

Review Article

Targeted Drug Delivery to Endothelial Adhesion Molecules

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Endothelial cells represent important targets for therapeutic and diagnostic interventions in many cardiovascular, pulmonary, neurological, inflammatory, and metabolic diseases. Targeted delivery of drugs (especially potent and labile biotherapeutics that require specific subcellular addressing) and imaging probes to endothelium holds promise to improve management of these maladies. In order to achieve this goal, drug cargoes or their carriers including liposomes and polymeric nanoparticles are chemically conjugated or fused using recombinant techniques with affinity ligands of endothelial surface molecules. Cell adhesion molecules, constitutively expressed on the endothelial surface and exposed on the surface of pathologically altered endothelium—selectins, VCAM-1, PECAM-1, and ICAM-1—represent good determinants for such a delivery. In particular, PECAM-1 and ICAM-1 meet criteria of accessibility, safety, and relevance to the (patho)physiological context of treatment of inflammation, ischemia, and thrombosis and offer a unique combination of targeting options including surface anchoring as well as intra- and transcellular targeting, modulated by parameters of the design of drug delivery system and local biological factors including flow and endothelial phenotype. This review includes analysis of these factors and examples of targeting selected classes of therapeutics showing promising results in animal studies, supporting translational potential of these interventions.

1. Introduction: Targeting Therapeutics to Endothelium

Most therapeutic agents do not naturally accumulate in intended targets in the body, which limits their efficacy and creates issues associated with off-target and systemic side effects and repetitive and complex administration regimens and costs. Utility of many drugs suffers from unfavorable solubility, pharmacokinetics, and permeability across cellular barriers. In order to overcome these issues of pharmacotherapy, drug targeting strategies emerged in the seventies, focusing primarily on delivery of antitumor, antimicrobial, and other toxic agents [1–3].

Advances in biotechnology yielded a new type of drugs, biotherapeutics, with wide utilities beyond oncology and infectious diseases, across diverse medical disciplines—cardiology, pulmonology, transplantation, rheumatology, and

so forth. These “natural” therapeutic agents include recombinant therapeutic proteins including antibodies, enzymes, inhibitors, decoy receptors, as well as diverse nucleic acid formulations—gene therapies, siRNA, miRNA, and so forth. Many of these agents offer natural biological catalytic mechanisms for elimination, synthesis or modification of their molecular targets in the body. They promise new level of potency, specificity, and precision of the effect. However, biological drugs are labile, costly, and potentially immunogenic and require precise delivery to desired sites of action in the target cells—plasmalemma, cytosol, and intracellular organelles.

Endothelial cells lining the vascular lumen play a key role in control of vascular tone, blood fluidity, and extravasation of blood components including white blood cells, WBC [4–6]. Endothelial dysfunctions and damage caused by pathological factors including inflammatory mediators, oxidants, and

abnormal blood flow is the key factor of pathogenesis of many human health maladies [7–9]. In particular, vascular inflammation, oxidative stress, thrombosis, and ischemia are intertwined mutually propagating processes involving endothelium and implicated in the pathogenesis of ischemia-reperfusion (e.g., acute myocardial infarction, stroke, and transplantation injury), as well as acute and chronic inflammation including sepsis and acute lung injury [10–15]. Many systemic conditions such as metabolic and genetic diseases involve and affect endothelium, which in turn worsens the disease and its prognosis. Endothelial cells represent an important target for therapeutic interventions [16–19].

Endothelium is accessible to drugs circulating in blood [20]. Nevertheless, most drugs including biotherapeutics have no endothelial affinity, and only a minor fraction of the injected dose is taken up by these cells. In order to provide targeted delivery to endothelium, drugs or their carriers can be conjugated with affinity ligands of endothelial surface determinants. Using antibodies and their fragments directed to endothelial determinants “vascular immunotargeting” or natural endothelial ligands represents examples of this strategy [18, 19, 21–24]. Since the late eighties this approach is being explored by several labs in diverse experimental models and a few clinical studies [18, 25–33].

Advanced drug delivery systems (DDS) including liposomes, polymeric carriers, protein chemical conjugates, and recombinant fusion constructs have been devised for drug delivery to normal and pathological endothelium [34–37]. Many candidate target molecules have been identified and explored including endothelial surface receptors and enzymes, structural elements of glycocalyx and specific domains in plasmalemma, and cell adhesion molecules [31, 38–40]. Numerous studies of the last decade indicate that using this approach for targeted delivery of biotherapeutics to endothelial cells in animal models of human pathology provides therapeutic effects superior to nontargeted interventions and in many cases enables novel mechanisms of drug action. In particular, cell adhesion molecules ICAM-1 and PECAM-1 represent versatile candidate determinants for site-specific delivery of diverse drugs to selected endothelial compartments [9, 20, 41].

2. Principles of Endothelial Drug Delivery

Generally, targeting is achieved by conjugating affinity ligands with drugs or drug carriers [42–44]. There is an arsenal of types of nanocarriers for targeted delivery of drugs and imaging agents to endothelial cells (Figure 1). The roster of carriers includes classical liposomes, arguably the most extensively characterized type of nanoparticles that are already in clinical use and more novel formulations such as dendrimers and polymersomes that are currently at relatively early translational phases. Each type of nanocarriers has its own benefits and shortcomings that will be discussed below in the context of their specific use.

