

Development of Vasculature Targeting Strategies for the Treatment of Chronic Inflammatory Diseases

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Abstract: The pathogenesis of a number of chronic inflammatory processes can be attributed to prolonged neovascularization. This article reviews recent studies on the vasculature targeting strategies for the treatment of chronic inflammatory diseases. Targeting of the vasculature of inflamed organs could underlie a new pharmacological approach in the treatment of inflammatory diseases by taking advantage of formulations that deliver drugs to blood vessels specifically located at disease sites and to inflammatory cells.

Keywords: Antiangiogenesis; chronic inflammatory diseases; vascular targeting

INTRODUCTION

The adult human endothelium measures from 1 to 7 m² and is composed of 1-6 x 10¹³ cells [1] that form a continuous monolayer between the blood and the interstitial fluid, are typically elongated (approx. 30 μm long, 12 μm wide and 0.3 μm deep) and carry a negatively-charged glycosaminoglycan layer (approx. 10 nm deep) on their surface.

Quiescent endothelial cells (EC) generate an active antithrombotic surface that facilitates the transit of plasma and cellular constituents throughout the vasculature, whereas perturbations, such as those at inflammation sites, induce their creation of a prothrombotic and antifibrinolytic microenvironment.

Blood flow is also partly regulated through the EC secretion and uptake of vasoactive substances. Cessation of blood flow into a capillary causes its regression, whereas an increase in pressure induces local recruitment of smooth muscle cells and leads to its differentiation into an artery or vein.

EC have membrane-bound receptors for numerous molecules including proteins (e.g., growth factors, coagulant and anticoagulant proteins), lipid transporting particles (e.g., low-density lipoprotein), metabolites (e.g., nitrous oxide and serotonin), and hormones (e.g., endothelin-1), as well as specific junctional proteins and receptors that govern cell-cell and cell-matrix interactions.

EC play a prominent role in the induction and progression of chronic inflammation through their involvement in angiogenesis and leukocyte recruitment, both of which contribute to its maintenance and exacerbation.

ANGIOGENESIS

A number of inflammatory diseases are the outcome of prolonged neovascularization. Examples are diabetic retinopathy, rheumatoid arthritis and a variety of autoimmune processes. Inflammatory cells, such as macrophages, lymphocytes, mast cells and fibroblasts, and the angiogenic factors they produce, stimulate vessel growth. Increased blood flow itself can stimulate angiogenesis through shear stresses and the endothelium. It is thought that angiogenesis maintains inflammation by allowing recruitment of these and supplying nutrients and oxygen to the inflamed tissue. The increased endothelial surface boosts the production of cytokines, adhesion molecules and other inflammatory stimuli [2].

Local dilation of blood vessels and exudation of fibrinogen are usually the first responses to an inflammatory stimulus. Fibrinogen is converted into fibrin by the procoagulant activities of EC and resident macrophages and a migratory and chemotactic matrix for EC and inflammatory cells is thus established. The inflammatory infiltrate is initially composed of granulocytes, soon followed by a growing number of monocytes. In addition, platelets adhere to the vessel wall and release platelet-derived growth factor (PDGF). Activated EC activate granulocytes and macrophages by their secretion of interleukin-1 (IL-1), IL-8 and granulocyte macrophage colony stimulating factor (GM-CSF). Macrophages are also activated by low pO₂ within the inflamed tissue.

As granulocytes contain high concentrations of proteases, angiogenesis may involve alteration of the extracellular matrix and mobilization of its fibroblast growth factor-2 (FGF-2) stores. Macrophages are not angiogenic per se, but appear to require stimulation [3]. Moreover, their angiogenic activity is associated with their secretory activity and since their half-life is much longer than that granulocytes or platelets, they must be regarded as the principal angiogenic agent after the initial phase.

LEUKOCYTE RECRUITMENT

EC are pivotal in the leukocyte recruitment that accompanies chronic inflammation. They facilitate the transmigration of leukocytes by expressing cell adhesion molecules and producing cytokines and chemokines. Leukocytes overcome the endothelial barrier by adhering to the EC surface and then extravasate through the EC layer. Adhesion usually requires a cascade of steps mediated by selectins, leukocyte activating chemotactic factors and leukocyte integrins [4]. It is commonly accepted that leukocyte docking and transmigration requires the active participation of EC. Leukocyte binding probably leads to signals in these cells that open their contacts and junctions to facilitate the passage of leukocytes [5].

SELECTINS

The selectin family consists of three molecules. Each has an aminoterminal lectin domain primarily responsible for its adhesive activity [6]. This is followed by an epidermal growth factor (EGF) motif, from 2 to 9 repeated complement regulatory domains, a transmembrane domain and a cytoplasmic tail. The major structural difference between the three is in the number of regulatory domains: L-selectin, expressed by leukocytes, has two; E-selectin, whose expression is limited to activated EC, has six; P-selectin, expressed by platelets and EC, has nine [7].

The strongly enhanced expression of both E- and P-selectin on EC in inflamed sites in both animals and humans [8] and allows specific drug targeting to these cells, and leukocyte recruitment to these sites is greatly reduced by selectin-blocking antibody or peptides.

Selectins initiate, but cannot maintain leukocyte endothelial interactions powerful enough to permit transmigration. Various stimuli are needed to activate leukocytes and enable them to bind more tightly to the endothelial surface, transmigrate and home to specific microenvironments within the extracellular tissue. Activation of β1- and β2-integrins is an essential step in this process [9]. Selectins are directly involved in the transmission of such stimuli. However, the primary players in signal transducing are chemokines at the EC surface and their receptors on leukocytes.

ICAM AND VCAM

Activated leukocyte integrins bind to their ligands or counter-receptors on the endothelium, intercellular adhesion molecules 1 and 2 (ICAM-1 and ICAM-2). Together with vascular cell adhesion molecule-1 (VCAM-1), they make high-affinity adhesions with the leukocyte integrins. This allows them to stop rolling and begin crawling on the EC surface, the next step in their migration [10].

ICAM-1 is expressed on leukocytes, EC, fibroblasts, and epithelial cells, and interacts with β-2 integrins [9]. It is strongly upregulated during

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Table 1. Main Chronic Inflammatory Disease Based on Angiogenetic Process

Disease	Main inflammatory component	Pro-angiogenic factor Involved	Pathological findings
<i>Rheumatoid Arthritis</i>	<i>VCAM-1, E-selectin, $\alpha\beta 3$</i>	<i>VEGF, FGF-2, IL-8</i>	<i>New immature microvessels in synovial pannus</i>
<i>Psoriasis</i>	<i>Infiltration of activated T cells, E-selectin, ICAM-1</i>	<i>VEGF, VEGFR-2, IL-8, IL-15, IFN-γ, TNF-α, IL-17</i>	<i>Dermal angiogenesis new typical microvessels in psoriatic plaque</i>
<i>Mice airway inflammation (by pseudomonas aeruginosa)</i>	<i>Lymphocytes, Macrophages</i>	<i>IL-1β, TNF-α, IL-8</i>	<i>Pulmonary vascular remodeling angiogenesis in the intra-acinar tissue</i>
<i>Murine air pouch granuloma model</i>	<i>Up-regulation of adhesion molecules</i>	<i>IL-1β, TNF-α, IL-8 metalloproteinase</i>	<i>New microvessels in inflammatory tissue</i>
<i>Atherosclerosis</i>	<i>CD40-CD40 ligand up-regulation, T cell, foam macrophages</i>	<i>VEGF, VEGFR-1, VEGFR-2, TNF-α TGF-β</i>	<i>New microvessels in atherosclerotic plaque</i>
<i>Diabetic retinopathy</i>	<i>E-selectin, ICAM-1</i>	<i>Link between insulin-like growth factor-1 and VEGFR</i>	<i>Retinal neovascularization VEGF in retinal cells</i>
<i>Corneal disease</i>	<i>CD18, ICAM-1</i>	<i>VEGF</i>	<i>Corneal neovascularization</i>
<i>Alzheimer's disease</i>	<i>β-amyloid plaque</i>	<i>VEGF, TNF-α, TGF-β</i>	<i>Increased microvessel density, vascular loop formation in brain</i>
<i>Bowel Disease</i>	<i>Mucosal cytokines, lymphocytes, macrophages</i>	<i>VEGF, b-FGF, TGF-$\beta 2$ and TGF-$\beta 3$, TNF-α</i>	<i>Increased vascular bed in the intestinal wall</i>
<i>Scarred Kidneys Secondary to Urinary Tract</i>	<i>T cells, followed by macrophages</i>	<i>PD-ECGF/TP, 2-deoxy-D-ribose, β-Amino-iso-butyric acid, VEGF, TNF-α</i>	<i>At tubulo-interstitial site: increased microvessel density, increased TP expression, fibrosis</i>

