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Targeted delivery of therapeutics to endothelium

Eric Simone[1], **Bi-Sen Ding**[2], and **Vladimir Muzykantov**[2,3]

[1] Departments of Bioengineering and Pharmacology and Program in Targeted Therapeutics of the Institute for Translational Medicine and Therapeutics, University of Pennsylvania School of Engineering and Applied Sciences, Philadelphia, PA, USA

[2] Departments of Bioengineering and Pharmacology and Program in Targeted Therapeutics of the Institute for Translational Medicine and Therapeutics, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

[3] IFEM, University of Pennsylvania Medical Center, 1 John Morgan Building, 3620 Hamilton Walk, Philadelphia, PA 19104–6068, USA e-mail: muzykant@mail.med.upenn.edu

Abstract

The endothelium is a target for therapeutic and diagnostic interventions in a plethora of human disease conditions including ischemia, inflammation, edema, oxidative stress, thrombosis and hemorrhage, and metabolic and oncological diseases. Unfortunately, drugs have no affinity to the endothelium, thereby limiting the localization, timing, specificity, safety, and effectiveness of therapeutic interventions. Molecular determinants on the surface of resting and pathologically altered endothelial cells, including cell adhesion molecules, peptidases, and receptors involved in endocytosis, can be used for drug delivery to the endothelial surface and into intracellular compartments. Drug delivery platforms such as protein conjugates, recombinant fusion constructs, targeted liposomes, and stealth polymer carriers have been designed to target drugs and imaging agents to these determinants. We review endothelial target determinants and drug delivery systems, describe parameters that control the binding of drug carriers to the endothelium, and provide examples of the endothelial targeting of therapeutic enzymes designed for the treatment of acute vascular disorders including ischemia, oxidative stress, inflammation, and thrombosis.

Keywords

Vascular pathology; Endothelium; Pharmacology; Thrombosis; Ischemia

Introduction

The endothelium represents an important therapeutic target in inflammation, ischemia, edema, thrombosis, diabetes, oxidative stress, atherosclerosis and hypertension, and metabolic and oncological diseases. Unfortunately, most drugs have no endothelial affinity leading to adverse effects or sub-optimal effectiveness. Inadequate delivery into a desirable site of action represents an especially acute concern for the utility of those biotherapeutics that require precise sub-cellular localization to exert their required activity, i.e., enzymes, proteins, genetic materials, and other biological compounds.

The endowment of these agents and their carriers with an affinity for endothelial surface determinants holds promise to localize therapeutic interventions in desired endothelial

Correspondence to: Vladimir Muzykantov.

compartments in vascular areas of interest. Thus, targeted drug delivery strategies may help optimize the treatment of diverse vascular disorders. Here, we discuss several aspects of this multifaceted area of research, including a brief overview of the design of drug carriers, the molecular features of endothelial cells useful for drug targeting, an analysis of parameters that control the anchoring of drug carriers to the endothelium, and examples of strategies for the endothelial targeting of drugs for the treatment of acute vascular pathologies.

Drug carriers

The blood stream is a natural route for endothelial drug delivery. However, drugs are rapidly eliminated from blood by renal clearance and hepatic uptake and by the reticuloendothelial system (RES; Moghimi and Szebeni 2003). Drug carriers are being designed in order to overcome this obstacle and (1) to optimize drug pharmacokinetics, protect drugs against inactivation, and limit premature activity *en route* to the target, (2) to control drug release kinetics, (3) to deliver drugs to target cells, and (4) to optimize their sub-cellular delivery.

Pharmacokinetics can be improved by conjugating polyethylene-glycol (PEG) to drugs or their carriers. PEG forms a hydrated shell that enhances the solubility of carriers or the drugs themselves and hinders interaction with RES and other eliminating systems in the body, thereby prolonging drug circulation (Abuchowski et al. 1977). For example, PEG-coated liposomal drug vehicles circulate in the vasculature for hours, relative to only minutes for their non-PEGylated counterparts (Discher and Eisenberg 2002).

Phospholipid-based liposomes arguably represent the most extensively studied drug vehicles (Moghimi and Szebeni 2003; Mainardes and Silva 2004). Amphiphilic phospholipids form bilayers in aqueous media to provide capsular vehicles. The internal aqueous space of the capsule can be used for the delivery of hydrophilic drugs, whereas the lipid bilayer can be loaded with small hydrophobic drugs. Liposomes can be made within a homogeneous and narrow size distribution; liposomes with a diameter of 100–200 nm are most suitable for intravascular delivery.

Polymersomes, the polymer analog of phospholipid liposomes, consist of amphiphilic diblock copolymers such as degradable PEG-poly(caprolactone). Polymersomes are more robust than liposomes and circulate for days (Discher et al. 1999). The extent of stealth effects depends on the molecular weight and surface density of the PEG on the carrier. Liposomes can only contain up to ~15 mol% PEG, whereas higher extents of hydrophilic PEG grafting destroys the phospholipid bilayer (Discher and Eisenberg 2002; Discher et al. 1999). In contrast, each polymer chain in a single polymersome can contain one PEG group; hence, 100 mol% surface coverage is possible, resulting in a circulation half life of days vs. hours for PEG-liposomes. Moreover, the membrane of polymersomes is thicker than that of liposomes (~8 nm compared with ~3 nm), providing highly durable carriers that are able to resist deforming forces that destroy liposomes (Discher and Eisenberg 2002; Discher et al. 1999). However, the harsh encapsulation conditions required to form polymersomes are more likely to affect the stability and activity of sensitive bioactive agents such as therapeutic enzymes.

By changing the ratio between the hydrophobic and hydrophilic polymer blocks (e.g., 42%–50% PEG content in co-polymers), the polymer chains can self-assemble into cylindrical flexible structures known as worm micelles, with widths of ~40 nm and lengths of up to 20–40 μm (Discher and Eisenberg 2002). These species have been only recently conceived as a new prospective class of drug carriers. A unique and highly attractive feature of worm micelles is their ability to align with flow (Croce et al. 2005), which might enhance circulation even further by avoiding collisions with vascular cells.

Solid PEG-copolymer nanoparticles formed by modified water/oil/water double emulsions can be loaded with either hydrophobic or hydrophilic drugs that can be incorporated into the polymer matrix or into internal aqueous domains, respectively (Dziubla and Muzykantov 2006). Therapeutic proteins encapsulated in polymer nanoparticles are protected against proteolysis (Dziubla et al. 2005). In general, nanocarriers with diameters in the 50 nm to 500 nm range, i.e., a size permitting circulation through capillaries and delivery into endothelial cells (Muro et al. 2004), have been employed for targeted drug and gene delivery in the vasculature.

The main requirement for any nanocarrier material is biocompatibility, which means that they can be injected intravenously without toxicity and overt side effects (Anderson and Langone 1999). These materials should be able to degrade into soluble components that are below 50 kDa in size and non-toxic. For example, PLGA hydrolyses into lactic and glycolic acid monomers in aqueous conditions, providing metabolizable and excretable degradation products.

To enhance the binding of drugs and their carriers to the endothelium, molecules recognizing endothelial surface determinants are used as affinity moieties (Muzykantov and Danilov 2005). Drugs and their carriers can be conjugated with such affinity moieties (antibodies, their fragments, peptides, aptamers; Muro et al. 2004) or fused with targeting peptides by using recombinant techniques (Ding et al. 2005).