Interaction of targeted drug delivery system with cells of interest includes distinct phases of molecular recognition and anchoring, followed by either residence on the plasmalemma

or internalization, and concluded eventually by either intracellular degradation or shedding from the plasmalemma.

These complex and rather partially understood dynamic processes are controlled by several factors pertinent to features of the target cell and its microenvironment (including but not limited to surface density and accessibility of the anchoring determinant molecules and their epitopes, parameters of flow, and functional and phenotypic characteristics of the cell), as well as features of the ligand (affinity and number and accessibility of binding sites), its configuration in the drug delivery system (valence, surface density, and interactive freedom), and features of the drug delivery system (size, shape, and pharmacokinetics). Effects of targeting additional to the action of the drug cargo also represent an important consideration pertinent utility of the strategy.

2.1. Target Determinant Accessibility. Endothelial determinants must be sufficiently accessible to the circulation to be able to anchor biotherapeutics, which size ranges from few to tens of nanometers, or their carriers, which size ranges from tens to hundreds of nanometers. Inaccessibility disqualifies intracellular molecules, unless they are exposed on the surface of pathologically altered cells (see below).

Even epitopes localized within the extracellular moiety of the same surface determinants may differ in their accessibility to affinity carriers. Epitopes located more proximally to the plasmalemma are less suitable for harboring carriers than distal epitopes [46]. Carrier dimensions represent an important factor: epitopes buried under the glycocalyx or in invaginations of the plasmalemma are accessible to small ligands such as antibodies and are not accessible to submicron carriers [46, 47].

In reality, “target epitope accessibility” is a collective rather than individual characteristic of exposure of binding epitopes to the circulation from the blood vessel lumen. With exception of monovalent ligands and their fusion constructs, congruent accessibility for multivalent interaction with target cell is necessary to anchor ligand-drug or drug-carrier conjugates with size ranging from tens to hundreds nanometers, which experience detaching hydrodynamic force of blood, proportional to their size [48].

Under pathological conditions some determinants normally expressed on the endothelial surface are masked (e.g., by adherent blood elements) or disappear due to shedding, which may impede their use as targets for therapeutic delivery in these pathologies [49, 50]. For example, ischemia, oxidants, cytokines, and other pathological agents suppress luminal surface density and/or accessibility of determinants including endothelial peptidases (see below) [51, 52]. This suppresses targeting to these determinants, thereby hindering therapeutic interventions in these conditions [53].

2.2. Constitutive versus Inducible and Panendothelial versus Domain-Specific Endothelial Determinants. Numerous molecules localized on the surface of endothelial cells of diverse phenotypes have been identified by high-throughput approaches [54] including selective proteomics of the endothelial plasmalemma [21, 55] and *in vivo* phage display

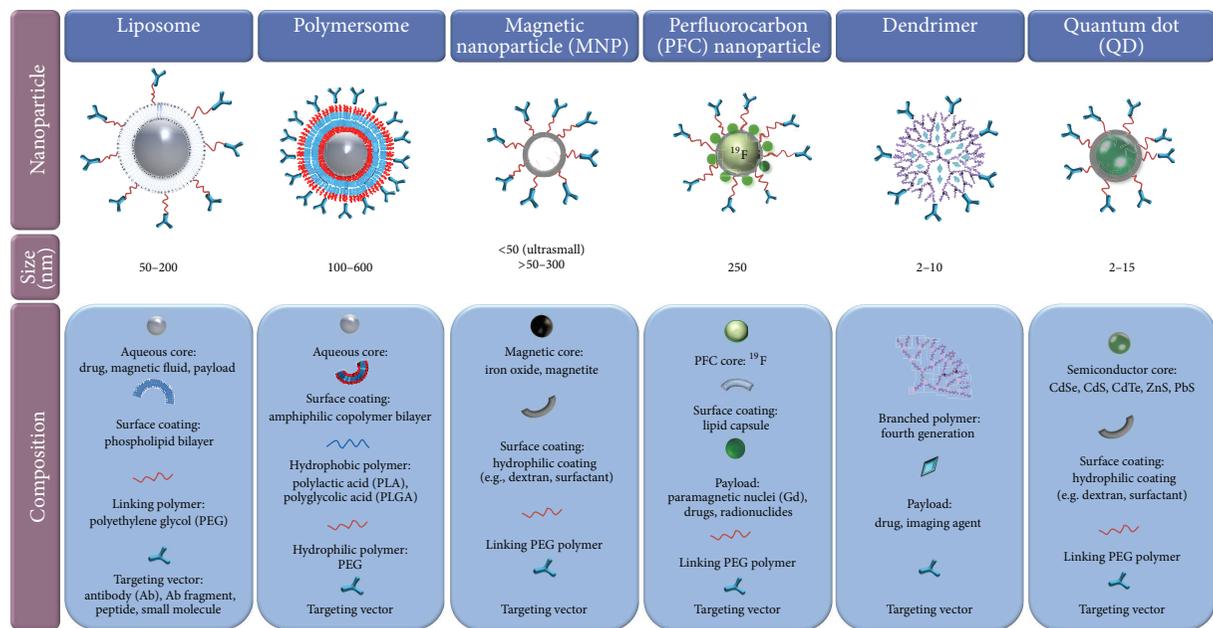


FIGURE 1: Nanocarriers for vascular delivery of imaging and therapeutic agents. Schematic representation of targeted nanoparticles engineered for biomedical imaging and therapeutic drug delivery applications. The components of a multifunctional nanocarrier can include a ligand for cellular targeting and an encapsulated payload for delivery of the therapeutic agents. The imaging probe (e.g., radioisotope) can be incorporated in the payload, on the targeting ligand, or associated with the nanoparticle shell, for example. From Chacko et al. [45].