inflammation. Anti-ICAM-1 antibodies have proved very effective in several animal chronic inflammation models and clinical arthritis trials. ICAM-2 is expressed on leukocytes, EC and monocytes. By contrast with ICAM-1, it is constitutively present and not upregulated during inflammation. VCAM-1 is expressed at low levels on EC in the absence of inflammation and induced by cytokine-mediated stimulation [11]. VCAM-1 and ICAM-1 are the main adhesion molecules for leukocyte recruitment. Anti-VCAM-1 antibody is efficacious in delayed-type hypersensitivity and rheumatoid arthritis.

PLATELET ENDOTHELIAL CELL ADHESION MOLECULE-1 (PECAM-1) AND CD99

PECAM-1 is constitutively expressed on EC at high levels and strongly involved in leukocyte transmigration [12]. Its expression is only slightly enhanced by stimulation with cytokines and mainly confined to the intercellular junctions. High basal levels of expression of anti-PECAM-1 antibody exert a therapeutic effect in inflammation.

Muller et al. have identified CD99 as a second surface protein at EC contacts that seems to be important for leukocyte extravasation. PECAM-1 and CD99 inhibition apparently blocks leukocyte transmigration in sequential steps [13,14].

CD40-CD40 LIGAND

Interactions between CD40 ligand and CD40 establish an important link between inflammation and vascular endothelial growth factor (VEGF)-induced angiogenesis. CD40, a 50 kd type I transmembrane glycoprotein member of the tumour necrosis factor receptor (TNF-R) gene family, is expressed by monocytes, macrophages and EC. CD40 ligand, a 33 kd type II membrane protein member of the TNF family, is predominantly expressed by activated CD4 lymphocytes and platelets. Signaling via CD40 mediates the activation of monocytes and the expression of ICAM-1 and several cytokines, such as TNF- α , transforming growth factor beta (TGF- β) and VEGF. Blockade of CD40-CD40 ligand inhibits chronic inflammation and angiogenetic lesions [15].

CHEMOKINES

Chemokines are a family of related cytokines with potent chemotactic activity towards leukocyte subsets. Due to their large number and both selective and overlapping activities, they are ideal regulators of leukocyte transmigration and trafficking within lymphoid organs and in inflamed

tissues [16]. Their functions, however, are not confined to the regulation of leukocyte transmigration. They are involved in hematopoietic development, angiogenesis and T cell and NK cell activation [17].

THE ROLE OF ANGIOGENESIS IN CHRONIC INFLAMMATION

The relationship between angiogenesis and inflammation has received extensive attention in recent years. Increased permeability and endothelial activation accompany vessel dilation in the initial stages of inflammation. Capillaries and venules then remodel at a high endothelial mitotic rate [18] and their density increases in the later stages [19]. Local angiogenesis is a feature of several chronic inflammatory diseases (Table 1).

RHEUMATOID ARTHRITIS

Here angiogenesis is an essential prelude to formation of the synovial pannus. EC soon activate *c-myc* and *c-fos* genes and markers of EC proliferation, such as Ki-67 antigen or proliferating cell nuclear antigen (PCNA), are regulated in rheumatoid synovitis [20-22]. Proliferation increases to levels found in tumors. Several features typical of newly formed microvessels are evident in inflamed synovia: i) lack of pericytes and/or smooth muscle cells; ii) up-regulation of E-selectin and $\alpha\beta 3$; iii) incomplete nerves and receptors for vasoregulatory neuropeptides, such as substance P [23-25]. In the pannus, the endothelium is subjected to continuous remodeling and is also active through its secretion of several pro-inflammatory cytokines. As a complementary feature, synoviocytes display some characteristics of neoplastic cells, including somatic mutations in regulatory genes such as *Ha-ras* and *p53*. The rheumatoid synovium can thus be compared to a tumor-like tissue that invades and destroys its local environment and is enriched in proangiogenic cytokines such as VEGF, FGF-2, IL-8, along with VCAM-1 and E-selectin [26].

PSORIASIS

Psoriasis is a chronic inflammatory disease of the skin characterized by epidermal hyperplasia, dermal angiogenesis, infiltration of activated T cells and increased cytokine levels. Typical new microvessels are observed in the skin at the same time as the active plaque. Increased VEGF production by keratinocytes, endothelial VEGFR-2 overexpression, high IL-8 and IL-15 levels are also evident [27-29]. IL-15 triggers inflammatory cell recruitment, angiogenesis and production of other inflammatory cytokines, including IFN- γ , TNF- α and IL-17, which are all

upregulated in psoriatic lesions. EC activation and proliferation play a key role in the development and maintenance of psoriasis [30].

THE RODENT AIRWAY INFLAMMATION MODEL

Chronic rat lung infection with *Pseudomonas aeruginosa* leads to angiogenesis or compensatory enlargement of the pulmonary vessels [31,32]. Chronic airway infection also causes pulmonary vessel remodeling and intra-acinar angiogenesis. Angiogenesis can thicken the airways in asthma and the lung parenchyma in pulmonary fibrosis and contribute to the growth of sarcoid granulomas. Invasion by granulation tissue may also be angiogenesis-dependent in bronchi after lung transplantation, in bronchioles in bronchiolitis obliterans organising pneumonia, and in alveoli after acute lung injury or other forms of pulmonary fibrosis [33]. These changes maintain the lumen cross-section and provide new vascular pathways through the lung to prevent pulmonary hypertension despite significant thickening of the vessel wall. Pulmonary angiogenesis may thus play an important role in lung disease and chronic inflammatory disorders [33].

MURINE AIR POUCH GRANULOMA MODEL

Murine air pouch granuloma displays a chronic inflammatory progression with a profound angiogenic component [34] and it provides a model for quantification of the modulation of angiogenesis in an inflammatory bed. During the chronic inflammatory phase, high IL-1 β and TNF- α are accompanied by intense angiogenesis [35]. Their numerous activities include upregulation of IL-8, upregulation of adhesion molecule expression, stimulation of matrix metalloproteinase expression and increased prostaglandin production [36], many of which may contribute to their angiogenic activity.

ATHEROSCLEROSIS

Atherosclerotic plaques display chronic inflammation with concomitant VEGF overexpression and CD40-CD40 ligand upregulation. VEGF levels are high in smooth muscle and macrophage-derived foam cells, and the T cell infiltrate near the macrophage is a source of VEGF. In addition overexpression of VEGFR-1 and -2 on EC is often observed [37]. Activation of the CD40-CD40 ligand leads to angiogenesis and vascular bed optimisation, while its interaction results in expression of TNF- α , TGF- β and VEGF via a transcriptional mechanism.

