. This demonstrates that endometriosis is a disease with a proangiogenic character, which suggests that novel new medical treatments for endometriosis can be aimed at the inhibition of angiogenesis (Rogers and Gargett, 1999).

Intraocular neovascularization causes a functional disorder of the eye and contributes to loss of vision. It is associated with diseases such as diabetic retinopathy, retinal vein occlusion, and age-related macular degeneration. In diabetic retinopathy and other retinal disorders, vitreous levels of soluble E-selectin and ICAM-1 were significantly elevated, indicating a strong inflammatory component in these diseases (Esser et al., 1995; Limb et al., 1999). Furthermore, several types and layers of retinal cells can produce VEGF, the major medi-

ator of intraocular neovascularization and permeability (Brown et al., 1997). Proangiogenic factors such as placenta growth factor and TGF-β1 may also be involved in deposition of other proangiogenic proteins in the blood vessel walls leading to neovascularization (Spirin et al., 1999). Recently, a hierarchical relationship between insulin-like growth factor-1 and VEGFR signaling in retinopathy was identified. This can (partially) explain the observation in diabetic patients who are treated with insulin that a rise in insulin-like growth factor-1 levels is followed by VEGF-induced retinopathy (Smith et al., 1999).

In various tumor types, inflammatory cells are present in the stromal areas. These cells may account for the production of cytokines that attract additional inflammatory cells but can have a proangiogenic activity themselves as well (Chen et al., 1999b; Goede et al., 1999). In wound healing, inflammation is followed by angiogenesis before resolution of the wound occurs (Witte and Barbul, 1997). Similarly, in foreign body reactions against biomaterial implants, neovascularization and giant cell formation take place in concert. Recently, a macrophage-derived peptide, PR39, was shown to inhibit proteasome-mediated degradation of HIF-1. This resulted in accelerated vasculature formation in mice (Li et al., 2000). All these observations indicate that in a variety of conditions, angiogenesis and inflammation exist at the same time. The differences in the expression of molecules such as CD40 and in the composition of cellular infiltrates between, e.g., rheumatoid arthritis lesions and solid tumors are highly suggestive of pathology-related variations in the angiogenic and inflammatory processes. Possibly, mechanisms driving the angiogenic cascade are differentially regulated depending on the disease pathology. Future studies will provide clues on whether regulatory fine tuning of angiogenic processes under the various pathologic conditions can be exploited therapeutically. Such an approach seems justified when undesired side effects in physiologic angiogenesis occur during therapy directed against pathologic angiogenesis.

C. Inhibition of Angiogenesis in Chronic Inflammation

In theory, several advantages of antiangiogenic therapy for chronic inflammatory diseases can be envisioned, similar to those of tumor growth-directed antiangiogenic strategies. First of all, suppression of blood vessel growth leads to diminished nutrient supply to the metabolically active cells present in inflamed tissue. Second, by preventing blood vessel formation, the entry route of inflammatory cells into the tissue becomes blocked. A third potential advantage of inhibiting endothelial cell activation, proliferation and vascular remodeling in chronically inflamed lesions is the inhibition of the production of endothelial cell-derived soluble factors such as MMPs and cytokines.

One of the first studies on the pharmacologic effects of angiogenesis inhibition in chronic inflammatory disease was reported by Peacock et al. (1992). They showed that the antiangiogenic fumagillin analog AGM-1470 (TNP-470) could prevent and even reverse established arthritis in rats. In combination with taxol, a microtubule inhibitor interfering with cell mitosis, migration, chemotaxis, and intracellular transport functions, an even greater reduction of arthritis was observed (Oliver et al., 1994).

Recent research on atherosclerosis investigated the role of angiogenesis (Carmeliet and Collen, 1998). In apolipoprotein E-deficient mice, neovascularization similar to that observed in human atherosclerotic lesions occurs. Treatment with either endostatin or AGM-1470 for 16 weeks inhibited plaque growth by 85 and 70%, respectively, whereas intimal smooth muscle cell content did not change during the treatment period (Moulton et al., 1999). This study clearly demonstrated that neovascularization is involved in the promotion of plaque formation and that antiangiogenic therapy in this disease may be an effective strategy.