[29] and low throughput individualized approaches such as tracing of ligand molecules [20]. Constitutively expressed determinants can be used for both prophylactic and therapeutic drug delivery, while those expressed in pathological sites are ideal for therapeutic interventions and imaging, for example, to the endothelium of inflammation sites [18, 22, 27, 56, 57].

Carriers targeted to panendothelial determinants expressed throughout the vasculature can be injected intravenously to achieve systemic delivery of drugs to treat generalized conditions (e.g., sepsis and disseminated intravascular coagulation) or infused in the conduit vessels to enrich accumulation in the downstream vascular area. Determinants preferentially expressed in certain vascular areas, types of blood vessels, or in sites of pathology support local delivery.

The pulmonary vasculature is the major capillary network containing ~30% of the endothelial surface in the body and receiving more than 50% of the entire cardiac output. As a result, agents with an endothelial affinity accumulate in the lungs after intravenous (IV) injection, even if their panendothelial target determinants are relatively evenly distributed throughout all types of endothelial cells in the body [20]. This vascular bed is an important target for treatment of acute lung injury, oxidative stress, thrombosis, and inflammation, among other conditions.

For example, angiotensin-converting enzyme (ACE), a glycoprotein constitutively expressed at the endothelial luminal surface, is a good target candidate [49, 53, 58]. ACE converts Ang I into Ang II, a vasoactive peptide that exerts constricting, pro-oxidant, prothrombotic, and pro-inflammatory activities [59]. Endothelial cells internalize ACE antibodies

(anti-ACE) and anti-ACE conjugates [60]. Labeled anti-ACE selectively accumulates in the lungs after IV injection in rats, mice, cats, primates, and humans [36, 49, 61, 62]. Pilot tests did not reveal harmful effects of anti-ACE in animals [25, 58] and humans [25]. Anti-ACE formulations are being used for targeting to the pulmonary endothelium of biotherapeutics such as antioxidant enzymes [32, 63, 64] and genetic materials including “retargeted” viruses [33, 65, 66] and show impressive therapeutic effects in animal studies [67–69]. Pilot studies in human organs support the notion of translation potential of ACE-directed drug delivery to endothelial cells [70].

Specific domains in the endothelial plasmalemma are enriched in certain molecules [71, 72]. For example, rat glycoprotein GP85 is predominantly localized on the luminal surface of the plasmalemma domain that belongs to a thin part of the endothelial cell body lacking organelles and separates alveolar and vascular compartments [73]. Antibodies to GP85 accumulate in rat pulmonary vasculature without internalization and deliver conjugated antithrombotic enzymes into the pulmonary vasculature [74]. Determinants localized in the endothelial caveoli including aminopeptidase P (APP) provide the pathway for transendothelial delivery of antibodies and small protein conjugates [75].

2.3. Potential Side Effects of Endothelial Targeting. In most clinical scenarios, drug delivery to endothelium should be free of adverse effects on the target cell and other cell types taking the drug (e.g., renal and hepatic cells), as well as systemic side effects such as activation of complement and

other host defense systems in the bloodstream. One specific aspect of this problem, sometimes overlooked, is that biocompatibility of the drug delivery system is not equal to that of its components [76]. Loading a relatively safe agent into a relatively safe carrier decorated by innocuous ligands may yield a toxic combo with pro-inflammatory or adjuvant features.

Furthermore, ligands and especially ligand-driven carriers may activate endothelial cells or induce shedding and/or internalization of target determinants, change their functionality, or otherwise disturb the endothelium. For example, targeting to thrombomodulin, a very useful model in animal studies [50, 77], is unlikely to find clinical use because of the high risk of thrombosis and inflammation [78] caused by inhibition of thrombomodulin protective functions [79]. Inhibition of endothelial enzymes ACE and APP results in elevation of level of one of their common peptide substrates, bradykinin, which may lead to side effects associated with enhanced vascular permeability, a known and generally tolerable side effect of ACE inhibitors.

Criteria of safety are different in targeting tumors and tumor endothelium versus targeting drugs for management of cardiovascular, pulmonary, neurological, and metabolic maladies [80]. Toxic effect to the tumor cells is often viewed a bonus, whereas the specificity of targeting must be maximal to avoid collateral damage. In contrast, endothelial disturbance must be minimized to avoid aggravation of oxidative stress, inflammation, and thrombosis. However, the criteria of specificity are less stringent in this case, because drugs alleviating these conditions (often associated with systemic pathologies) are less likely to cause systemic harmful effects; therefore, pan-endothelial delivery of antioxidant, anti-inflammatory, or anti-thrombotic agents throughout the vasculature is a suitable option.