DIABETIC RETINOPATHY

Retinal angiogenesis occurs in a variety of diseases including diabetic retinopathy, the most common cause of blindness in the developed world. Aberrant, ischemia-induced angiogenesis produces vessels that protrude beyond the retinal inner limiting membrane into the vitreous, causing severe loss of vision and frequently leading to retinal detachment. Significant elevation of E-selectin and ICAM-1 is indicative of a strong inflammatory component [38,39] and VEGF is produced by retinal cells. The intriguing relationship recently discerned between insulin-like growth factor-1 and VEGFR signaling in retinopathy may partly explain why a rise in insulin-like growth factor-1 levels is followed by VEGF-induced retinopathy when diabetics are treated with insulin [40].

CORNEAL DISEASE

Angiogenesis is a common feature of inflammatory, infectious and traumatic diseases of the cornea and the limbal stem cell barrier. It is a severely disabling condition that deprives the cornea of its immunological capabilities and results in visual impairment [41]. VEGF may be of importance in this process since exogenous VEGF stimulates corneal neovascularization [42]. Even so, CD18 and ICAM-1 are required as mediators for the VEGF-dependent corneal angiogenesis that follows limbal injury [42] and their small-molecule targeting may prove useful in its treatment when topically applied [43].

ALZHEIMER'S DISEASE

In this disease, the brain endothelium secretes the precursor substrate for the β -amyloid plaque, a neurotoxic peptide that selectively kills cortical neurons. Angiogenesis occurs in response to cerebral hypoperfusion and inflammation and is revealed by increased microvessel density, vascular loop formation and overexpression of VEGF, TNF- α and TGF- β . Epidemiological studies have shown that long-term use of non-steroidal anti-inflammatory analgesics, statins, histamine H2 receptors blockers or

calcium-channel blockers seems to prevent Alzheimer's disease, perhaps because they inhibit angiogenesis [44]. Antiangiogenic drugs targeting abnormal brain EC could perhaps provide a means of both prevention and treatment.

BOWEL DISEASES

Inflammatory bowel disease (IBD) comprises ulcerative colitis and Crohn's disease, and is increasingly thought to be the result of dysfunctional immunoregulation manifested by inappropriate production of mucosal cytokines. Abnormal angiogenesis has also been implicated in its pathogenesis. Serum VEGF and FGF-2 levels are significantly higher in active IBD than in healthy controls. Immunohistochemical studies have shown an increased vascular bed in the intestinal wall. Overexpression of VEGF and FGF-2 by EC and the presence of TGF- β 2 and TGF- β 3 are other features of IBD [45], while regulation of TNF- α , a key mediator in IBD, is interconnected with mitogen-activated protein kinase pathways. Vasoconstriction provoked by the reciprocal reactions of these cytokines may encourage angiogenesis by inducing intestinal ischemia [46].

SCARRED KIDNEYS SECONDARY TO URINARY TRACT DISEASES

Tubulointerstitial disorders are marked by inflammation, tubular atrophy and interstitial fibrosis. Injured tubular cells releases a variety of growth factors and cytokines that promote peritubular inflammation, scar formation and angiogenesis. T cells predominate, followed by macrophages [47,48]. The latter may release angiogenic factors including VEGF, TNF- α , FGF-2 and PDGF [49-51]. PDGF is an EC mitogen originally purified to homogeneity from human platelets [52] that stimulates EC chemotaxis *in vitro* and displays an angiogenic activity *in vivo*. It is now known to be the same as thymidine phosphorylase (TP) [53], which catalyzes the reversible phospholysis of thymidine to 2-deoxy-D-ribose-1-phosphate and thymine. Furthermore, 2-deoxy-D-ribose derived from this phosphate has chemotactic and angiogenic activities, while thymine is metabolized to dihydrothymine. This, in turn, is converted to β -amino-iso-butyric acid, which stimulates microvessel formation and elongation *in vitro* [54]. PDGF is important in the progression of human cancers, and its overexpression is associated with increased vessel count [55-58]. Immunostained PDGF has been observed in T cells, macrophages, fibroblasts, renal tubules and the fibrotic areas of scarred kidneys [51].

EC AS TARGETS IN THE TREATMENT OF CHRONIC INFLAMMATORY DISEASES

Administration of angiogenesis inhibitors in inflammation will be primarily undertaken to prevent the clinical and pathological consequences of aberrant vascular growth and normalize vascular reactivity though their inhibition of tissue growth and remodeling, and impairment of the formation of anastomoses, shunting and mismatches between blood flow and metabolic demand [59]. EC are easily accessible as treatment targets since they line the blood vessels and are in direct contact with the blood [60-63].

A rationale approach to the development of drug carriers specifically targeting EC in chronic inflammation is to identify epitopes preferably expressed on the diseased endothelium alone as opposed to resting EC [9]. The characteristics of the epitope and the carrier molecule will determine whether the carrier is internalized and the drug acts inside the cell (intracellular delivery), or is bound to the cell surface and the drug acts outside the cell (extracellular delivery) [59]. Prolonged circulation of the carrier is probably required to deliver sufficient amounts of drugs into the EC.

Both particle and soluble drug carriers are being developed (Table 2). Carriers must be stable *in vivo*, protect the drug from degradation and the body from harmful side effects, and allow for specific interaction with and drug delivery to the target cell. This in turn must be accessible and display surface molecules allowing selective targeting and efficient drug delivery [59-63].

VASCULAR TARGETING

Long-Circulating Particles

The engineered carriers called long-circulating particles described below are used for vascular drug delivery and release, and site-specific targeting.