Thalidomide is a drug that has now been tried in humans for various diseases, among which are rheumatoid arthritis and gastrointestinal ulcerations. It is believed to act primarily as an inhibitor of TNF expression by destabilization of TNF mRNA. It may, however, also impair angiogenesis, possibly by down-regulating endothelial integrin expression (Marriott et al., 1999; Sands and Podolsky, 1999). In an arthritis rat model, on the other hand, inhibited collagen induced arthritis by mechanisms other than TNF or VEGF down-regulation (Oliver et al., 1998). Also, thrombospondin-1 has been studied in arthritis models. Surprisingly, this angiogenesis inhibitor augmented the severity of the disease (Koch et al., 1998). These findings may be explained by interference in the numerous angiogenesis-independent functions of thrombospondin-1. Alternatively, it may reflect disadvantageous features of antiangiogenic treatment options for arthritic disease.

The observation that synovial blood vessels from rheumatoid arthritis patients have an increased expression of integrin $\alpha_v\beta_3$ prompted Cheresh and collaborators (Storgard et al., 1999) to study the effects of inhibition of this integrin on arthritic disease in rabbits. Intra-articular administration of a cyclic peptide antagonist of $\alpha_v\beta_3$ induced vascular apoptosis and inhibition of synovial angiogenesis. This was paralleled by a reduction of joint swelling, synovial infiltrate, and pannus formation in both early and well established arthritis. Moreover, the antagonist was able to protect against cartilage erosions.

Antiangiogenic strategies have enormous potential for clinical application in the treatment of arthritis and other chronic inflammatory diseases accompanied by overt neovascularization (Firestein, 1999). Furthermore, application of antiangiogenic strategies will allow

us to study in more detail the functional role of angiogenesis during chronic inflammation. Initiation of the angiogenic cascade during inflammation is most likely caused by activated and proliferating cells that are in continuous demand of nutrients and oxygen. It may well be that during a prolonged inflammatory response, cells involved in the angiogenic cascade start to function autonomously. Continuous production of cytokines involved in angiogenesis as well as leukocyte recruitment and activation may be the consequence. These issues need to be studied to better understand the role of angiogenesis in the pathology of chronic inflammation and to design tailor made drugs to interfere at specific levels of regulation of the disease.

D. Clinical Trials with Inhibitors of Angiogenesis for Noncancerous Diseases

At present, data on the effects of drugs in patients suffering from chronic inflammatory diseases with respect to their antiangiogenic activity are limited. One study on interference with macular degeneration was announced to be initiated soon. The neovascular form of age-related macular degeneration is the leading cause of blindness and vision impairment in people aged 60 years and older. A phase II clinical trial will assess the safety of the MMP inhibitor AG3340 in approximately 100 patients aged 50 years and older affected by this disease and determine the optimal dose and regimen to use in subsequent phase III trials.

In rheumatoid arthritis patients treated with TNF-α-blocking antibodies, a decrease in synovial vascularity was observed. This effect is likely to be mediated by VEGF, because treatment with anti-TNF-α antibodies significantly decreased serum VEGF levels. Studies with human rheumatoid arthritis synovial membrane cultures furthermore demonstrated that a combined neutralization of TNF-α and IL-1 elicited a more pronounced reduction of VEGF production than separate inhibition of either cytokine. Whether IL-1 also participates in in vivo effects exerted by anti-TNF-α antibodies is not clear yet (Paleolog et al., 1998).

Thalidomide was shown to be efficacious in some patients with refractory Crohn's disease. In an open label trial, 9 of 22 patients with either luminal disease or fistulas achieved clinical remissions (Ehrenpreis et al., 1999). Although at present the mechanism through which thalidomide induced these remissions remains to be elucidated, it is believed to act via a combination of direct inhibition of TNF production and angiogenesis.

V. Back to the Drawing Board

An enormous research effort in the last decade has resulted in important advances in our understanding of the role of angiogenesis in health and disease. Still, several issues will have to be addressed in the coming years before we can fully appreciate the options and limitations of modulation of angiogenesis as a therapeutic tool.