3. Endothelial Cell Adhesion Molecules: Targets for Drug Delivery

Endothelial adhesion molecules are being actively pursued as candidate targets to deliver drugs, biotherapeutics, and imaging agents to vascular endothelium [9]. These molecules are involved in vascular adhesion of activated white blood cells (WBC) in the pathological sites and therefore seem good markers (detection), targets (inhibition of leukocyte migration), and drug delivery destination (anchoring of drug carriers) to treat vascular inflammation, thrombosis, and oxidative stress.

3.1. Inducible Endothelial Cell Adhesion Molecules. Inducible vascular cell adhesion molecule-1 (VCAM-1), P-selectin, and E-selectin are exposed on the endothelial surface in pathologically altered vasculature. Pathological factors including cytokines, oxidants, and abnormal flow cause mobilization of P-selectin from the intracellular storage organelles (Weibel-Palade bodies) to endothelial surface within 10–30 min [81] and within several hours induce *de novo* synthesis and surface expression of E-selectin [82] and VCAM-1 [83]. Selectins and VCAM-1 facilitate rolling phase of the adhesion of leukocytes to endothelial cells [84].

Ligands of inducible adhesion molecules are explored for drug delivery to activated endothelium. Conjugation with antibodies to these molecules facilitates drug delivery to cytokine-activated endothelium in cell culture and animal models of inflammation [7, 23, 27, 85–87]. Endothelial cells internalize selectins via clathrin-coated pits [88–90]. This feature supports intracellular delivery into endothelial cells of anti-E-selectin targeted liposomes [91], anti-inflammatory drugs [91, 92], and genetic materials [93]. Anti-VCAM also enters endothelial cells via clathrin endocytosis [94, 95]. Selection of epitope-specific VCAM-1 ligands further activates endocytosis [85, 86, 95], enhancing vascular VCAM-1 imaging in animal models of inflammation [85, 86].

Of note, these inducible adhesion molecules are exposed on the surface of pathologically activated endothelium at surface density level of $<10^4$ copies per cell, fairly modest comparing with more robust determinants (see below). P-selectin targeted compounds also bind to activated platelets [96]. The regional, temporal, and stimulus-specific parameters of expression of E-selectin and VCAM-1 are still not fully understood even in animal models; for example, they seem to be expressed by activated endothelium in arteries and skin microvasculature at higher extent than in the pulmonary vasculature [85]. In models of acute inflammation, selectins disappear from the luminal surface within time intervals varying from minutes to a few hours [97]. Due to rapid natural lysosomal traffic of materials entering cells via clathrin endocytic pathway, inactivation in this degrading compartment may restrict duration of therapeutic effects.

The utility of positron emission tomography (PET) and other imaging modalities with high sensitivity generally is less dependent on the ability of a drug delivery system to concentrate large doses in the target site, whereas the tissue selectivity is the key objective. In this context, inducible adhesion molecules represent excellent determinants for visualization of activated endothelium in inflammation foci by delivery of conjugated isotopes [56] or ultrasound contrasts [96, 98] (see below).

3.2. Constitutive Cell Adhesion Molecules (CAMs): Platelet-Endothelial Adhesion Molecule-1 (PECAM) and Intercellular Adhesion Molecule-1 (ICAM). PECAM-1 (CD31, or PECAM thereafter) and ICAM-1 (CD54, or ICAM thereafter) are type I transmembrane glycoproteins that belong to the Ig-like superfamily, sharing similar composition: a large extracellular region composed of several Ig-like C2-type domains, a transmembrane segment, and a cytoplasmic tail mediating signal transduction pathways [99, 100]. They are present in several cell types, for example, platelets (PECAM), epithelial cells (ICAM), and leukocytes (both). However, their surface density is orders of magnitude higher in endothelial cells. PECAM is stably expressed at level of $0.2\text{--}2 \times 10^6$ copies per cell [83], whereas ICAM is expressed in vessels by quiescent and activated endothelium at levels of $\sim 0.2\text{--}1 \times 10^5$ versus $0.5\text{--}3 \times 10^5$ copies per cell, respectively [101].

PECAM and ICAM are expressed on endothelial surface throughout the vasculature. PECAM is localized predominantly in the interendothelial borders, whereas ICAM is

localized in the luminal membrane and tends to concentrate in lipid rafts, where it may exist either as a monomer or oligomer form [102]. Unlike other endothelial constitutive determinants (thrombomodulin, APP, or ACE), PECAM density is not suppressed in pathological states [103, 104]. In contrast, ICAM constitutive expression is further upregulated in pathologically altered endothelia and other cells types [105]. Quiescent confluent endothelial cells in culture express very low amounts of ICAM and treatment with cytokines or thrombin leads to 50–100-fold upregulation [106]. Endothelial cells in the vasculature express ICAM at a surface density of 2×10^4 – 2×10^5 surface copies per cell, and this level roughly doubles upon pro-inflammatory challenge [102]. In contrast with ICAM expressed by other cell types, the ICAM molecules located on endothelial luminal surface are directly accessible to the bloodstream.