Table 2. Main Anti-Angiogenetic Vascular Targeting Strategy

Target	Vehicle	Experimental set	Comments
<i>Endothelial cells</i>	<i>Polymeric Nanospheres</i>	<i>Mice, rat</i>	<i>Gene delivery, drugs administration, encapsulated contents</i>
<i>Endothelial cells</i>	<i>Micelles</i>	<i>Mice, rat</i>	<i>Gene delivery, drugs administration, encapsulated contents</i>
<i>Endothelial cells</i>	<i>Liposomes: classical liposomes; sterically stabilized liposomes; pH-sensitive liposomes, cationic liposomes</i>	<i>HUVEC, mice, rat</i>	<i>Gene delivery, drugs administration, encapsulated contents</i>
<i>ELAM on endothelial cells</i>	<i>Immunoliposomes (bearing the anti-ELAM-1 MAb H18/7)</i>	<i>HUVEC</i>	<i>Genes delivery, drug administrations such as doxorubicin</i>
<i>E-selectin on endothelial cells</i>	<i>Dexamethasone-anti-E-selectin conjugate</i>	<i>TNF-α-stimulated endothelial cells</i>	<i>Down-regulation of the proinflammatory gene IL-8</i>
<i>E-selectin on endothelial cells</i>	<i>Immunoconjugate Hirudin- MoabH18/7</i>	<i>HUVEC</i>	<i>Protection from thrombin-induced event</i>
<i>VCAM on endothelial cells</i>	<i>Immunoliposomal</i>	<i>Watanabe rabbits</i>	<i>Atherosclerosis treatment</i>
<i>VCAM on endothelial cells</i>	<i>Phosphatidylserine liposome</i>	<i>In vitro</i>	<i>Thrombogenesis of blood vessels</i>
<i>ICAM-1 on endothelial cells</i>	<i>Small peptides</i>	<i>In vitro</i>	<i>Injury of blood vessels</i>
<i>ICAM-1 on endothelial cells</i>	<i>Murine MAb to human ICAM-1 (R6.5) coated with fluorescent microspheres biotinylated and complexed to streptavidin</i>	<i>HUVEC</i>	<i>Drugs administration, injury of blood vessels</i>
<i>PECAM-1 on endothelial cells</i>	<i>Anti-PECAM antibodies-conjugates multivalent anti-PECAM- conjugates</i>	<i>Endothelial cells in culture Pulmonary vessels in mice</i>	<i>Injury of blood vessels</i>
<i>PECAM-1 on endothelial cells</i>	<i>Conjugation of anti-PECAM with streptavidin</i>	<i>HUVEC</i>	<i>Improved endothelial cells targeting: administration of drugs, genes, and toxins and blood vessels destruction</i>
<i>PECAM-1 on endothelial cells</i>	<i>Biotinylated anti-PECAM with streptavidin</i>	<i>Mice, rat</i>	<i>Vascular immunotargeting of bioactive drugs and blood vessels destruction</i>
<i>PECAM-1 on endothelial cells</i>	<i>Conjugating of cationic polymer, with anti-PECAM-DNA complex (Adenoviral plasmide vector)</i>	<i>Mouse</i>	<i>Suitable gene delivery in mice lung endothelium</i>
<i>PECAM-1 on endothelial cells</i>	<i>Anti-PECAM-Streptavidin-β-galactosidase</i>	<i>Animal models</i>	<i>Intracellular delivery of an active enzyme</i>
<i>Tthrombomodulin</i>	<i>Anti-thrombomodulin with enzyme glucose oxidase</i>	<i>Mice</i>	<i>Intravascular thrombosis of lung endothelium by H₂O₂</i>
<i>Angiotensin-converting enzyme</i>	<i>Anti-ACE MAb 9B9</i>	<i>Ppulmonary capillary endothelium of rat</i>	<i>Offers a high selectivity of pulmonary targeting in vivo</i>
<i>αvβ3 on activated endothelial cells</i>	<i>Cyclic peptides antagonist of integrin αvβ3</i>	<i>Rabbits</i>	<i>Apoptosis of endothelial cells and regression of rheumatoid arthritis</i>
<i>IL-15</i>	<i>Anti-IL15 MAb 146B7</i>	<i>Human psoriasis xenograft model</i>	<i>Decreased IL-15 and TNF-α-production, decreased the inflammatory cells in the psoriatic lesion</i>
<i>p38 mitogen-activated protein kinase (MAPK)</i>	<i>SB 220025 (pyrimidyl imidazole) specific inhibitor of p38 MAPK</i>	<i>Murine air pouch granuloma Murine collagen-induced arthritis</i>	<i>Decreased inflammatory angiogenesis, decreased: IL-1β, TNF-α, IL-6, IL-8</i>
<i>Caveolae on endothelial cells</i>	<i>Monoclonal antibody specific for lung caveolae (TX3.833)</i>	<i>Rat lung</i>	<i>Gene therapy</i>
<i>Tie2 specific tyrosine kinase receptor on endothelial cells</i>	<i>Hypoxanthine phosphoribosyltransferase (Hprt) gene locus</i>	<i>Murine chimeric animals</i>	<i>Gene therapy</i>
<i>Survivin</i>	<i>Antisense oligonucleotide to Survivin</i>	<i>In vitro cell death assays</i>	<i>Regression of three-dimensional vascular capillary network by pro-apoptotic activity</i>
<i>Glycoprotein (GP) Ib, on endothelial cells mediates the binding of von Willebrand factor vWF</i>	<i>Agkistin blocks the interaction of vWF with GPIb</i>	<i>HUVEC Embryo chick chorioallantoic membrane</i>	<i>Anti-angiogenic effect</i>
<i>αvβ3 on endothelial cells</i>	<i>Agkisti bloks αvβ3</i>	<i>HUVEC Embryo chick chorioallantoic membrane Matrigel</i>	<i>Apoptosis of endothelial cells</i>
<i>Pigment epithelium-derived factor (PEDF)</i>	<i>Adenoviral vectored (PEDF) gene transfer</i>	<i>Transgenic mice</i>	<i>Apoptosis of endothelial cells</i>
<i>Combretastatin-A4 (CA-4)</i>	<i>Anti-vascular tubulin-binding</i>	<i>In vitro, transgenic mice</i>	<i>Multiple microthrombi</i>
<i>bFGF</i>	<i>TNP-470: inhibition of bFGF</i>	<i>Rat Mouse</i>	<i>Anti-angiogenic effect on: corneal neovascularization, arthritis, atherosclerosis</i>
<i>TNF, Integrin, VEGF, FGF</i>	<i>Thalidomide, inhibitor of: TNF, Integrin, VEGF, FGF</i>	<i>Arthritis rat model Clinical trial I in Crohn's disease</i>	<i>Anti-angiogenic effect</i>

a) Polymeric Nanospheres

Long-circulating particles entered the scene in the late sixties when a pilot experiment demonstrated that i.v. injected lipid emulsions prepared with high-molecular-weight POE/POP copolymer nonionic surfactants (poloxamers and poloxamines) as emulsifiers remained in the blood for relatively long periods [64]. Poloxamers consist of a central POP block flanked on both sides by two hydrophilic POE chains, whereas poloxamines are tetrafunctional copolymers with four POE/POP blocks joined together by a central ethylene diamine bridge. Poloxamine-908, poloxamine-1508, poloxamer-238 and poloxamer-407 have proved the most effective for prolonging the circulation time of hydrophobic 15-150 nm nanoparticles in mice and rats. PEG is a linear polyether diol with low immunogenicity and antigenicity [65]. Its polymer backbone is chemically inert and its terminal primary hydroxyl groups are available for derivatization. These groups are usually activated and then reacted with the chosen molecule. Surface modification of nanoparticles with PEG and its derivatives can be performed by adsorption, incorporation during the production of nanoparticles or covalent attachment [66]. Nanoparticle engineering programmes have synthesized and used dextranox-methoxy-PEG for cross-linking to albumin nanospheres. PEG-ylated poly(isobutyl 2-cyanoacrylate) nanoparticles have also been produced by emulsion/polymerization [67]. Covalent attachment of semitelechelic poly[N-(2-hydroxypropyl) methacrylamide]s of different molecular weights to nanospheres based on methyl methacrylate, maleic anhydride and methacrylic acid is another attractive approach [68].

b) Micelles

Multiblock copolymers such as POE-poly(L-lysine), POE-poly(-benzyl-L-aspartate), POE-poly(-caprolactone) and poly(acrylic acid)-poly(methyl methacrylate), as well as those used in particle coatings (e.g., poloxamers, poloxamines, PEG-PLA, PEG-PLGA), also self-disperse in water to form spherical polymeric micelles 15-80 nm in diameter [69]. Some of these micelles have been suggested as promising long-circulating carriers of poorly water-soluble and amphiphilic drugs, because of their small size and hydrophilic shell [70]. The effectiveness of such carriers, however, will depend on their micelle concentration. When injected i.v., micelles are often diluted to less than their minimum concentration, and polymer molecules are known to behave in dramatically different ways.

c) Liposomes

Liposomes are bilayer lipid vesicles that can be modified to obtain other desirable properties, including prolonged circulatory half-life and the ability to complex with nucleic acids and mediate gene delivery or genetic regulation, and to deliver encapsulated contents to the cytosol through the endosome/lysosome pathway [71,72]. Since the lipid composition of classic liposomes closely resembles that of the outer monolayer of the plasma membrane, they do not destabilize under physiological conditions, but are rapidly cleared from the peripheral circulation by the reticuloendothelial system. Sterically stabilized liposomes contain lipids that provide a steric barrier to opsonization and eventual uptake by the reticuloendothelial system, and thus have a prolonged circulatory half-life. pH-sensitive liposomes are composed of lipids that form a stable bilayer at neutral or basic pH, but destabilize at acidic pH. Lastly, cationic liposomes contain a lipid that is cationic under physiological conditions, and are capable of condensing and carrying relatively large amounts of nucleic acid for delivery to target cells. Destabilization of a liposome in an acidifying endosome facilitates the delivery of its contents to the target cell cytosol [73].