Surface determinants for endothelial targeting

In order to be useful for endothelial drug delivery, an endothelial determinant must be present on the endothelium lumen, viz., accessible to carriers circulating in the blood stream. This key selection criterion excludes intracellular epitopes that represent a major “catch of molecular fishing” that employs vascular genomics and proteomics. Selective proteomics of the endothelial plasmalemma (Durr et al. 2004; Oh et al. 2004) and in vivo phage display (Rajotte et al. 1998) are attractive methods for defining new targets, as they identify binding sites accessible from the circulation in normal and pathological vascular areas (Hajitou et al. 2006). These methods also allow the identification of endothelial surface determinants in the pathological vasculature (Nicklin et al. 2000); such determinants are being explored for vascular targeting of imaging probes and drugs (Kelly et al. 2005). Some determinants are masked by the glycocalyx or hidden in plasmalemma structures. Access to these determinants may be limited by the size of the drug delivery system. For example, caveoli are accessible to antibodies and small peptides, but not to carriers larger than 100 nm. The surface density of certain endothelial molecules is reduced upon disease conditions, thereby hindering targeting to these determinants.

Target determinants must be safe and provide specific targeting. First, unless designed for such a goal, targeting should not cause harmful side effects to endothelial cells. This aspect represents a difference between targeting intentionally toxic agents to tumor endothelium and non-toxic therapeutics for the treatment of acute vascular disorders. In the first paradigm, the secondary toxic effect of targeting is a bonus. The specificity of targeting, i.e., preference of delivery to tumor vs. non-tumor endothelium, must be maximal in this case in order to avoid collateral damage. In the second paradigm, contrary to tumor targeting, safety is the major concern since endothelial disturbance is typically devastating in conditions associated with vascular oxidative stress, inflammation, and thrombosis. However, the criterion of specificity of targeting is less stringent in this case, because drugs alleviating these conditions are less likely to cause harmful effects in the normal vasculature. These conditions are often associated with systemic involvement of the vasculature in the

pathological process; thus, the endothelial delivery of antioxidants, anti-inflammatory, or anti-thrombotic therapeutics throughout the vasculature is a suitable option.

The binding of targeted drugs to the endothelium may activate cells and induce shedding and/or internalization of target determinants or change their functionality. Ideally, the binding of an antibody-drug complex to a target antigen should cause therapeutically beneficial side effects. Second, docking to a surface determinant should result in the ultimate proper sub-cellular localization of a drug. Dependent on the therapeutic goal, a drug should either be retained on the cell surface or transported to the proper sub-cellular compartment. This section provides a brief overview of endothelial determinants suitable for these goals (Table 1).

Constitutive determinants enriched in pulmonary endothelium

Angiotensin-converting enzyme (ACE; Muzykantov et al. 1991; Danilov et al. 1994, 2001) and thrombomodulin (TM; Kennel et al. 1990) are prime examples of this determinant class. The pulmonary vasculature is the major capillary network containing ~30% of the endothelial surface in the body and receives the entire cardiac output. As a result, agents with an endothelial affinity accumulate in the lungs after intravascular injection, even if their target determinants are relatively evenly distributed throughout all types of endothelial cells in the body (Muzykantov 2005). However, ACE and TM are enriched in this vascular bed, an important target for treatment of acute lung injury, pulmonary hypertension, edema, thrombosis, and inflammation, among other conditions.

ACE, a transmembrane glycoprotein expressed on the endothelial luminal surface, converts Ang I into Ang II, a vasoactive peptide that exerts vasoconstricting, pro-oxidant, pro-thrombotic, and pro-inflammatory activities (Heitsch et al. 2001). Some ACE antibodies (anti-ACE) inhibit ACE and cause its shedding (Danilov et al. 1994; Balyasnikova et al. 2002), which might be beneficial in conditions associated with hypertension, oxidative stress, and inflammation (Muzykantov 2001, 2002; Atochina et al. 1998). However, ACE also inactivates substance P and bradykinin; an elevated level of these peptides attributable to ACE inhibition may cause adverse effects including hypotension, cough, and edema.

The pulmonary vasculature is enriched in ACE; ~100% vs. <15% of endothelial cells are ACE-positive in the alveolar vs. the extra-pulmonary capillaries, respectively (Danilov et al. 2001). Endothelial cells internalize anti-ACE and anti-ACE conjugates, which thereby deliver drugs intracellularly (Muzykantov et al. 1996a). Oxidants, cytokines, and other pathological agents suppress ACE expression and thus inhibit targeting to ACE (Watanabe et al. 1992; Atochina et al. 1992). Monoclonal anti-ACE with species specificity including rat, mouse, cat, primate, and human ACE selectively accumulate in the pulmonary vasculature after IV injection in these species (Danilov et al. 2001, 1989; Balyasnikova et al. 2005, 2006). Pilot safety tests have not revealed overt harmful effects of injection of anti-ACE in normal animals and humans (Danilov et al. 1994; Muzykantov and Danilov 1995).

Thrombomodulin (TM), another transmembrane glycoprotein expressed on the luminal surface of endothelium, converts thrombin into an anti-thrombotic and anti-inflammatory enzyme (Esmon 1989). Anti-TM accumulates in the lungs after intravenous (IV) injection and has been employed for the targeting of liposomes, genetic materials, model enzymes, and isotopes to the pulmonary endothelium in laboratory animals (Christofidou-Solomidou et al. 2002). However, interventions that affect TM activity may compromise important endothelial functions and provoke thrombosis (Guermazi et al. 2004).

Constitutive cell adhesion molecules

Platelet endothelial cell adhesion molecule-1 (PECAM-1) and inter-cellular adhesion molecule-1 (ICAM-1) are transmembrane glycoproteins constitutively expressed on endothelial cells throughout the vasculature. Their ligands, e.g., anti-ICAM and anti-PECAM or conjugates of these antibodies, when infused locally into a conduit artery, accumulate in the downstream vascular areas including cardiac (Scherpereel et al. 2001), cerebral (Danielyan et al. 2007), and mesenteric (Garnacho et al. 2008; Muro et al. 2006a) vasculature. Anti-PECAM and anti-ICAM tend to accumulate in the lungs after IV injection and may be used for drug targeting to either normal and/or pathologically altered pulmonary and systemic endothelium (Muro et al. 2005).

PECAM (CD31) is predominantly localized in inter-endothelial borders (Albelda 1991). Platelets express CD31 at levels that are orders of magnitude lower than endothelial cells and bind a minor fraction of circulating anti-PECAM. PECAM is stably expressed on the endothelium at the level of a million copies per cell, permitting robust drug targeting to normal and pathologically altered vasculature, for either prophylaxis or therapy. The multivalent binding of anti-PECAM/nanocarriers enhances targeting (Danilov et al. 2001; Muzykantov et al. 1999). Diverse reporter (Wiewrodt et al. 2002), enzymatic (Scherpereel et al. 2001; Nakada et al. 2000; Scherpereel et al. 2002), and genetic (Li et al. 2000) materials conjugated to anti-PECAM accumulate and display their functional activity in the endothelium as quickly as 10 min after IV injection in mice and pigs.