CAMs are involved in endothelial signaling [107]. Clustering of PECAM or ICAM by multivalent ligands including leukocytes initiates signal transduction mediated by alteration of phosphorylation state in their cytosolic domains [108]. Adhesion and signaling induced by ligand binding to extracellular domains of CAMs are involved in maintenance of dynamic integrity of endothelial monolayer, endothelial activation leading to release of inflammatory mediators, cytoskeleton remodeling and change of cellular shape, and leukocyte mobilization in sites of inflammation [107, 109].

Via its extracellular domain, endothelial PECAM engages in heterophilic binding to heparin-containing proteoglycans and $\beta 1$ and $\beta 3$ integrins of leukocytes and in homophilic PECAM-PECAM interactions, maintaining the monolayer integrity [108, 110, 111]. The extracellular region of ICAM binds ligands including fibrin, certain pathogens and β -2 integrins of activated leukocytes, mediating their firm adhesion to endothelial cells [112, 113]. Therefore, PECAM and ICAM are involved in mechanisms of cellular recognition, adhesion, and trans-endothelial migration of leukocytes [114]. Interference in pro-inflammatory functions of these molecules by CAM antibodies and other pharmacological means may be beneficial in treatment of inflammation [115].

3.3. Affinity Ligands for CAM Targeting. Antibodies to PECAM and ICAM (anti-ICAM and anti-PECAM) and anti-CAM conjugates bind to endothelial cells and accumulate in vascularized organs after intravascular injection [17, 78, 116–121]. Systemic intravenous injection favors pulmonary accumulation [118], whereas infusion in a conduit artery offers local accumulation in the downstream vascular areas including cardiac [122], cerebral [123], and mesentery [124, 125] vasculature.

“Designer” affinity ligands have been devised for targeting endothelial CAMs [41]. They include monoclonal antibodies and their scFv fragments [118, 126, 127] as well as affinity peptides selected using phage display library [128]. A humanized monoclonal antibody binding to human ICAM with 50-times higher affinity than original mouse anti-ICAM has been produced [129], as well as multivalent Fab fragments of a monoclonal antibody to human ICAM [130].

Some of these recombinant proteins devised in attempts to develop anti-inflammatory and anti-infectious treatments based on ICAM blocking have been clinically tested and showed generally acceptable safety [131, 132]. More recently, a short 17-mer linear peptide derived from one of natural ICAM ligands, fibrinogen, has been devised and showed excellent targeting features *in vitro* and in animal studies, providing binding and internalization of nanoparticles on pair with ICAM antibodies [133]. This type of ligands offers advantages of lowering risk of immune reactions and utility in diverse animal species.

Monomolecular ligand-directed therapeutics may interact with their target determinants either in bivalent (e.g., antibodies themselves) or monovalent fashion (e.g., Fab-fragment conjugates and scFv-fragment fusion proteins). Bivalent binding of an antibody to glycoprotein(s) on the cell surface offers higher affinity yet requires higher freedom and congruency of carrier-target interaction. Ligands binding to distinct epitopes on the same target molecule may influence each other, for example, inhibiting binding to adjacent epitopes. The competitive inhibition of binding to overlapping epitopes has been described for antibodies to ACE [134–136].

Recently, it has been found, however, that distinct monoclonal antibodies directed to adjacent epitopes in the distal domain of the extracellular moiety of PECAM, rather stimulate binding of each other, both in cell cultures and *in vivo* [137]. The endothelial binding of PECAM-directed mAbs is increased by coadministration of a paired mAb directed to adjacent, yet distinct PECAM-1 epitopes. The “collaborative enhancement” of mAb binding was affirmed in mice, manifested by enhanced pulmonary accumulation of intravenously administered radiolabeled PECAM-1 mAb when coinjected with an unlabeled paired mAb. This unusual finding, which can be explained by unmasking conformational changes induced by a paired “stimulatory” ligand, may find utility in vascular immunotargeting. This phenomenon provides a novel paradigm for optimizing the endothelial-targeted delivery of diagnostic agents and therapeutics.

3.4. CAM-Directed Endothelial Targeting of Drug Carriers. Viewed as a translational drug delivery platform, nanocarriers provide a way to configure molecules of ligands, which may or may not have sufficient individual affinity for effective targeting, into multimolecular compounds, which avidity may be greatly elevated by multivalent binding. For example, studies in cell cultures and in animal models revealed fairly consistent elevation of an effective endothelial avidity of anti-ICAM/nanocarriers versus free anti-ICAM [125, 138].

Quantitative measurements and computational analysis of binding of anti-ICAM/nanocarriers to endothelial cells under static and flow conditions revealed that in order to achieve productive anchoring interaction, several antibodies coupled to the carrier should engage simultaneously in binding to endothelial cells [48, 139, 140]. At the present time, quantitative parameters of ligand affinity and surface density on a carrier have to be determined empirically, at least in part because the surface density and clustering of CAMs in the

vasculature remain to be characterized quantitatively. Generally, the multivalent binding of CAM-targeted nanocarriers boosts endothelial drug delivery [49, 116].