Cationic liposomes have some advantages. Angiogenesis was compared in pancreatic islet cell tumors of RIP-Tag2 transgenic mice, chronic airway inflammation in *Mycoplasma pneumoniae* infected C3H/HeNcr mice, and normal mouse ovaries. EC in these three models avidly bound and internalized fluorescently labeled cationic liposomes (1,2-dioleoyl-3-trimethylammonium-propane [DOTAP]/cholesterol or dimethyldioctadecyl ammonium bromide [DDAB]/cholesterol) or liposome-DNA complexes [74]. Confocal microscopy showed that angiogenic EC averaged 15-33-fold more uptake than normal EC. Cationic liposome-DNA complexes were also avidly taken up, but anionic, neutral or sterically stabilized neutral liposomes were not. Electron microscopy showed that 32% of gold-labeled liposomes associated with tumor EC were adherent to the luminal surface, 53% were internalized into endosomes and multivesicular bodies and 15% were extravascular 20 min after injection.

The above experimental data indicate that angiogenic endothelial cells in these models avidly bind and internalize cationic liposomes and liposome-DNA complexes but not other types of liposomes. This

preferential uptake of cationic liposomes and liposome-DNA complexes suggests that these liposomes can be used to selectively deliver diagnostic or therapeutic agents to angiogenic blood vessels in chronic inflammation sites [75]. Moreover, all the immunoliposome formulations developed display some degree of activation-dependent, antigen-specific targeting and may be suitable for application *in vivo* [71-73].

Targeting to Selectins

a) Immunoliposomes to ELAM-1

Cultured human umbilical vein endothelial cells (HUVEC) activated by pro-inflammatory stimuli, such as the cytokine IL-1, show increased expression of ELAM-1, VCAM-1, and ICAM-1 in temporally well-characterized patterns [76,77]. ELAM-1 displays the most distinct activation-dependent and endothelial-selective pattern. It is not detectable in normal vessels, but appears rapidly in EC in spatially circumscribed patterns in response to local inflammatory stimuli [78,79]. Because of its highly activation-dependent pattern of induction, ELAM-1 may provide a useful target for the site-specific delivery of agents to distinct regions within the vascular bed. Targeted liposomes or another targeting moiety (e.g., a specific peptide or lipid) have several advantages over simple monoclonal antibody-drug conjugates for specific drug delivery [80-82]. Various types of immunoliposomes, each bearing the anti-ELAM-1 monoclonal antibody H18/7, bound to activated cultured HUVEC at levels 13 to 275-fold higher than to unactivated HUVEC. Classic ELAM-1-targeted immunoliposomes bound to activated cells at relatively high levels and showed negligible nonspecific binding to unactivated cells. ELAM-1-targeted, sterically stabilized liposome binding to activated HUVEC was consistently lower than classic immunoliposome binding [82]. However, the extremely low levels of nonspecific binding of these sterically stabilized liposomes resulted in a 145-fold activation-dependent binding ratio. The cationic immunoliposomes had a relatively high level of nonspecific binding due to interactions with the anionic cell surface. Finally, ELAM-1-targeted pH-sensitive liposomes did not exhibit as high an activation-dependent binding ratio as classic or sterically stabilized liposomes [80-82]. E-selectin-targeted immunoliposomes appeared in acidic, perinuclear vesicles 2-4 hr after binding to the cell surface, consistent with internalization via the endosome/lysosome pathway. Activated HUVEC incubated with E-selectin-targeted immunoliposomes, loaded with the cytotoxic agent doxorubicin, displayed significantly decreased cell survival, whereas unactivated HUVEC were not affected.

b) Dexamethasone-Anti-E-Selectin Conjugate

In another approach, an antibody to E-selectin (CD62E) was used to deliver the covalently coupled anti-inflammatory drug dexamethasone into activated EC. The ultimate goal of this drug targeting approach is to selectively block EC activation at the site of inflammation. It was demonstrated that the dexamethasone-anti-E-selectin conjugate, like the unmodified anti-E-selectin antibody, selectively bound to TNF- α -stimulated and not to resting EC. After binding, the conjugate was internalized and routed to multivesicular bodies, which is a lysosome-related cellular compartment. After intracellular degradation, pharmacologically active dexamethasone was released, as shown in EC transfected with a glucocorticoid-responsive reporter gene. Furthermore, intracellularly delivered dexamethasone downregulated the proinflammatory gene IL-8. [83].

c) Targeting of an Anti-Thrombin Agent to E-Selectin

An immunoconjugate was developed to deliver hirudin, a potent and specific inhibitor of thrombin, to the surface of activated EC. Hirudin was covalently cross-linked to the H18/7 monoclonal antibody, which recognizes the extracellular domain of E-selectin [84]. The hirudin-H18/7 immunoconjugate selectively bound to interleukin-1-activated, but not to unactivated cultured HUVEC with a temporal profile similar to that of inducible cell-surface procoagulant activity. When bound to activated EC, the immunoconjugate significantly inhibited endogenous thrombin activity generated by cocultured human plasma and fibrin clot formation on the monolayer surface [85,86]. Cellular responses mediated via the thrombin receptor, such as increases in cytoskeletal F-actin content, were also significantly downregulated, and monolayers were protected from thrombin-induced disruption by this treatment. Selective antagonization of thrombin-dependent processes at the endothelium-blood interface may provide new insights into complex processes, such as thrombosis, inflammation and atherogenesis [87]. These studies also demonstrate the general feasibility of selective targeting of therapeutic agents on EC based on recognition of an activation-dependent surface phenotype.

Targeting on VCAM-1

a) Radiolabeled Anti-VCAM-1 Antibody

Experimental animal models have revealed that the expression of VCAM-1 and of atherosclerosis associated-ELAM (ATHERO-ELAM) is one of the earliest detectable molecular changes in EC in the vicinity of a developing atheromatous plaque [88]. Radiolabeled monoclonal antibodies directed against rabbit VCAM-1, infused into Watanabe (low density lipoprotein-receptor-deficient, atherosclerosis-prone) rabbits localize selectively and specifically (compared with isotopic matched, nonbinding control monoclonal antibody) at sites of early lesion formation. Thus, VCAM-1, or an analogous human AATHERO-ELAM, would be a potential molecular target for immunoliposomal intervention in atherosclerosis. In addition to activation-dependent phenotypic markers, there is increasing evidence that EC in different regions of the vasculature can be distinguished by the constitutive expression of tissue-specific cell surface antigens [89].

b) Thrombogenic Phosphatidylserine Liposome Targeting to VCAM-1

Membrane phosphatidylserine (PS) exposure plays an important role in blood coagulation. Elaboration of a liposome formulation containing PS could be useful if it were designed to achieve selective vessel thrombosis. A thrombogenic PS liposome targeted to VCAM-1 via attachment of an anti-VCAM-1 monoclonal antibody has been obtained experimentally. Binding of the anti-VCAM-1 antibody-conjugated PS liposomes to VCAM-1 using two *in vitro* models has recently described. These liposomes catalyzed blood coagulation reactions when exposed to the thrombogenic PS membrane. Surface PS exposure was checked with exchangeable PEG-derivatized phosphatidylethanolamines (PE-PEG): 97% of clotting activity was covered after PE-PEG exchanged out the procoagulant liposomes that selectively target thrombogenesis in blood vessels [89]. Recognition and exploitation of these markers for selective liposomal targeting need not be limited to monoclonal antibody and could involve cognate ligands and even gene delivery [90,91].