PECAM is involved in mechanisms of cellular recognition, adhesion, and trans-endothelial migration of leukocytes (Nakada et al. 2000). Thus, drug targeting to PECAM may inhibit leukocyte trafficking (Murohara et al. 1996). Leukocyte infiltration is generally viewed as an injurious factor (Kumasaka et al. 1996), and the attenuation of leukocyte infiltration by PECAM targeting would be a bonus during the treatment of inflammation.

Endothelial cells bind single anti-PECAM antibodies without internalization but internalize multivalent anti-PECAM complexes with diameters of 100–500 nm (Muzykantov et al. 1999; Wiewrodt et al. 2002), regardless of the chemistry of formation of such multivalent conjugates (Muro et al. 2004). The cross-linking of PECAM by multivalent anti-PECAM conjugates induces signaling via the PECAM-1 cytosolic tail (Garnacho et al. 2008), leading to endocytosis (Muro et al. 2003a). In contrast, monomeric anti-PECAM and anti-PECAM scFv cause no such effects (Muzykantov et al. 1999; Muro et al. 2003a).

ICAM-1 (CD54) is another Ig superfamily surface glycoprotein. Quiescent confluent endothelial cells in culture do not express appreciable amounts of ICAM-1, but expression is increased 20–50 times after cytokine treatment (Muro et al. 2003b). However, ICAM-1 in the vasculature is normally expressed by endothelial cells at a surface density of 2×10^4 to 2×10^5 surface copies per cell (Almenar-Queralt et al. 1995). Other cell types also express ICAM, although blood-accessible ICAM is located predominantly on endothelial cells. Anti-ICAM and anti-ICAM conjugates bind to the vascular endothelium after IV administration in animals (Panes et al. 1995; Murciano et al. 2003). Pathological stimuli including oxidants, cytokines, and abnormal shear stress stimulate the de novo synthesis and surface expression of ICAM by endothelial cells (Beck-Schimmer et al. 1997) and thereby facilitate anti-ICAM endothelial targeting (Panes et al. 1995; Murciano et al. 2003; Vaporciyan et al. 1995; Sasso et al. 1996). The conjugation of anti-ICAM to therapeutics (Murciano et al. 2003; Villanueva et al. 1998), liposomes (Bloemen et al. 1995), or polymer carriers (Muro et al. 2006a) providing multivalent binding to the endothelium further enhances drug delivery.

ICAM supports leukocyte adhesion to the endothelium (Kishimoto and Rothlein 1994). Anti-ICAM inhibits this process, providing anti-inflammatory effects in animal models of

vascular pathologies (Kavanaugh et al. 1996; Rothlein et al. 1993). ICAM may also serve for the docking of pathogens (Sarantos et al. 2005) and as a signaling molecule (Zimmerman et al. 1996). Adverse effects of ICAM blocking in animal and human studies are rare and confined to aggravated airway infection by anti-ICAM delivered via airways and to adverse reaction to heterologous anti-ICAM used to inhibit inflammation in stroke (Muro et al. 2005; Rothlein et al. 1993; Zivin 1998).

Internalization of anti-ICAM follows the same pattern as described for PECAM: endothelial cells internalize multivalent anti-ICAM conjugates, but not monomeric anti-ICAM (Muro et al. 2003a), via a unique internalization pathway termed CAM-mediated endocytosis and described in detail elsewhere (Muro et al. 2003a, b, 2004). Therefore, monomolecular anti-ICAM carriers anchor drugs to the endothelial surface (Murciano et al. 2003), whereas polyvalent anti-ICAM conjugates deliver drugs intracellularly (Muro et al. 2004, 2005). After internalization, ICAM-1 dissociates from the anti-ICAM carriers and recycles to the plasma membrane, supporting multiple cycles of intracellular delivery (Muro et al. 2005). The intracellular trafficking of cargoes internalized via PECAM-1 and ICAM-1 can be regulated by auxiliary pharmacological agents. For example, alteration of the proton-sodium balance in endosomal-lysosomal compartments decelerates the degradation of proteolysis-susceptible cargoes and permits their recycling to the plasma membrane (Muro et al. 2003b, 2005). The disruption of endothelial microtubules blocks lysosomal trafficking and prolongs the duration of activity of internalized drugs (Muro et al. 2003b).

The list of ICAM-targeted drug delivery systems includes immunoliposomes (Bloemen et al. 1995; Mastrobattista et al. 1999), polymer nanocarriers (Muro et al. 2005, 2006a; Karnik et al. 2008), protein conjugates (Atochina et al. 1998; Muro et al. 2003b), imaging agents (Sasso et al. 1996; Rossin et al. 2008), and acoustic micron-size bubbles used as contrast agents for ultrasound imaging (Villanueva et al. 1998). These systems have been characterized in cell culture (Muro et al. 2005; Bloemen et al. 1995; Mastrobattista et al. 1999; Karnik et al. 2008) and animal models (Muro et al. 2003b; Sasso et al. 1996; Villanueva et al. 1998; Rossin et al. 2008).

Inducible endothelial cell adhesion molecules: selectins and VCAM-1

These are molecules that are normally absent on the vascular lumen but are exposed on pathological endothelium. For example, pathological mediators cause the mobilization of intracellular P-selectin to the endothelial surface within 10–30 min (Kumasaka et al. 1996) and induce de novo synthesis and surface expression of E-selectin (Kishimoto and Rothlein 1994) and VCAM-1 (Albelda 1991) within several hours. Selectins and VCAM-1 facilitate the adhesion of leukocytes to endothelial cells (Springer 1990).

Selectin and VCAM ligands represent attractive affinity moieties for delivery diagnostics and therapeutic agents to activated endothelium. The attenuation of leukocyte adhesion is a potential secondary benefit of this approach. Experiments in cell culture and animals show that anti-selectins permit drug targeting to cytokine-activated endothelium (Spragg et al. 1997; Kiely et al. 1995). Endothelial cells constitutively internalize selectins via clathrin-coated pits (Kuijpers et al. 1994; Straley and Green 2000; von Asmuth et al. 1992), permitting entry into endothelial cells of anti-E-selectin targeted liposomes (Kessner et al. 2001), anti-inflammatory drugs (Kessner et al. 2001; Kok et al. 2002), and genetic materials (Harari et al. 1999).

However, even at maximal activation, selectins and VCAM-1 are exposed at surface densities lower than PECAM-1 and ICAM-1; hence, targeting robustness may be sub-optimal for therapies requiring the delivery of large doses of drugs. P-selectin targeted compounds also bind to activated platelets (Lindner et al. 2001). Interestingly, E-selectin

and VCAM-1 seem to be more readily expressed in activated endothelium outside the pulmonary vasculature, e.g., in arteries and skin microvasculature (Kelly et al. 2005). These determinants seem to be useful for the diagnostic visualization of activated endothelium in inflammation foci by the delivery of conjugated isotopes (Keelan et al. 1994) or ultrasound contrasting materials (Lindner et al. 2001, 2003).

Determinants of specific endothelial domains

Specific domains in the endothelial plasmalemma are enriched in certain molecules. For example, rat glycoprotein gp85 is predominantly localized on the luminal surface of the plasmalemma domain and belongs to a thin part of the endothelial cell body that lacks major organelles and separates alveolar and vascular compartments (Ghitescu et al. 1999). Anti-gp85 accumulates in rat pulmonary vasculature without internalization and delivers conjugated cargoes into the pulmonary vasculature (Murciano et al. 2001). A human counterpart of this antigen could be an interesting candidate for drug delivery to the surface of alveolar capillaries.