A considerable attention has been paid in the last decade to optimization of affinity interactions of CAM-targeted carriers with endothelia of interest. One intriguing idea explored by several labs is that combining on the surface of the carrier affinity ligands that bind to different determinants may boost the selectivity and efficacy of drug delivery. For example, combinations of anti-ICAM with antibodies to inducible adhesion molecules (selectin, VCAM-1, ELAM) have been tested *in vitro* in models that employ coimmobilized antigens [141] or cytokine-activated cells [142]. The practical utility of these studies performed in experimental models remote from physiological context remains to be more fully understood.

Of note, a new dual-targeting strategy employing spherical 100–200 nm carriers carrying antibodies to both ICAM and transferrin receptor has recently been tested *in vivo* and showed promising results: each of the ligands apparently promoted targeting to the vascular area of its destination, that is, nanocarriers could be directed to the inflamed pulmonary vasculature via ICAM and to cerebral vasculature via transferrin receptor [143].

Liposomes were the first delivery system employed in midnineties for drug targeting to endothelial ICAM in cell culture models [144]. Concomitantly, therapeutic enzymes chemically conjugated with antibodies to ICAM (anti-ICAM) have been devised and tested in animal models [63]. Since then, diverse drug carriers and drug conjugates targeted to CAMs have been devised and tested in animal studies including lipid particles [145, 146], polymersomes [147] and polymeric nanocarriers of diverse geometries [148–150], protein conjugates [116, 151, 152], and recombinant fusion proteins [127]. Diverse reporter [153] and enzymatic [118, 122, 151] and genetic materials [154] conjugated to anti-PECAM accumulate and display their functional activity in the endothelium as soon as 10 min after IV injection in mice, rats, and pigs. Similarly, conjugation of anti-ICAM to therapeutics [120, 155], liposomes [144], or polymer carriers [125] providing multivalent binding to the endothelium and enhances drug delivery.

3.5. Intracellular Delivery via CAMs. Endothelial cells internalize ligands by phagocytosis and endocytosis via caveoli [57, 156, 157] and clathrin-coated [158] and uncoated vesicles [159–161] and use pinocytosis for fluid phase uptake [57]. In contrast to determinants and receptors involved in these endocytic pathways, PECAM and ICAM are stably anchored in the endothelial plasmalemma and turnover slowly via proteolytic shedding, with low level of endocytic turnover [9, 162]. Within the reasonable time intervals, from minutes to an hour, internalization levels of antibodies to these CAMs are just ~10% higher than the background uptake on ice [116, 163].

However, endothelial cells internalize multivalent anti-ICAM and anti-PECAM conjugates and carriers coated by multiple copies of anti-CAM [78, 116, 120, 163] (Figure 2).

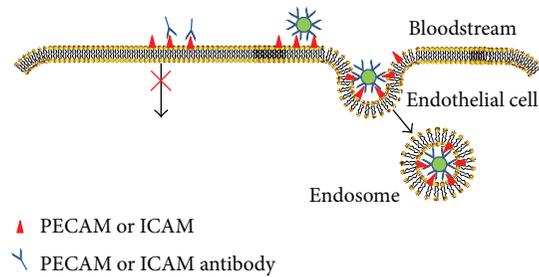


FIGURE 2: Anti-CAM nanocarriers: intracellular delivery in endothelial cells. Monomolecular antibodies bound to PECAM-1 or ICAM-1 do not undergo internalization, thereby providing mechanism for anchoring conjugated cargoes in the vascular lumen. In contrast, multivalent anti-CAM/nanocarriers and conjugates binding to and clustering cell surface CAMs activate internalization via a specific endocytic pathway [43].

Multivalent binding of these artificial ligands causes redistribution, cross-linking, and clustering CAM, triggering endothelial uptake via a unique pathway, CAM-mediated endocytosis [57, 163]. Uptake of anti-CAM conjugates and carriers is relatively rapid, with a $T_{1/2}$ varying from 5 to 20 min for different formulations and effective, with total level of the uptake achieving 85–90% of the total amount of particles bound to the cells [106].

The studies using isotope tracing, multilabel fluorescent and electron microscopy in static and flow-adapted cell cultures and in animals revealed that the mechanism of CAM-mediated internalization is distinct from canonical endocytic pathways via clathrin-coated pits or caveoli, as well as phagocytic and pinocytosis mechanisms [106]. Molecular signaling in CAM-mediated endocytosis is unique and involves Ca^{2+} and a series of kinases and second messengers mediating reorganization of the cytoskeleton driving the uptake of CAM-anchored conjugates [106, 163].

Internalized anti-CAM conjugates initially reside in the nascent intracellular vesicles negative for endosomal markers for about 1 h, subsequently traffic to the endosomal compartment (1 to 2 h postinternalization), and reach lysosomal compartments 3 h after internalization within endothelial cells [106]. Therefore, most (but not all) anti-CAM conjugates arrive in lysosomes several hours after uptake [57, 106, 117, 163, 164]. This pace of vesicular traffic is fairly slow comparing with the classical endocytic pathways delivering their ligands to the lysosomes within minutes after internalization in endothelial cells [106].