Targeting to ICAM-1

EC internalize natural ligands and artificial macromolecular ligands designed as carriers for specific drug and gene delivery. Murine monoclonal antibodies to human ICAM-1 were coated with fluorescent microspheres to originate immunobeads ranging from 180 to 250 nm in diameter. Anti-ICAM-1 immunoconjugates were biotinylated and complexed to streptavidin (SA) [92]. The ratio of biotinylated-ICAM-1 to SA was varied to generate immunoconjugates with a diameter of less than 500 or more than 1000 nm. Conjugates < 500 nm were internalized by HUVEC, whereas those > 1000 nm displayed little, if any, internalization. The immunobeads were internalized by HUVEC when incubated at 37° C but not at 4° C. Internalization was followed by further clustering of conjugates and immunobeads, probably due to endosome fusion while the particles were transported along the endocytic pathway. Only multimeric anti-ICAM-1 are internalized. Amiloride and protein kinase C, which inhibit macropinocytosis, reduced the internalization of clustered ICAM-1. Binding of the conjugates stimulated the formation of actin stress fibers by HUVEC. Latrunculin, radicicol and Y27632 also inhibited internalization, suggesting that actin rearrangements requiring Src kinase and Rho kinase are needed. Interestingly, these kinases are part of the signal transduction pathways activated when circulating leukocytes engage EC adhesion molecules. CAM-mediated endocytosis may thus be regulated by using comparable signaling pathways. Combination of these agents with pharmacologically active, enzyme-carrying anti-ICAM conjugates could be used to target the vascular bed [93].

Targeting to PECAM

a) Antibody to PECAM-1

A promising target determinant is PECAM abundantly, stably and ubiquitously expressed on the EC surface [94]. Active reporter enzymes and genetic materials coupled to PECAM antibody (anti-PECAM conjugates) bind selectively to EC *in vitro* and to mouse lung pulmonary vessels after *i.v.* administration [94-97]. Monomeric antibodies to PECAM-1 are poorly internalized by EC [95]. Multivalent anti-PECAM-1 conjugates with a diameter of 100-300 nm are readily internalized, but the efficiency of internalization decreases with increasing conjugate size [95]. By contrast, anti-PECAM antibody serve as carriers that effectively target active protein to vascular EC in large animals [98]. Selective catheterization of pulmonary vessels augments the conjugate uptake in target lung lobes, whereas coronary artery infusion results in selective uptake in downstream cardiac EC. Theoretically, administration of

antibody-conjugated drugs via a catheter placed in the vessel of interest could augment local or regional delivery to the endothelium in this organ and increase vascular targeting.

b) Conjugation of Ab to PECAM-1 with SA in the *in vitro* HUVEC Model

Endothelium poorly internalizes certain candidate carriers for vascular immunotargeting, such as antibodies to PECAM-1, whereas conjugation with SA facilitates their intracellular uptake. To determine whether uptake is a function of conjugate size, anti-PECAM/SA and anti-PECAM/bead conjugates from 80 to 5000 in diameter were produced [95]. HUVEC and PECAM-transfected mesothelioma cells internalized 80 to 350-nm anti-PECAM conjugates, but not those > 500 nm. Size also governs the intracellular targeting of active therapeutic cargoes *in vitro* and *in vivo*. Small (350 nm) anti-PECAM/DNA conjugates transfected target cells in culture 5-fold more effectively than their large (4200 nm) counterparts. To evaluate the practical significance of size-controlled subcellular addressing, glucose oxidase (GOX) was coupled to anti-PECAM and antithrombomodulin [99]. Both types of conjugates had equally high pulmonary uptake after *i.v.* in mice, yet only 200-250 nm and not 600-700 nm GOX conjugates caused deep oxidative injury the lung vessels, presumably owing to intracellular generation of H₂O₂. Thus, engineering of affinity carriers of specific size permits intracellular delivery of active cargoes to endothelium *in vitro* and *in vivo*, a paradigm useful for the targeting of drugs, genes and toxins [100].

c) Conjugation of Biotinylated Anti-PECAM with SA *In vivo*

¹²⁵I-labeled anti-PECAM bound to endothelial cells in culture, but was poorly internalized, and also accumulated poorly after *i.v.* administration in mice and rats. However, conjugation of four biotinylated anti-PECAM: i. "Houston", a polyclonal rabbit IgG against human and rat PECAM-1; ii. Monoclonal antibody 62, reacting with human and rat PECAM-1; iii. Monoclonal antibody 4G6, reacting with the most membrane-proximal, sixth Ig-like loop of human PECAM-1; iv. Monoclonal antibody 390, reacting with murine PECAM-1/CD31 [99,101,102], facilitate the process of internalization.

Murine monoclonal antibody 1045 recognizes a chondroitin sulfate-dependent epitope of human thrombomodulin (TM) [102]. Complex anti-PECAM with SA markedly stimulated uptake and internalization of anti-PECAM by EC and cells expressing PECAM. In addition, conjugation with SA markedly stimulated uptake of ¹²⁵I-labeled anti-PECAM in perfused rat lungs and in those of intact animals after *i.v.* intra-arterial injection. Thus, modification of a poor carrier antibody with biotin and SA offers a way of facilitating antibody-mediated drug targeting. Anti-PECAM/SA is a promising candidate for vascular immunotargeting of bioactive drugs [103].

d) Targeted Gene Delivery to the Lung Endothelium

Adenoviral vectors are relatively inefficient in gene transfer to the intact pulmonary circulation as a result of either brief transit times or the absence of endothelial adenovirus receptors [104]. An experimentally efficient vector for systemic gene delivery was developed by chemically conjugating a cationic polymer, polyethylenimine (PEI), with anti-PECAM antibody [105]. Transfection of mouse lung EC with a plasmid expression vector with cDNA to luciferase (pCMVL) complexed with anti-PECAM antibody-PEI conjugate was more efficient than that with PEI-pCMVL complexes. Furthermore, the anti-PECAM antibody-PEI conjugate mediated efficient transfection at lower plus-to-minus charge ratios. Conjugation of PEI with a hamster IgG did not enhance transfection of mouse lung EC, suggesting that the cellular uptake of anti-PECAM antibody-PEI-DNA complexes and subsequent gene expression were governed by a receptor-mediated process rather than a nonspecific charge interaction. Conjugation of PEI with anti-PECAM antibody also significantly increased lung gene transfer to intact mice after *i.v.* administration [106]. This increase was associated with a decrease compared with PEI-pCMVL with respect to circulating TNF- α levels. These results indicate that targeted gene delivery to the lung endothelium is an effective strategy to enhance gene delivery to the pulmonary circulation while simultaneously reducing toxicity.

e) Targeting to Anti-PECAM-SA- β -Galactosidase

i) Anti-PECAM/SA- β -Gal recognizes the vascular endothelium and accumulates preferentially in the lungs after *i.v.* administration; ii) Endothelium-associated β -Gal displays high enzymatic activity detectable within several minutes after a single bolus injection and lasts for at least 8 h; iii) Some injected β -Gal conjugates accumulate in EC *in vitro* and probably *in vivo*. This finding demonstrates that vascular immunotargeting permits cell-selective, intracellular delivery of an active foreign enzyme to the endothelium of intact animals [97].

ENZYME GLUCOSE OXIDASE (GOX) TO THE PULMONARY ENDOTHELIUM

Vascular immunotargeting of GOX to the pulmonary endothelium causes acute oxidative lung injury in mice [104]. The immunotargeting to the pulmonary endothelium with specific anti-EC antibodies provides such a model. The pathological features of the lung injury caused by GOX targeted to endothelial antigens PECAM and thrombomodulin (TM) are an increase in vascular permeability, EC damage, hemorrhage and accumulation of products of the oxidative modification of lipids and proteins. Specifically GOX targeting to TM results in intravascular thrombosis. Therefore, GOX immunotargeting-based models offer a means of testing new strategies for treatment of chronically inflamed airways associated with pathological angiogenesis [105].