Phage display library selection in vivo has yielded several candidate peptides that bind to and provide the homing of phages into the pulmonary (e.g., peptides with an affinity to aminopeptidase P; APP) and other endothelia including tumor endothelium (e.g., peptides with an affinity to aminopeptidase N; APN; Rajotte et al. 1998; Kolonin et al. 2006). Endothelial proteomics have shown that APP is enriched in the pulmonary vasculature and represents an interesting model target determinant in animal studies (Oh et al. 2004). This enzyme, however, plays important physiological roles including the degradation of bradykinin (Skidgel 1992); hence, its inhibition may cause side effects including edema. APN is involved in angiogenesis, and its inhibition may provide a beneficial side effect in the context of anti-cancer therapy (Hajitou et al. 2006).

The pulmonary endothelium contains cholesterol-rich plasma-membrane flask-shaped invaginations named caveoli (Schnitzer 2001; Stevens et al. 2000). Antibodies of determinants localized to caveoli including APP and glycoproteins gp60 and gp90 accumulate in the pulmonary vasculature after IV injection into rats, enter endothelial intracellular vesicles, and traverse endothelial barriers (McIntosh et al. 2002). Caveoli-mediated endocytosis and transcytosis are involved in endothelial transport functions (Schnitzer 2001; Predescu and Palade 1993; Stan 2002). Interaction of a protein ligand (e.g., antibodies) leading to receptor clustering in caveoli further activates endocytosis and transcytosis (Minshall et al. 2000; Vogel et al. 2001). Caveoli seem to be involved in trans-endothelial transport of albumin (John et al. 2003), a process further stimulated by albumin nitration that may take place during oxidant stress (Predescu et al. 2002).

Caveolar transcytosis is envisioned as a pathway for the transcellular delivery of drugs targeted to caveolae-located receptors. For example, IV injection of tracers conjugated with antibodies directed against the specific antigen gp90 localized in pulmonary endothelial caveolae undergo transport through the pulmonary endothelium (McIntosh et al. 2002). Recently, the extremely rapid trans-endothelial transport of caveoli-targeted antibodies and affinity peptides identified by a phage-display has been demonstrated in mice (Oh et al. 2007). The caveoli neck diameter is ~50 nm. Therefore, the size of the drug carriers that can employ this pathway is limited to <100 nm: larger phages and nanoparticles targeted to these caveolar determinants do not accumulate in the lungs because of steric hindrances (Oh et al. 2007). Caveoli represent endothelial transport and signaling organelles, and therefore, any side effects of targeting caveolar determinants must be rigorously characterized.

Interaction of affinity carriers with endothelial determinants

Factors controlling the binding of drug carriers to the vascular endothelium include carrier affinity, surface density, and distribution of target determinants in the plasmalemma, their accessibility to carriers, flow parameters, carrier size, geometry, and binding stiffness. For the sake of simplicity, “antigen-antibody” terminology will be used in this review. However, the considerations outlined in this section are applicable to many types of affinity pairs including antibody fragments, peptides, hormones, and other natural and artificial ligands.

Carrier affinity and antigen surface density

Binding affinity, the measure of the interaction strength between a targeting moiety such as an antibody and its antigen, controls the effectiveness of the initial arrest and firm retention of a carrier on endothelial cells. Both antibody affinity and its surface density on the carrier dictate the resultant carrier affinity (Fig. 1a). For example, spherical polymer nanocarriers (100 nm in diameter) decorated with a high-affinity TM antibody demonstrate more effective endothelial targeting and local effect of a model enzymatic cargo after IV injection in mice vs. the same carrier decorated with a lower affinity antibody (Shuvaev et al. 2007a). An increase in the surface density of both an antibody on the carrier and an antigen on the endothelium augments synergistic multivalent binding favoring arrest and firm adhesion. A combination of several affinity moieties on the carrier, e.g., selectin and ICAM-1 ligands, facilitates the endothelial binding of polymer microspheres (Omolola Eniola and Hammer 2005).

Enhanced surface density of an antigen on the target cell, such as is seen with inducible cell adhesion molecules in pathological vascular areas (Pober et al. 1986), facilitates multivalent high-affinity carrier binding (Fig. 1b). ICAM-1 and other determinants cluster upon binding of antibody-coated carriers, assisting multivalent binding (Muzykantov et al. 1999; Muro et al. 2003a; Wiewrodt et al. 2002). Computational models describing the endothelial binding of drug carriers and leukocytes reveal the key role of multivalent interaction, dependent on antigen surface density (Chang and Hammer 2000; Hammer and Apte 1992). These models utilize the adhesive dynamics method, which approximates the binding of ligand-coated spheres of a given size (e.g., anti-ICAM-coated particles) to a planar surface covered with the complimentary receptor (Bhatia et al. 2003). Adhesive dynamics studies have demonstrated that increasing either the number of antigen copies on the cell surface or the antibody density on the targeting particle enhances binding (Hammer and Apte 1992). The simulation results are comparable with experimental results that have studied these parameters in mice (Forlow et al. 2000). Similar experimental results have further verified the importance of adequate antibody coating on both micron-scale carriers (Omolola Eniola and Hammer 2005) and nanoscale carriers (Muro et al. 2006a).

Shear stress

Effect of flow conditions on vascular drug delivery is being addressed both in vitro by using flow chambers that allow the controlled modulation of shear stress and in vivo by using intravital microscopy to monitor binding in a vessel of interest (Sakhalkar et al. 2003; Garnacho et al. 2008; Smith et al. 2007; Kiani et al. 2002). Particle migration velocities have been investigated as functions of different shear rates: high and low velocities indicate rolling and firm adhesion, respectively. For example, low-affinity selectin-targeted particles roll upon endothelial contact and exhibit higher migration velocities, whereas low velocities are observed for firm adhesion molecules such as CAM-targeted particles (Smith et al. 2007; Omolola Eniola and Hammer 2005). Increased shear stresses result in a reduction in the firm adhesion of a carrier to the endothelium and to immobilized endothelial antigens.

Binding of weaker affinity carriers, such as carriers decorated with the selectin-targeted ligand, sialyl Lewis^X, appears to follow a more complex scenario. In this type of binding, which is associated with rolling as opposed to firm adhesion, a shear threshold effect has been noted, whereby lower shear stresses result in low binding, and maximum binding occurs at an intermediate shear stress (Alon et al. 1997; Chen and Springer 1999; Finger et al. 1996; Lawrence et al. 1997; Puri et al. 1998; Sarangapani et al. 2004; Yago et al. 2004). As flow is increased above this intermediate value, binding reduces again. This is in contrast with the binding seen with both high and low affinity antibodies, whereby binding is maximal at low shear stresses and simply decreases with increasing flow. The adhesive dynamics method takes into account the forces and torques acting on the targeting molecule as it passes over its receptor-coated surface under flow and is thus a logical approach for determining the mechanism behind this shear threshold. From this technique, the mechanism responsible has been suggested to be a catch-slip bond in which the off rate initially decreases and then increases with increasing shear stress (Caputo et al. 2007). This same catch-slip phenomenon has been observed for selectins via flow chamber and atomic force microscopy dissociation studies (Yago et al. 2004; Dwir et al. 2003; Marshall et al. 2003) and is illustrated in Fig. 1c.