Target ICAM cointernalized with bound anti-ICAM conjugates dissociates from the immune complex in the endosomes [117]. The conjugates traffic further to lysosomes, while CAM recycles to the EC surface allowing multiple cycles of intracellular delivery *in vitro* and *in vivo* [117]. However, once in lysosomes, the labile protein content in anti-ICAM conjugates, such as enzyme cargoes and anti-ICAM itself, is degraded by acidic proteases [106].

Therefore, both the mechanism of uptake of anti-CAM conjugates (called CAM-mediated endocytosis) and the

intracellular trafficking differ from classical endocytic pathways [9]. A large body of evidence accumulated in the last decade in experiments *in vitro* and in animal models indicates that PECAM and ICAM represent highly unusual endothelial targets providing either surface anchoring or effective internalization, and the choice can be controlled by the parameters of design of drug delivery system, that is, valence of binding. This feature permits both targeting of drugs that need to be retained on the cell surface (e.g., anti-thrombotic agents) or delivered inside the cell.

Using pharmacological agents interfering with vesicular transport and cytoskeleton permits to deflect the vesicular transport from lysosomal destination and facilitate recycling of the internalized carriers to the cell surface. Finally, recent studies revealed that nanocarriers targeted to ICAM can even go across the cellular barriers (see below). Therefore, CAM-directed targeting provides unprecedented diversity of subcellular destinations in the target cells, which can be controlled by rational design of the drug delivery system and cellular functional status (see below).

3.6. Transcellular Delivery via CAM-Endocytosis. In most vascular beds, with notable exception of organs of the reticuloendothelial system (liver, spleen, and bone marrow), where large openings connect blood vessels with tissue sinuses, endothelial monolayer is a barrier for extravascular drug delivery. The endothelial cells exert their barrier function differently in the distinct regional and phenotypic domains: for example, the blood-brain barrier is notoriously difficult to permeate, whereas postcapillary venules in dermal microcirculation are more permissive for pericellular transport, especially in pathological sites [57, 165–168].

Some ligands of receptors involved in endocytosis via clathrin-coated pits, such as transferrin receptor, [169] and caveoli, such as APP [170–172], are capable of crossing the endothelial barrier. These pathways provide an opportunity for trans-endothelial transport of drug delivery systems with size suitable of these endocytic vesicles (<100 nm). For example, antibodies to caveolar APP undergo fast transport across endothelium, but particles >100 nm do not enter this pathway [75]. There is a discussion whether caveoli merge into “caveosomes” supporting uptake of large particles and at which extent data obtained in static cell cultures reflect this aspect of endothelial physiology *in vivo* [173–175]. However, potential side effects of engaging caveolar determinants must be more fully understood in order to define biomedical utility of this transcellular pathway [28, 176, 177]. In addition, many disease conditions, including inflammation, may affect this pathway [57, 165–168, 178].

In this context, it is intriguing to explore endothelial transport opportunities offered by CAM-endocytosis, the pathway not restricted by size of the objects entering the cell up to the several microns. Dr. Muro and coworkers have recently reported that gastrointestinal epithelial cells, which normally express ICAM, take up anti-ICAM/nanocarriers (~100 nm diameter spheres) via CAM-endocytosis and transport the carriers across the cellular monolayer without cell damage or disruption of intercellular junctions

[179]. Further, this team reported that orally administered anti-ICAM/nanocarriers enter endocytic pathway(s) in the epithelial cells in the gastrointestinal tract and that this process may be differentially modulated by auxiliary drugs that regulate intestinal digestion and peristalsis, opening an opportunity for oral delivery of polymeric nanocarriers into the vascular compartment [180]. It is quite plausible that this transcellular transport pathway operates in the vascular endothelium as well and can be modulated by rational carrier design taking into account parameters of its geometry and affinity, discussed below.

3.7. Targeting Modulation by Parameters of Carrier Design and Biological Factors. Endothelial targeting is governed by hemodynamics, binding parameters of nanocarriers and cellular phenotype. The effect of hydrodynamic factors on targeting has been so far studied mostly for adhesive interactions of spherical particles of given size coated with ligands with immobilized anchoring molecules or cells [139, 141, 181–185]. These models allow quantitative measurements under well controlled conditions at cost of ignoring many physiological factors. Trends revealed in these oversimplified models *in vitro* (e.g., that the binding is optimal at shear stress levels close to that in veins [139]) remain to be systematically and quantitatively validated in the relevant types of vessels [186] and vascular areas in animal studies [187, 188].

Factors controlling binding include ligand affinity, surface density, spatial organization, and orientation of ligand molecules on the carrier surface, as well as surface density, accessibility, and spatial organization of target determinants. Enhancing ligand density beyond saturation level, that is, already providing maximal binding avidity may be problematic economically and negatively impact pharmacokinetics via interfering with the masking effects of polymeric coating. Furthermore, studies with ICAM targeted particles revealed that the highest surface density of a ligand on the carrier may or may not provide optimal congruency with natural clusters of target determinant [189].

The efficacy of targeting of anti-ICAM/nanocarriers to endothelial cells, either quiescent or cytokine-activated is proportional to the antibody surface density in the range of ~20 to ~150 molecules per particle of 100 nm diameter, both *in vitro* [139, 140] and *in vivo* [48]. However, recent study in animals showed that reduction of anti-ICAM density on the nanocarrier somewhat paradoxically increases the selectivity of targeting to the inflamed endothelium, via suppression of basal binding to quiescent endothelial cells expressing a lower level of the target determinant [190]. Figure 3 illustrates this finding that may be of general utility in targeting to cells expressing determinants with rather limited selectivity relative to nontarget tissue.