ANTI-ANGIOTENSIN-CONVERTING ENZYME MONOCLONAL ANTIBODIES TO THE PULMONARY ENDOTHELIUM

The monoclonal antibodies to angiotensin-converting enzyme (ACE) offer more selective *in vivo* targeting than other carriers presumably because of the very high ACE content in the pulmonary capillary endothelium. ACE (peptidyl-dipeptidase A) is a transmembrane ectopeptidase found on the luminal surface of vascular EC in the lungs and elsewhere [106]. Anti-ACE monoclonal antibody 9B9 recognizes rat and human ACE and accumulates in rat lung with high specificity and effectiveness [107]. ACE is very heterogeneously distributed in EC along the human vascular tree. It is strongly expressed in small arteries and arterioles, but poorly expressed or even absent in the aorta, large arteries and veins [108]. ACE monoclonal antibodies allows more selective targeting *in vivo*, presumably because there are many ACE-positive capillaries in the lungs [109,110].

PEPTIDE ANTAGONIST OF INTEGRIN $\alpha_v\beta_3$

Rheumatoid arthritis (RA) is an inflammatory disease associated with intense angiogenesis and vascular overexpression of integrin $\alpha_v\beta_3$. Early intra-articular administration of the cyclic peptide integrin $\alpha_v\beta_3$ antagonist EMD 66203 to rabbits with antigen-induced arthritis inhibited synovial angiogenesis and reduced synovial cell infiltrate, pannus formation and cartilage erosion [111,112]. These effects were not associated with lymphopenia or impairment of leukocyte function.

TARGETING TO IL-15

IL-15 is a proinflammatory cytokine whose expression is upregulated under inflammatory conditions. It acts early in the inflammatory response and induces the production of as TNF- α , IFN- γ and IL-17 [113], amplifies inflammation by the recruitment and activation of T lymphocytes and other inflammatory cells, and induces angiogenesis. These effects may be of pivotal importance in the pathogenesis of RA, psoriasis and other chronic inflammatory diseases [100]. *In vitro* and *in vivo* studies indicate that monoclonal antibody 146B7 inhibits IL-15-induced T cell proliferation and TNF- α production, reverses IL-15-mediated protection against monocyte apoptosis and decreases the number of inflammatory cells in psoriatic lesions. It also reduced the severity of psoriasis in a human xenograft model [31].

TARGETING TO P38 MITOGEN-ACTIVATED PROTEIN KINASE

In the murine air pouch granuloma model, SB 220025, a pyrimidyl imidazole compound that specifically inhibits human p38 mitogen-activated protein (MAP) kinase, reduces inflammatory angiogenesis. At 30 mg/kg b.i.d. p.o., it greatly reduces the expression of both IL-1 β and TNF- α . Other factors, such as inducible cyclooxygenase, IL-6 and IL-8 also are downregulated by p38/CSBP MAP kinase [114,115]. A study of the effects of p38/CSBP MAP kinase inhibition by SB 220025 in angiogenesis-dependent chronic inflammatory disease has shown that it prevents the progression of murine collagen-induced arthritis.

TARGETING TO CAVEOLAE

Caveolae are flask-shaped invaginations in the plasma membrane open to the entry of circulating molecules from the luminal blood vessel space. They may provide a pathway for the traffic of macromolecules into and possibly across cells [116,117]. An antibody- and subfractionation-based strategy has been used to generate a monoclonal antibody specific for lung caveolae (TX3.833) that targets rat lungs after i.v. injection (up to 89% of dose in 30 min). Unlike control antibody (nonbinding or to lipid

rafts), TX3.833 targets caveolae that bud to form free vesicles for selective transendothelial transport to underlying tissue cells *in vivo*. Rapid sequential transecytosis occurs to the alveolar air space via epithelial caveolae. Conjugation to TX3.833 increases drug delivery to the lung up to 172-fold and achieves rapid, localized bioefficacy. Published data indicate that the molecular heterogeneity of the endothelium and its caveolae permits vascular targeting to achieve theoretical expectations of tissue-specific delivery and bioefficacy. Targeting caveolae offers exciting possibilities of achieving site-directed drug and gene therapy of various diseases [118].

TARGETING THE HYPOXANTHINE PHOSPHORIBOSYLTRANSFERASE LOCUS

The EC-specific tyrosine kinase receptor Tie2 is involved in the remodeling of blood vessels, interactions of EC with the extracellular matrix and perivascular cells, angiogenesis and vessel maintenance [119]. The discovery that Tie2 is upregulated during pathological angiogenesis has aroused great interest in its biology [120]. Transcriptional regulation of the murine Tie2 gene during pathological and physiological angiogenesis has been investigated as a step towards the elaboration of a targeting strategy [121]. This has been done by targeting the hypoxanthine phosphoribosyltransferase (HPRT) gene locus in embryonic stem cells, generation of chimeric animals, and germ line transmission of the targeted transgenes, and has allowed direct comparison of the *in vivo* activity of two Tie2 promoter/enhancer β -galactosidase (LacZ) gene/LacZ cassettes by integration of a single copy in the same genomic locus. HPRT targeting has shown that the 2.1-kb murine Tie2 promoter drives EC-specific expression in vessels both in embryonic development and in the brain and kidney of adult mice. Targeting the HPRT locus with EC-specific sequences is thus a feasible way of treating pathological angiogenesis [122].

TARGETING TO SURVIVIN

The protective genes that mediate EC survival during angiogenesis have not been completely characterized. Antisense oligonucleotide to the apoptosis inhibitor survivin suppresses its *de novo* expression in EC by VEGF, whereas the antisense oligonucleotide did not affect anti-apoptotic bcl-2 levels in the endothelium [123]. Antisense targeting of survivin abolished the anti-apoptotic function of VEGF against TNF- α or ceramide-induced cell death, enhanced caspase-3 activity, promoted the generation of a 17-kd active caspase-3 subunit, and increased cleavage of the caspase substrate, polyADP ribose polymerase. In contrast, the antisense oligonucleotide had no effect on EC viability in the absence of VEGF. Antisense oligonucleotides to PECAM-1, lymphocyte function-associated molecule-3 and ICAM-1 did not reduce VEGF's anti-apoptotic function. When tested on other angiogenic activities mediated by VEGF, survivin antisense treatment induced rapid regression of three-dimensional vascular capillary networks, but did not affect EC migration/chemotaxis [124]. The anti-apoptotic properties of VEGF during angiogenesis are primarily mediated by the induced expression of survivin in EC. Manipulation of this pathway may increase EC viability in compensatory angiogenesis or facilitate EC apoptosis and promote vascular regression during pathological angiogenesis.

AGKISTIN

Glycoprotein (GP) Ib, an adhesion receptor expressed on EC, mediates the binding of von Willebrand factor (vWF). The interaction vWF with GPIb is not fully understood, though it has been demonstrated that the receptor mediates EC endothelial cell migration during wound repair [125]. Agkistin, a snake venom protein, selectively blocks the interaction of vWF with human GPIb and inhibits angiogenesis. It specifically blocks HUVEC adhesion to immobilized vWF in a concentration-dependent manner. Fluorescein isothiocyanate (FITC)-conjugated agkistin bound to HUVEC in a saturable manner. AP1, a monoclonal antibody raised against GPIb, specifically inhibits binding of FITC-conjugated agkistin to HUVEC in a dose-dependent manner, whereas anti-integrin monoclonal antibody had no effect. However, neither agkistin nor AP1 reduced HUVEC viability. Both agkistin and AP1 displayed a profound anti-angiogenic effect *in vivo* in a 10-day-old embryo chick chorioallantoic membrane (CAM) assay. These results suggest that GPIb plays a role in spontaneous angiogenesis *in vivo*, and that the anti-angiogenic effect of agkistin may be due to disruption of the interaction of vWF with GPIb [126]. GPIb antagonists could thus be of use

in the treatment of angiogenesis-related diseases, such as chronic inflammation and tumour metastasis.