Carrier geometry

Within the realm of vascular targeting, carrier size and geometry can have profound, yet complicated, effects on the ultimate binding. For instance, with larger particle size, assuming an equal surface coating density of antibodies on the carriers, more potential binding contacts exist. This is an intuitive result as the larger the carrier, the higher the resultant surface area on the particle is available for antibody coating. However, larger particles experience an effectively higher detachment force relative to smaller carriers when subjected to a high shear environment such as the flow conditions encountered in much of the vasculature upon injection. Larger particles, by virtue of their larger diameters and thus displacement from vessel walls upon binding, have larger moment arms along which the flow force can act. The result is the higher torque (torque = radius×force) and thus greater effective detachment force that larger carriers experience under shear. This effect is convoluted further with non-spherical carriers, such as nanofilaments, and is a topic currently under investigation (Geng et al. 2007).

Additional parameters in carrier binding

Additional parameters governing drug carrier binding to the endothelium are more difficult to study experimentally, often being studied only by computational methods. Examples of such parameters include the flexural rigidity of receptors and receptor-ligand bond stiffness and strength (Hammer and Apte 1992; Agrawal and Radhakrishnan 2007). In addition, the glycocalyx, a polysaccharide coating on cells, is a potential barrier to drug targeting (Sabri et al. 2000; Mulivor and Lipowsky 2002). Thermodynamic modeling of nanocarrier binding and experimental data suggest that removal of the glycocalyx can enhance the binding of spherical carriers (Agrawal and Radhakrishnan 2007; Mulivor and Lipowsky 2002). Of note, some vascular conditions including inflammation and ischemia-reperfusion have been reported to be associated with glycocalyx shedding (Mulivor and Lipowsky 2004), which may naturally enhance drug delivery to pathologic vasculature.

Examples of drug targeting to vascular endothelium

During the last decade, the targeting of therapeutic, reporter, and genetic cargoes to the vascular endothelium has been translated from studies in artificial systems in vitro to multiple investigations in laboratory animal and limited human settings. Targeting tumor endothelium, an important area on its own, is not reviewed in this article. Instead, we focus

on approaches that demonstrate promise for the diagnosis and treatment of acute vascular pathologic conditions including ischemia, inflammation, thrombosis, oxidative stress, and hypertension.

Imaging

The visualization of vascular compartments has great potential for the improvement of disease diagnostics by using modalities such as gamma-scintigraphy, computer tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), and combinations thereof. In particular, partially because of the extensive nature of vascularization in pulmonary tissues and the degree of severity of pulmonary pathologies, the imaging of the lungs has been pursued with great vigor. For example, anti-ACE labeled with radioactive ^{125}I and ^{99}Tc isotopes accumulate in the lungs after IV injection in diverse animal species including primates (Danilov et al. 2001; Muzykantov and Danilov 1995). Pulmonary accumulation of anti-ACE has been visualized in real time with a gamma camera in these animal species (Danilov et al. 1989) and in humans (Muzykantov and Danilov 1995). A reduction in the pulmonary uptake of isotope-labeled anti-ACE is a sensitive indicator of lung endothelial disturbance in rat models of endotoxemia, edema, and ischemia-reperfusion (Muzykantov et al. 1991; Atochina et al. 1992, 1997). Clinical studies with gamma-camera thoracic imaging have revealed that the pulmonary uptake of isotope-labeled anti-ACE is reduced in patients with sarcoidosis, in comparison with healthy volunteers (Muzykantov and Danilov 1995). Similarly, the pulmonary accumulation of ^{125}I-anti-TM is reduced to 50% of basal level in mice exposed to hyperoxic lung injury (Shuvaev et al. 2007a), consistent with the loss of TM in pathologically altered pulmonary endothelium (Perkowski et al. 2003).

^{125}I-labeled anti-ICAM accumulates preferentially in the lungs and mesentery and, to a lesser extent, in other highly vascularized organs after IV injection in rats (Panes et al. 1995) and mice (Komatsu et al. 1997). In contrast to anti-ACE and anti-TM, isotope-labeled anti-ICAM shows elevated vascular accumulation under oxidative stress and during inflammation. Enhanced pulmonary uptake of ^{125}I-anti-ICAM has been shown in the lungs of rats and mice challenged intratracheally with immune complexes (Vaporciyan et al. 1995), the bacterial endotoxin lipopolysaccharide (LPS; Beck-Schimmer et al. 1997), cytokines (Komatsu et al. 1997, Mulligan et al. 1993), and hyperoxia (Perkowski et al. 2006). Increased uptake has also been shown in lungs challenged with LPS systemically (Muro et al. 2003b; Panes et al. 1995, 1996) and in a model of chronic hypertension (Komatsu et al. 1997). Gamma-camera imaging has shown that the lungs represent the main target organ after injection of ^{111}In-labeled anti-ICAM in rats (Sasso et al. 1996). Pulmonary uptake of ^{111}In-anti-ICAM is enhanced in allograft rat lungs during acute rejection (Ishida et al. 2003) in rat models of acute lung injury caused by oleic acid injury (Weiner et al. 2001) and bleomycin-induced lung injury (Weiner et al. 1998). Expression of other cell adhesion molecules in the pulmonary vasculature in mice has also been probed by using radiolabeled antibodies. For example, elevated pulmonary accumulation of ^{125}I-labeled antibody to P-selectin has been reported within a few minutes post-injection in mice treated with histamine (Langley et al. 1999), several hours after treatment with LPS (Langley et al. 1999), and a day after exposure to hyperoxia (Perkowski et al. 2006).

Polymer nanoparticles labeled with positron emission isotopes including ^{64}Cu and Cd^{125m}Te, targeted to anti-ICAM (Rossin et al. 2008) and anti-TM (Woodward et al. 2007), respectively, have been employed to visualize the pulmonary vasculature in real time in mice. Consistent with other studies that have employed labeled anti-ICAM, the pulmonary accumulation of anti-ICAM/^{64}Cu-PNC is markedly enhanced in mice treated with bacterial LPS, reflecting enhanced exposure of ICAM-1 on the endothelial lumen (Rossin et al.

2008). The combination of PET or SPECT with micro-CT has augmented the topographical localization of isotope signals in the chest in these studies.

Monoclonal antibodies, recombinant derivatives of these antibodies, and affinity peptides binding to endothelial determinants localized in the caveoli accumulate within minutes in the pulmonary vasculature after IV injection in rats and permit lung imaging with gamma-scintigraphy (Valadon et al. 2006). For example, a monoclonal antibody to APP (^{125}I-labeled anti-APP) has provided discernable lung images within minutes after IV injection in rats and maintains a strong signal from the lungs without major cardiac signal in the thorax for the ensuing 48 h (Oh et al. 2007). ^{99}Tc-labeled anti-APP has allowed lung imaging during whole body SPECT/CT in rats, whereas fluorescently labeled anti-APP gives high-resolution real-time microscopic imaging of its permeation across rat lung endothelium (Oh et al. 2007).

Phage display-defined affinity peptides to pulmonary endothelial determinants including APP also permit targeting to and visualization of the lung vasculature, e.g., by optical imaging of injected peptide-decorated quantum dots (Akerman et al. 2002). Hybrid viral vectors decorated by vascular-addressing peptides offer new modalities for targeted interventions and imaging, capitalizing on the local expression of enzymes converting latent agents into imaging probes (Hajitou et al. 2006).