A. Angiogenesis Stimulation

The first results of the angiogenesis stimulation trials in patients with ischemic disease are encouraging. However, the company that developed VEGF protein for this purpose recently announced that they would not pursue further clinical trials with this protein. This decision followed disappointing results with a phase II trial in 178 patients. The 60-day results of this trial showed no difference in clinical improvement compared with placebo, and although the 120-day follow-up was more promising, the trial was halted. Because the protein was very effective in animal models, it has now taken back into the laboratory to improve its effectiveness An enormous research effort in the last decade has resulted in important advances in our understanding of the role of angiogenesis in health and disease. Still, several issues will have to be addressed in the coming years before we can fully appreciate the options and limitations of modulation of angiogenesis as a therapeutic tool. An important obstacle in this respect is the fact that the responses of patients suffering from ischemia and those of healthy animals with a relatively short-term ischemic insult are likely to be incomparable (Ferrara and Alitalo, 1999).

As with angiogenesis inhibition, it may well be that the effects of angiogenesis induction also takes months to become evident. Although the clinical studies on the naked plasmid VEGF administration in myocardial ischemia patients challenges this, it is important to note that these were not placebo-controlled studies. The development of a catheter that can inject the plasmid without the need for opening the chest now allows placebo-controlled studies and proper evaluation of the effects. If indeed prolonged levels of proangiogenic factors will be required for treatment success, the effects of prolonged treatment on tumor dormancy will be an issue to reconsider. Even though it is believed that the angiogenic factors alone do not affect a tumor's angiogenic switch, this belief has been based on relatively short-term studies in animals. Markers other than VEGF or FGF-2 plasma levels used to measure the occurrence of tumor-specific angiogenesis would be enormously useful in this respect.

At present, all studies on angiogenesis induction made use of a single proangiogenic factor. Given the complexity of the angiogenic cascade, this may result in incompletely functioning or unstable endothelial channels with defects in differentiation (Ferrara and Alitalo, 1999).

B. Antiangiogenic Strategies in Cancer Therapy

One of the main messages during the angiogenesis symposia at the 1999 Annual Meeting of the American Association of Cancer Research was that "we need to go back to the drawing board". Although many antiangio-

genic therapies for treating cancer were highly active in animal models, clinical results so far are disappointing. This may either be a result of the most promising antiangiogenic compounds having not been tested in the clinic yet or that the read-out systems available for measuring clinical efficacy of antitumor drugs are not suitable for measuring antiangiogenic effects. For a better understanding of the pharmacologic effects of antiangiogenic therapies in cancer patients, methods need to be developed that are able to determine the response(s) of the body to the therapy. In the clinical trials recently performed on angiogenesis stimulation with ph-$VEGF_{165}$, markers of blood flow, imaging techniques, and simply increased limb viability nicely demonstrated clinical improvement. For antiangiogenic therapy for tumor growth or chronic inflammatory diseases, the read-out system is less clear. In addition to this, biological agents need a longer period of time to induce a response, in contrast to, e.g., cytotoxic drugs. An example of this has been described by Folkman and colleagues (Ezekowitz et al., 1992), who treated a child with hemangioma with IFN-α for almost a year before disease improvement was visible. Furthermore, biological agents are likely to exert their effects in a concentration-dependent way. At higher concentrations, antagonistic effects or loss of effects may occur, as has been described for TNF-α and granulocyte macrophage-colony-stimulating factor, for example. A complete inventory on cytokine levels in sera/plasma of patients before and after treatment with antiangiogenic drugs would be ideal to see whether it is possible to define a set of (surrogate) markers for antiangiogenic effects. In several tumor types, VEGF plasma levels, for example, can be of value in predicting tumor vascularity and disease outcome, and hence serve as a parameter of treatment effectiveness. These levels are, however, not applicable to all tumor types and disease stages (Salven et al., 1998; Gadducci et al., 1999). It is likely, therefore, that tumor type-specific sets of markers need to be defined. In this respect, it would be of enormous help, but extremely difficult to achieve, to define markers related to endothelial cell death and decreased endothelial cell proliferation rate, because these are *the* common denominators of antiangiogenic activity.