THE DISINTEGRIN FAMILY

Accutin, a new member of the disintegrin family derived from the venom of *Akistrodon acutus*, potently inhibits human platelet aggregation caused by thrombin, collagen and adenosine diphosphate (ADP) through blockade of fibrinogen binding to platelet glycoprotein IIb/IIIa. Accutin specifically inhibits binding of monoclonal antibody 7E3, which recognizes integrin $\alpha v\beta 3$, to HUVEC, but not that of other anti-integrin monoclonal antibodies [127]. Moreover, accutin, but not the control peptide GRGES, dose-dependently inhibited the 7E3 interaction with HUVEC. Both 7E3 and GRGDS, but not GRGES or Integrelin, significantly blocked FITC-conjugated accutin binding to HUVEC. In functional studies, accutin inhibited HUVEC adhesion to immobilized fibrinogen, fibronectin and vitronectin, and the capillary-like tube formation on Matrigel in a dose- and RGD-dependent manner. In addition, it displayed an antiangiogenic effect *in vivo* in the chick CAM and induced HUVEC apoptotic DNA fragmentation, as shown by electrophoresis and flow cytometry. Accutin thus inhibits angiogenesis *in vivo* and *in vitro* by blocking integrin $\alpha v\beta 3$ of EC, and by inducing apoptosis [128]. The antiangiogenic activity of disintegrins could perhaps be exploited in the treatment of chronic inflammatory diseases.

ADENOVIRAL VECTORED PIGMENT EPITHELIUM-DERIVED FACTOR GENE TRANSFER

Several drugs reduce ocular neovascularization when administered before the onset of angiogenic stimuli, but none induce regression of already established vessels [129]. A recent study of the effect of adenoviral vectored pigment epithelium-derived factor (PEDF) gene transfer on established new vessels in transgenic mice with expression of VEGF in photoreceptors has indicated that its increased expression causes their regression by promoting apoptosis of cells in neovascular lesions and may be applicable for this purpose in humans [130,131].

COMBRESTATIN-A4

Cis combretastatin-A4 (CA-4) is a tubulin-binding agent originally isolated from the South African shrub *Combretum caffrum*. The water-soluble phosphate prodrug (CA-4P) is rapidly hydrolyzed by phosphatases *in vivo* to yield the parent drug. CA-4 elicits acute anti-vascular effects in established tumor blood vessels and is the subject of ongoing clinical trials for the treatment of solid tumors [132]. However, it is not tumor-specific, but disrupts non-neoplastic angiogenic with the formation of multiple microthrombi without hemorrhage [133]. The mechanism involved is not known. Since CA-4 binds to the colchicine binding site of tubulin, it may change the shape of tumor vessel EC in such a way as to impair the integrity of the luminal monolayer [134]. The effect of CA-4P has been quantitatively assessed in transgenic mice with retinal overexpression of VEGF (rho/VEGF mice) and mice with choroidal neovascularization (CNV) due to laser-induced rupture of Bruch's membrane. In the rho/VEGF mice, daily i.p. injections of CA-4-P starting at postnatal day (P)7, the time of onset of transgene expression, significantly reduced the number of neovascular lesions and total area of neovascularization per retina at P21, compared with vehicle-injected mice. In the CNV mice, similar injections of CA-4-P significantly reduced the area of CNV at rupture sites compared with vehicle-injected mice. In mice with established CNV, similar injections of CA-4-P for 1 week resulted in a significant reduction in CNV area at rupture sites compared with the baseline area before treatment, or the area of CNV in vehicle-treated mice [135]. In another study, ischemia-induced murine proliferative retinopathy was used to determine whether CA-4 inhibits pathological angiogenesis. Mice were exposed to hyperoxia, returned to ambient conditions and daily i.p. injection of CA-4P was commenced after 24 hours. A dose-dependent inhibition of retinal neovascularization was observed [136]. Taken as a whole, these data show that CA-4 elicits anti-vascular effects and suggest that it could be used to treat chronic inflammatory diseases.

TNP-470

TNP-470 (AGM 1470) is an artificial analog of fumagillin [137], the antibiotic protein secreted by *Aspergillus fumigatus* that inhibits angiogenesis and EC proliferation. TNP-470 displays the same antiangiogenic activity, but has fewer side-effects. It inhibits the growth in

human tumor xenografts [137], and is both effective and relatively safe for clinical use. The principal toxicity is reversible and dose-dependent neurotoxicity. However, the actual molecular targets of TNP-470 are unknown. TNP-470 has recently been shown to be effective via FGF-2 inhibition in rat and mouse CNV [138]. Its topical and systemic delivery in a murine model of inflammatory corneal angiogenesis resulted in direct inhibition of EC proliferation [139]. It may therefore become clinically useful for a variety of ocular diseases involving inflammatory neovascularization, such as wound- and inflammation-related corneal angiogenesis with limbal insufficiency, which is still pharmacologically untreatable [140]. It could also prevent and even reverse established arthritis in rats and inhibit atheromasic plaque growth in mice [141,142].

THALIDOMIDE

Thalidomide is a sedative whose teratogenic effects, mainly dysmelia, became evident in babies born during the 1960s whose mothers who used it during pregnancy. D'Amato et al. have demonstrated that orally administered thalidomide inhibit angiogenesis [143]. Thalidomide has displayed antitumor activity in phase II-III clinical trials. Its side-effects were: sedation, constipation and peripheral neuropathy. It has been experimented for the management of chronic inflammatory diseases, including RA and GI ulcers. It is believed to act primarily as an inhibitor of TNF- α expression by destabilization of TNF- α mRNA, though it may also impair angiogenesis by downregulating endothelial integrin expression [144,145]. In a rat model, on the other hand, it inhibited collagen-induced arthritis by mechanisms other than TNF- α or VEGF/FGF-2 downregulation. In an open label clinical trial, thalidomide was efficacious in some patients with refractory Crohn's disease [146].

CONCLUDING REMARKS

The endothelium is an important and privileged target for site-specific delivery of therapeutic agents or genes. Immunotargeting strategies are based on conjugation (chemically or via gene engineering) of a drug (enzyme, gene) with carrier antibodies that recognize specific antigens on the surface of the target cell. Substances can also be selectively delivered to angiogenic EC by targeting specific growth factor receptors and the employment of upregulated coagulant factors, key cell adhesion proteins, nanoparticles and several antiangiogenic drugs. The uptake of liposomes by angiogenic EC described in this review may provide an intriguing new delivery pathway. For example, substances that initiate coagulation and stasis in tumor vessels could be targeted transiently to the luminal surface of the endothelium. Alternatively, drugs, toxins, or antisense oligonucleotides could be delivered by endocytosis. In addition, plasmids encoding appropriate transgenes could be complexed to liposomes and delivered to angiogenic EC for expression. The employment of liposomes to deliver substances to tumor EC and the development of tools to modulate angiogenesis in tumors, chronic inflammation and other diseases require further investigation. In conclusion, because the vascular endothelium serves as an extensive interface between circulating blood and various tissues and organs of the body, it offers an accessible target for blood-borne pharmacological and genetic manipulations that can mediate both local and systemic effects. Targeting of the vasculature of inflamed organs could underlie a new pharmacological approach in the treatment of inflammatory diseases by taking advantage of formulations that deliver drugs to blood vessels specifically located at disease sites and to inflammatory cells.

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