Several types of pathologically altered vasculature outside of the lung have been imaged in animal models by using labeled targeted carriers. Anti-VCAM-1-coated microparticles made of iron oxide have been used for MRI imaging of acute brain inflammation in mice (McAteer et al. 2007). Superparamagnetic nanoparticles coated with a near infrared dye have been employed to visualize acute vascular inflammation in mice (Tsourkas et al. 2005). Peptides with VCAM-1 affinity have been designed and conjugated to these nanoparticles (Kelly et al. 2005) and subsequently used to visualize binding in vitro to endothe-lial cells and isolated human carotid arteries. VCAM-targeted peptides have also been utilized in vivo for intravital confocal microscopy and MRI (Kelly et al. 2006; Nahrendorf et al. 2006) in mouse models of acute vascular inflammation (unilateral injection of tumor necrosis factor-α in one ear) and atherosclerotic lesions in mouse aortas. Enhanced targeting to pathologically altered endothelium has been detected by both MRI and fluorescent-imaging modalities. An alternative targeting approach has been employed whereby small 20-nm magnetofluorescent particles are internalized upon injection by macrophages, which then migrate to inflammation-rich atherosclerotic plaques (Jaffer et al. 2006). Expanding upon this result, magnetofluorescent nanoparticles have been labeled with the PET isotope ^{64}Cu (Nahrendorf et al. 2008). These 20-nm particles are transported to macrophages and ultimately to atherosclerotic plaques. This multimodal imaging probe has demonstrated a marked increase in accumulation in atherosclerotic lesions in the aortic root and arch. Lesion locations have been determined by CT imaging. This imaging modality has also been utilized to diagnose aortic valve disease in mice (Aikawa et al. 2007). Magnetofluorescent nanoparticles coated with anti-VCAM-1 peptide are internalized by macrophages and target early stage valve-disease endothelium. Not only can the presence or absence of diseased valves be detected, but valvular proteolytic and osteogenic activities can be monitored with specific fluorescent and near-infrared probes linked to the carriers.

Similar MRI studies of atherosclerosis in the aortic arch have been performed with magnetic particles conjugated to whole VCAM-1 or P-selectin antibodies (McAteer et al. 2008). Further, the use of gas-filled microparticles, conjugated with ICAM-1 antibodies, has also been pursued for the ultrasound imaging of the blood brain barrier during autoimmune encephalomyelitis (Linker et al. 2005). Addressing another imaging modality, gas-filled lipid microbubbles have been targeted to leukocytes via the incorporation of

phosphatidylserine into their membranes for contrast echocardiography of myocardial reperfusion injury (Christiansen et al. 2002; Lindner et al. 2000).

From this brief overview, it is evident that vascular imaging utilizing reporter probes targeted to specific endothelial determinants represents an exponentially developing area of modern biomedicine.

Antioxidants

The most potent antioxidants in nature are enzymes. For example, one molecule of catalase, a 250-kDa protein, is capable of dissociating millions of molecules of toxic H_2O_2 per second. However, inadequate delivery and limited stability limit the therapeutic utility of enzymes. Targeted delivery of antioxidant enzymes to the endothelium may help solve these problems. Endothelial cells internalize multivalent solid nanoparticles surface-coated with anti-ICAM or anti-PECAM and catalase within 30 min (Muro et al. 2003b), similarly to the enzyme conjugated directly with these targeting antibodies (Atochina et al. 1998; Muzykantov et al. 1996b; Christofidou-Solomidou et al. 2003; Shuvaev et al. 2004). Since H_2O_2 easily diffuses through cellular membranes, including those that make up endo/lysosomal compartments, targeted catalase transported into endo/lysosomes degrades H_2O_2 and protects cells against oxidative stress without endo/lysosomal escape (Muro et al. 2003b). Likewise, the targeting of anti-PECAM/SOD conjugates to endothelial cells protects them against oxidative stress caused by either extracellular or intracellular generation of the superoxide anion (Shuvaev et al. 2007b). Furthermore, IV injection of anti-PECAM/catalase conjugates in animals protects them against acute oxidative stress in pulmonary vasculature in mice (Christofidou-Solomidou et al. 2003) and improves the outcome of lung transplantation in rats (Kozower et al. 2003). Lysosomal degradation of antioxidant enzymes (Muro et al. 2006b) terminates the protective effect within 3 h of binding (Muro et al. 2005). The use of auxiliary pharmacological agents affecting lysosomal trafficking and degradation prolongs the protective effects of anti-ICAM/catalase formulations by many hours (Muro et al. 2005, 2006b).

Alternatively, the employment of biocompatible polymer nanocarriers selectively permeable to H_2O_2, but not to proteases, permits a prolonged antioxidant effect (Fig. 2). These carriers thus act as a protective cage, capable of therapy without traditional “drug release”. A freeze-thaw modified double emulsion allows the encapsulation of active catalase into such protective nanocarriers with a controlled size and shape, appropriate for vascular delivery into endothelial cells (Dziubla et al. 2005;Simone et al. 2007). Typical carrier morphologies are nanospheres with diameters of <500 nm (Dziubla et al. 2005) or flexible nanofilaments that are a few microns in length and only ~50 nm in cross section (Simone et al. 2007). Morphology and loading of nanocarriers are controlled by the chemistry of the polymers utilized, e.g., the molecular weight and ratio of hydrophilic to hydrophobic domains in the used amphiphilic diblock copolymers (e.g., PEG-PLA). Not only does this approach enable significant loading of active catalase, but also the cargo enzyme is protected against external proteolysis. Catalase-loaded polymer nanocarriers targeted to PECAM-1 deliver active cargo to the pulmonary vasculature after IV injection in animals and protect endothelial cells against H_2O_2-induced injury for durations on the order of days (Dziubla et al. 2008).

Anti-inflammatory agents

Other protective treatments typically utilize smaller anti-inflammatory drugs such as corticosteroids. One classic example of an anti-inflammatory steroid is dexamethasone (Dex), which exhibits diminished effectiveness attributable to poor vascular delivery (Melgert et al. 2000). In order to overcome this obstacle, Dex has been conjugated with an anti-E-selectin. Studies in endothelial cell culture have shown anti-selectin/Dex binding and

trafficking to lysosomes, where proteolytic degradation of the conjugate subsequently occurs (Kok et al. 2002; Asgeirsdottir et al. 2003). Based on this outcome, Dex-loaded liposomes targeted to E-selectin have been tested in vivo and display binding and internalization in a mouse model of skin inflammation (Everts et al. 2003). In an injury model characterized by glomerular renal inflammation, E-selectin-targeted liposomes with encapsulated Dex not only accumulate in the inflamed kidney, but also reduce inflammatory gene expression (Asgeirsdottir et al. 2007, 2008). Furthermore, the typical side effects of free Dex, such as altered blood glucose levels, have not been detected with the Dex-immunoliposomes. Dex-liposomes coated with RGD peptides targeting $\alpha_v\beta_3$ integrins on endothelial cells have been tested for the treatment of inflammation in a rat arthritis model (Koning et al. 2006). Interestingly, a single injection of targeted Dex-carriers is able to reduce the arthritis score of injured animals for several weeks. Another similar RGD-targeted platform that utilizes albumin as the carrier particle has been used to deliver the p38MAP (mitogen-activated protein) kinase inhibitor SB202190 (Temming et al. 2006). This small molecule inhibitor has been utilized because p38MAP kinase inactivation suppresses the synthesis of inflammatory cytokines. This delivery platform partially inhibits gene expression and the secretion of multiple inflammatory cytokines (Temming et al. 2006).