Of great importance is the observation by Kennel et al. (1999) that a better antitumor response of targeted ^{213}Bi was observed in immunocompetent mice. Many antitumor studies, either exploiting "conventional" chemotherapeutic drugs or drugs with a pronounced antiangiogenic effect, are performed in immunodeficient mice. They accept human tumor grafts but lack additional antitumor mechanisms brought about by cells of the immune system. It is very possible, that upon tumor endothelial cell inhibition/killing or blood coagulation induction, an inflammatory response is initiated when an intact immune system is available. In contrast to cell killing via apoptosis, a mechanism aimed at eliminating the dying cells without eliciting an inflammatory reaction, target cell killing via necrosis is expected to be more effective in initiating such an inflammatory response. These effects are underestimated in immunodeficient animal models. In cancer patients, defects in the immune defense may exist, and additional immunologic responses on antiangiogenic therapies may or may not be present. To date, we are not capable of fully interpreting the consequences of the lack of certain immunologic specificities on the effects of antiangiogenic therapies due to lack of knowledge. Another major drawback of the use of xenograft mouse models is the xenogeneic interplay between the mouse vessels and the human tumor. It is unknown to what extent mouse vessels in a human microenvironment are more fragile than vessels in an entirely syngeneic mouse model.

An important issue, which has not been addressed to the full extent, is the potential occurrence of side effects during antiangiogenic treatment. Recently, Ferrara and colleagues (Gerber et al., 1999) demonstrated that VEGF-driven angiogenesis is an important feature of bone formation in the growth plate. This may have important implications for antiangiogenic therapies in the elderly when the growth plate undergoes active remodeling. From the clinical studies with MMP inhibitors, it was observed that at generally well tolerated doses, in most cases musculoskeletal- and joint-related events occurred approximately 4 weeks after the start of treatment. These events included joint stiffness and swelling, and, limited to a few patients, the mobility of certain joints. The side effects typically begin in a dose- and time-dependent manner and may be explained by unintentional inhibition of proteolytic processes of macrophages that are involved in bone and cartilage remodeling in the joints. The side effects are reversible and can be managed by treatment rests and subsequent dose reduction (Wilding et al., 1998). The recent withdrawal of two MMP inhibitors from clinical trials indicates the occurrence of toxicities that were not obvious in animal models (Anonymous, 1999b).

Because angiogenesis is a process that occurs during many normal physiologic processes, great care should be taken to address the issue of side effects of the tumor vasculature-directed antiangiogenic therapy in patients as well.

One of the advantages of antiangiogenic therapy is believed to be the lack of induction of resistance to therapy (Boehm et al., 1997). This idea is based on the fact that processes exerted by endothelial cells in angiogenesis are not a result of endothelial cell genetic alterations in the oncogene/tumor suppressor gene activity. In other words, the absence of drug resistance to angiogenesis inhibitors is most likely explained by the fact that endothelial cells are genetically stable cells that are considered not to mutate into drug-resistant variants. Although this is a highly promising feature of this kind of treatment, absence of resistance strongly depends on

the type of angiostatic therapy applied. If the therapy is aimed interference at the level of tumor cell produced (growth) signals, it can be expected that the therapy eventually will run into drug-induced resistance due to adaptation or mutation of the tumor cells. For example, when the approach consists of intervention at VEGF signaling, regardless of which approach (blocking antibodies, soluble receptors, or inhibition of signal transduction), the tumor will either switch to dependence on other growth factors, or selection of other growth factor-dependent cells will occur. The recent observation by Jain and coworkers (Hansen-Algenstaedt et al., 1999), that upon long-term blocking of VEGF activity a second wave of VEGF-independent angiogenesis occurred, shows the importance of this concept. Next to this phenomenon, the success of the use of angiogenesis inhibitors that are not specific for endothelial cells, such as MMP inhibitors and the calcium influx inhibitor CAI, may also very well be dependent on effects on the tumor cells. In that case, mutation of tumor cells will reduce the efficacy of the compound. Also, it may well be that the dual activity of these compounds dictates their efficacy and loss of one effector function reduces therapeutic outcome. A therapeutic consequence of these considerations is that treatment of excessive angiogenesis with only one angiogenesis inhibitor is not an option. Moreover, one should realize that in animal models of either tumor growth or inflammatory angiogenesis, the majority of the vasculature is in a proangiogenic state. In contrast, in human tumors the percentage of proangiogenic vessels is variable, often quite low, and hence antiangiogenic therapy may only affect the minority of vessels. Furthermore, fine-tuned strategies to target specific stages of the disease progression and hence angiogenesis were recently proposed (Bergers et al., 1999). Such strategies require an enormous preclinical research effort on the most potent formulations, dosing regimens, and so on, and therefore, clinical applications of these optimized strategies are not expected to be started soon.