Vascular-targeted anti-thrombotic interventions

Thrombosis is caused by intravascular thrombi. Fibrinolytic plasminogen activators (PAs) activate plasminogen to plasmin, which subsequently degrades fibrin clots and restores blood perfusion. However, the clinical utility of PAs is limited by inadequate delivery attributable to rapid elimination and side effects caused by collateral damage and hemorrhage. Several laboratories have conjugated fibrin-specific antibodies with PAs to deliver fibrinolytics to intravascular clots (Bode et al. 1991; Runge et al. 1991). However, delivery systems that target determinants present in thrombi are not suitable for prophylactic usage. Further, their therapeutic utility is limited by clot impermeability.

Theoretically, the enhancement of anti-thrombotic activity on the vascular luminal surface could be used for thromboprophylaxis. Gene transduction of anti-thrombotic proteins in the vasculature in laboratory animals supports this notion (Waugh et al. 1999), but this approach is impractical in acute settings. Immunotargeting of anti-thrombotic proteins to the endothelial surface might rapidly restrict and sustain their activity to the vascular lumen. This approach can potentially provide thromboprophylaxis in patients with a high propensity for thrombosis, particularly in settings where the risk of bleeding prohibits use of systemic anticoagulation (Topol et al. 1999).

Several endothelial determinants have been employed for this function. For example, tPA chemically conjugated with anti-ACE retains fibrinolytic and antigen-binding activities and exhibits sustained preferential accumulation in rat pulmonary vasculature, providing proof of principle for this strategy (Muzykantov et al. 1996c). Other endothelial determinants have also been explored with regard to this goal; thus, both tPA and urokinase-type PA (uPA) conjugated chemically with antibodies against antigens enriched in the pulmonary endothelium accumulate and facilitate thrombolysis in the pulmonary vasculature (Murciano et al. 2001; Ding et al. 2003). However, cell adhesion molecules ICAM-1 and PECAM-1, which are more stably expressed by endothelium and do not support endocytosis of single antibodies, represent the most attractive target for anchoring anti-thrombotic drugs on the endothelial luminal surface.

Radiolabeled tPA conjugated with anti-ICAM accumulates in the pulmonary vasculature of anesthetized rats with a high degree of specificity. After IV injection in rats, the uptake of anti-ICAM/tPA in the pulmonary vasculature is ~70 times higher than that of control IgG/tPA, which results in enhanced fibrinolysis of subsequent pulmonary emboli (Murciano et

al. 2003). Thus, anti-ICAM carriers are likely to provide robust and preferential targeting to the pulmonary endothelium in intact animals, on par with the best candidate carriers tested to date, including antibodies directed against ACE and the caveoli-associated antigen gp90 (Muzykantov et al. 1996c; Oh et al. 2004). However, in contrast to these endothelial determinants, ICAM-1 offers enhanced pulmonary targeting in the context of inflammation, which is frequently intertwined with thrombosis (Idell 2003; Levi and Schultz 2008). The observation that thrombin up-regulates the expression of ICAM-1 provides an additional rationale for its use as a target for delivering anti-thrombotic agents (Wu and Aird 2005).

One important technical note is that the production of chemical antibody/tPA conjugates provides heterogeneous formulations including multivalent conjugates that cause endocytosis. Genetically engineered antibody fragments, single-chain Fv (scFv, comprising variable domains of heavy chain V_H and light chain V_L) represent a more attractive alternative that allows the direct fusion of recombinant anti-thrombotic proteins. The result is a highly homogeneous and relatively small (50–70 kDa, hence low immunogenicity and lack of Fc-fragment-mediated side effects) bi-functional recombinant drug.

As a proof of principle for prophylactic thrombolysis by endothelium-targeted thrombolytic fusions, an anti-PECAM scFv has been fused with a uPA that exists as an inactive single-chain zymogen of 411 amino acid residues (single-chain uPA, scuPA; Ding et al. 2005). In the presence of fibrin, endogenous plasminogen activators convert plasminogen to plasmin, which in turn cleaves the Lys158-Ile159 peptide bond in scuPA and generates fully active two-chain uPA (tcuPA) consisting of two polypeptide chains linked by a Cys148-Cys279 disulfide bond (Stump et al. 1986).

The natural scuPA consists of three domains: the N-terminal domain homologous to the epidermal growth factor, the kringle domain, and the C-terminal catalytic domain. Urokinase binding to a cellular receptor via the “growth-factor-like domain” (GFD) activates vascular cells (Blasi and Carmeliet 2002), but deletion of the GFD provides a low-molecular-weight form of scuPA (MW: 32 kDa) that has enzymatic features similar to those of full-length scuPA.

The fusion protein composed of an anti-PECAM scFv connected with lmw-scuPA by using a linker of three Gly_4Ser repeats has been recently designed (scFv/uPA). After IV injection, scFv/uPA preferentially accumulates in the lungs of wild-type but not PECAM-deficient mice, persists in the lungs for at least 3 h, and remains on the endothelial surface. Compared with non-targeted uPA, scFv/uPA greatly augments the local lysis of pulmonary emboli in a mouse pulmonary thrombotic model. Further, scFv/uPA, but not wild-type uPA, accumulates in the cerebral vasculature after intra-arterial and IV injection. This construct dissolves cerebral clots without hemorrhagic complications and improves cerebral blood reperfusion, thereby mitigating post-thrombotic brain edema in a model of cerebral embolism (Danielyan et al. 2007). These data support the concept that scuPA can be delivered to the endothelial surface in effective concentrations and for a relevant duration, providing the necessary thromboprophylaxis and preventing tissue damage.

The scuPA zymogen (pro-urokinase) appears to possess low intrinsic peptidase activity that generates plasmin over time, which in turn converts scuPA to fully active tcuPA. tcuPA may cause adverse effects, including fibrinogen consumption and hemorrhage (Liu and Gurewich 1992). Since tcuPA is rapidly inactivated by PAI-1, the duration and effectiveness of prophylaxis would also be limited. Furthermore, thrombin inactivates uPA by cleaving Arg156-Phe157, negating its effect at sites of active thrombosis, the intended target area (Ichinose et al. 1986). These problems might be solved by deleting Phe157 and Lys158, thereby yielding a plasmin-resistant mutant activated by thrombin (uPA-T; Yang et al.

1994). This pro-enzyme is not activated by plasmin in vivo (thus avoiding systemic effects and premature PAI-1 inactivation), whereas thrombin can activate it locally at sites of nascent thrombosis within seconds of clotting.

Replacing the native plasmin activation site in the uPA moiety of scFv/uPA with a thrombin activation site produces thrombin-activated anti-PECAM scFv/uPA-T (Ding et al. 2008). This construct has also been found to contain an intrinsic thrombin-sensitive cleavage site in the anti-PECAM scFv moiety, providing a built-in mechanism for local drug release. The resultant scFv/uPA-T is latent and resists the PA inhibitor PAI-1 until activated by thrombin. After intravenous injection in mice, scFv/uPA-T does not consume plasma fibrinogen, in contrast with scFv/uPA, which has this liability. However, scFv/uPA-T binds to the endothelium and accumulates in the vascularized organs, particularly the lungs, similar to scFv/uPA. In a mouse model of thromboplastin-induced pulmonary thrombosis, scFv/uPA-T provides more potent and durable thromboprophylaxis than both plasmin-sensitive scFv/uPA and lmw-scuPA. Further, injection of scFv/uPA-T into mice 30 min prior to unilateral in situ lung ischemia/reperfusion (120 min/150 min) attenuates pulmonary fibrin deposition to a significantly greater extent than plasmin-sensitive scFv/uPA and restores arterial oxygen tension, whereas PAI-1-susceptible plasmin-activated scFv/uPA does not improve blood oxygenation. Therefore, endothelium-targeted thromboprophylaxis triggered by a pro-thrombotic enzyme illustrates a novel approach to the time- and site-specific regulation of “on demand” proteolytic reactions that can be modulated for therapeutic benefit (Fig. 3). In clinical settings, this strategy of targeting anti-thrombotic drugs to the endothelial surface may provide local thromboprophylaxis in patients with an acute risk of developing new or recurrent thrombi and thus might prevent clot extension.

Concluding remarks and perspectives

Endothelial targeting of active reporters and therapeutic cargoes including enzymes and genes has been achieved in intact animals and animal models of human pathologies. Recent animal studies have shown that determinants including ACE, APP, ICAM, and PECAM have a potential for drug targeting to the endothelium. Their functions are well enough understood to avoid unintentional side effects in a given pathological context. Targeting caveoli provides an avenue for intracellular and transcellular vascular drug delivery. Careful selection of targets and modulation of features of the antibody-drug conjugates, such as valency and size, provide powerful tools for the control of intracellular uptake and the trafficking of cargoes.

The industrial synthesis and quality control of targeted drug delivery systems with a standard level of homogeneity acceptable to national drug agencies represent a significant challenge for their commercial development and clinical utility. Recombinant fusion of protein drugs and pro-drugs with protein affinity moieties provides therapeutic agents that are suitable for good manufacturing practices, i.e., homogeneous and relatively easy to scale-up. The recombinant design of these constructs permits the deletion of unnecessary parts of molecules or the insertion of point mutations endowing products with novel favorable pharmacokinetics and/or functional features. Clinical testing of biotherapeutics targeted to endothelial cells has been recently conducted and has demonstrated promising levels of safety and efficacy. It is tempting to hope that, in the next decade, targeted interventions into endothelial cells will be translated from initial successes reported in laboratory animals to medical practice.

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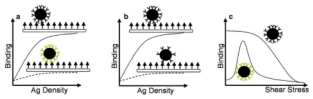

Fig. 1.

Determinants of drug carrier binding to vascular endothelium (*black Ys*, solid line, high affinity antibodies, *green Ys*, dashed line, low affinity antibodies, *arrows* endothelial antigens). **a** Effect of antibody affinity on carrier binding. **b** Effect of surface density coating of carriers with antibodies and of antigen (Ag) density (the number of antigens per unit area of endothelium). **c** Effect of shear stress on binding. The *upper curve* is relevant to firm adhesion targeting, such as CAM targeted carriers, whereas the *bottom curve* (bell-shaped) illustrates catch-slip binding and the shear threshold effect, typical of lower affinity selectin targeting. Binding is schematically depicted here as the number of carriers bound to the endothelium

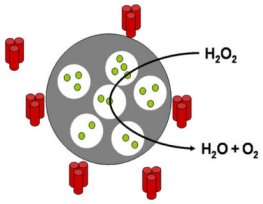

Fig. 2.

Selectively permeable antioxidant polymer nanocarriers (*large gray sphere* polymer nanocarrier with internal aqueous cavities containing catalase, *green circles* catalase). The *red cylinders* represent generic proteases that cannot penetrate the polymer material. However, the small molecule H_2O_2 easily permeates through the polymer matrix and can thus be dissociated into inert water and oxygen by the encapsulated catalase

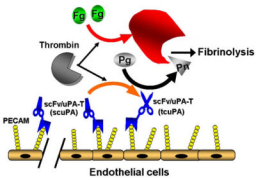

Fig. 3.

"On demand" fibrinolysis by vascular targeting of thrombin-activated anti-PECAM scFv/uPA (*scFv/uPA-T*). scFv-uPA/T circulates in a pro-drug form, viz., single-chain uPA (*scuPA*), binds to PECAM-1, and remains anchored on the endothelial luminal surface for at least several hours. Upon environmental stress or tissue injury, thrombin is generated to cleave fibrinogen (*Fg*) and form fibrin clots. While this causes in situ thrombosis, the generated thrombin also converts the endothelium-bound scFv/uPA-T to enzymatically active *tcuPA*, inducing local conversion of plasminogen (*Pg*) into plasmin (*Pn*). The active plasmin subsequently facilitates fibrinolysis, which restores the blood flow and mitigates tissue ischemia and injury

Table 1

Endothelial determinants: selected candidate targets for drug delivery (*EC* endothelial cells, *ACE* angiotensin-converting enzyme, *TM* thrombomodulin, *PECAM* platelet endothelial cell adhesion molecule, *ICAM* intercellular adhesion molecule, *gp* glycoproteins, *APN* aminopeptidase N, *APP* aminopeptidase P)

Target	Function and localization	Targeting advantages	Potential problems
ACE	Peptidase converts Ang I into Ang II and cleaves bradykinin. ACE enriched in the lung capillaries	Selective targeting to lung EC. Intracellular delivery. Vasodilating and anti-inflammatory effects of ACE inhibition	Inflammation suppresses targeting. ACE inhibition may be dangerous
TM	Binds thrombin and converts it into anti-coagulant enzyme. Enriched in the lungs	Intracellular delivery to EC useful for modeling of lung injury in animals	Inflammation suppresses targeting. Thrombosis attributable to TM inhibition
PECAM-1	Facilitates transmigration of leukocytes. Stably expressed in EC borders	Intracellular delivery of anti-PECAM conjugate may also suppress inflammation	PECAM-signaling and side effects are not fully understood
ICAM-1	Mediates leukocyte adhesion to EC. Stably expressed by EC and up-regulated by pathological agents	Similar to PECAM, but inflammation enhances targeting	Similar to PECAM
E-selectin	Supports leukocyte adhesion. Expressed only on altered EC	Intracellular targeting to EC in inflammation	Targeting is not robust. Transient expression. Surface targeting is not effective because of endocytosis
P-selectin	Similar to E-selectin	Similar to E-selectin	Similar to above. Limited specificity: platelets bind P-selectin ligands
gp90	Function unknown. Localized in EC caveoli	Transendothelial targeting in rat lungs	Human analog and side effects are not known
gp85	Function unknown. EC avesicular zone in alveolar capillaries	Targeting to the EC surface in rat lungs	Similar to above
gp60	Albumin-binding protein in EC caveoli	Transendothelial targeting	Side effects and specificity of targeting are not known
APN	Digestion of peptides by proteolysis	Upregulated in tumor vasculature	Expression in other cell types
APP	Cleavage of bradykinin and substance P	Localized in the caveoli, enriched in lungs, and offering highly effective and fast endothelial targeting	Inhibition of APP may cause side effects including edema
Integrin$\alpha_v\beta_3$	Cell adhesion to extracellular matrix and cell survival	Selectively expressed by tumor vasculature	Unclear targeting level, controversial in vivo function of α_v, and potential side effects of β_3 blockage