The heterogeneity in angiogenic stages in human tumor vasculature makes that antiangiogenic therapy alone is believed to be never sufficiently effective on its own. Combining antiangiogenic therapy with conventional, tumor cell-directed therapies seem contradictory; blockade of the formation of new blood vessels will hamper the availability of the chemotherapeutics in the tumor tissue. Whereas antiangiogenic therapeutics act on vessels in hypoxic tumor sites, chemotherapeutics would become available in sites where the tumor vasculature is at rest. One can ask the question whether the tumor cells in such a site are nondividing cells, requiring few nutrients *and* being unresponsive to chemotherapeutics acting on cell cycle regulatory processes. On the other hand, by lowering the tumor mass with antiangiogenic drugs, intratumoral pressure may concurrently drop. As a result, the availability of chemotherapeutics can increase. Furthermore, radiotherapy had an additional antitumor effect when combined with angiostatin in animals and hence may be considered for combination therapies as well (Mauceri et al., 1998). Still, one has to keep in mind that these therapies have all been tested in animal models with a homogeneous proangiogenic tumor vasculature make-up. Lack of proper knowledge on the mechanistic backgrounds of this potentiating effect prohibits any prediction on usefulness in cancer patients at this moment.

C. Antiangiogenic Strategies in Chronic Inflammation

Many of the above considerations on tumor-directed antiangiogenic therapies seem to be applicable to chronic inflammatory diseases. The codependence of angiogenesis and chronic inflammation (Jackson et al., 1997) seems to justify the development of inhibitors of chronic inflammation for specific tumor types and inhibitors of angiogenesis for the treatment of chronic inflammation. Still, the data obtained so far do not allow a definite conclusion about whether inflammation-induced angiogenesis and tumor growth-induced angiogenesis are analogous processes. For example, in animal models, thrombospondin-1 inhibited tumor-induced angiogenesis but worsened the disease parameters in adjuvant-induced arthritis (Koch et al., 1998). Therefore, care should be taken in just extrapolating the knowledge on tumor angiogenesis to the situation of chronic inflammation. And similar to anticancer therapy, knowledge on the stages of angiogenesis during chronic inflammation in human and animal models is a prerequisite for designing optimal treatment strategies.

Maintenance of the vascular wall integrity is a general function of endothelial cells. If the integrity of endothelium is lost, the integrity of the whole body will be negatively affected. Therefore, regulatory processes in endothelial cells are such that they acquire a phenotype that protects them from activation-induced damage, e.g., cytokine-induced apoptosis. With this function, the endothelium distinguishes itself from almost all other cell types in the body (Badrichani et al., 1999). This characteristic is believed to play a pivotal role in endothelial cell function and possibly dysfunction especially under proinflammatory conditions. This subject of protective gene expression has up to now only been extensively addressed in transplantation-related research. One can easily envision that under chronic inflammatory conditions, both proangiogenic and proinflammatory factors affect these specific functions of the endothelium. Moreover, they may significantly contribute to the outcome of therapeutic interventions aimed at proangiogenic processes in chronic inflammation. Addressing these issues in full detail and comparing the status of the endothelium in patient lesions and lesions in animal models with respect to these specific characteristics will provide insight based on which drugs can be developed.

Taking into account that inhibitors of angiogenesis may be quite toxic to normal tissues, it is worthwhile to consider the selective delivery of drugs to endothelial cells to block their proangiogenic behavior. But, in contrast to endothelial cell killing as an effector modality in tumor endothelial cell targeting, a more sophisticated approach will be required to obtain anti-inflammatory effects.

VI. Concluding Remarks

Angiogenesis is an important process during normal physiology and pathologic conditions such as ischemic diseases, chronic inflammatory diseases, and tumor growth. Important advances have been made in unraveling the regulatory pathways involved in the various steps that take place during angiogenesis. The cellular players, soluble factors, and environmental conditions that are able to affect one or more of the angiogenic steps have been identified in studies referred to in this review and in many others. In the coming years, it will be a great challenge to unravel the causal versus the consequential relation between the various disease pathologies and the spatiotemporal disbalance in pro- and antiangiogenic entities in human disease in light of the development of better therapies. In this respect, the advent of novel molecular biological techniques such as the isolation of single cells from patient biopsies and subsequent profiling of gene expression with DNA array technology will be a helpful tool.

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