Carrier size and geometry also modulate targeting. For example, specific uptake in the pulmonary vasculature increased with enlargement of anti-PECAM conjugates from <50 nm to approximately 300–400 nm diameter, likely due to higher avidity resulting from larger number of anti-PECAM copies per particle, but further increasing size of conjugates sharply reduced the ratio of the pulmonary accumulation of

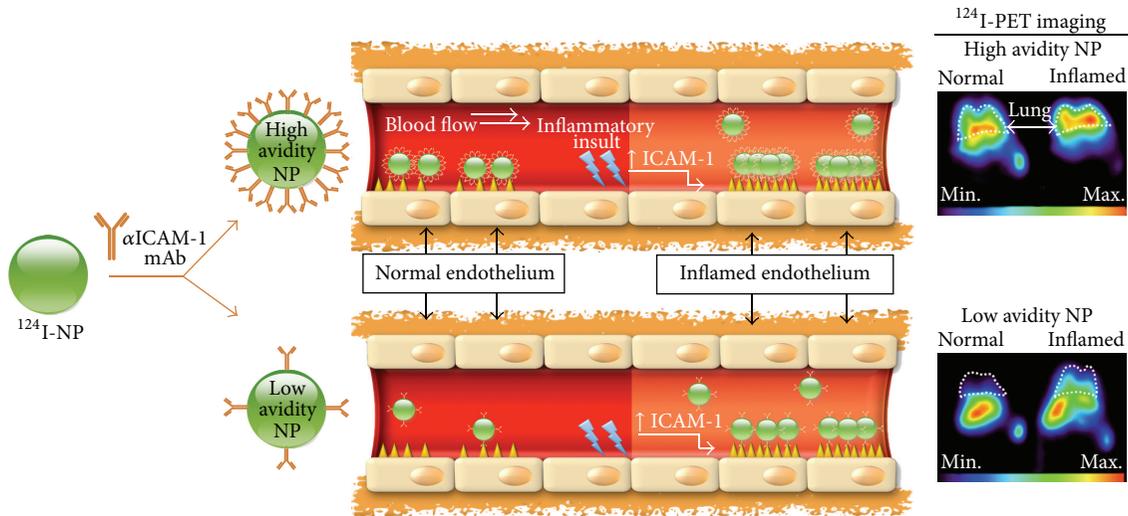


FIGURE 3: Enhanced selectivity of targeting to inflamed endothelium of nanocarriers with reduced surface density of ICAM antibody. One way to achieve the targeting nanoparticles (NPs) carrying drugs and probes to precise locations in the body is to couple to the surface of NPs affinity ligands that bind to target molecules. High ligand surface density favors multivalent binding of NPs to target, yet in some cases reduction of ligand density helps to optimize the targeting, as illustrated by detection of the inflamed pulmonary vasculature by isotope-labeled NPs directed to ICAM-1. After intravenous injection in mice, the specific pulmonary uptake of anti-ICAM/carrying NPs directly correlated with NP avidity controlled by ligand surface density. However, in comparison with high-avidity NPs, low avidity NPs exhibited several fold higher selectivity of targeting to the inflamed pulmonary vasculature of endotoxin-challenged versus naive mice. As a result, low avidity NPs provided more selective PET imaging detection of pulmonary vascular inflammation in endotoxin-challenged mice. Computational modeling revealed that the inflammation-induced elevation in surface density of ICAM-1 in the endothelium is critical for multivalent anchoring of NPs with low ligand density, while high-ligand density NPs anchor to both quiescent and inflamed endothelium. From Zern et al. [190].

CAM-targeted versus nontargeted carriers, which characterizes the specificity of targeting, presumably due to nonspecific entrapment in small vessels [191]. Of note, nonspherical carriers—disks and filomicelles have higher specificity of ICAM directed endothelial targeting in mice as compared to their spherical counterparts [149, 192].

Internalization is also modulated by carrier geometry and selection of epitopes on the target determinant. For example, ICAM targeted disks enter endothelial cells more slowly than spherical carriers of similar size, whereas pace of traffic through the vesicular compartments was controlled by size: smaller particles arrived to the lysosomes faster, regardless of their shape [149]. Spherical nanocarriers directed to certain PECAM epitopes do not enter the endothelium, whereas binding to adjacent epitopes results in rapid uptake [46]. Furthermore, the residence time in endosomal compartment varied dramatically among PECAM antibodies to several adjacent epitopes, providing equally effective endocytosis of spherical nanocarriers [46].

The functional status of endothelial cells and their microenvironment modulate CAM-endocytosis. Cytokine-activated endothelium internalizes ICAM-targeted nanocarriers more actively than quiescent cells [193]. Studies in flow chambers revealed that prolonged exposure to flow leads to partial, yet significant inhibition of endocytosis of nanocarriers targeted to ICAM and PECAM, likely due to reorganization of the cytoskeleton associated with cellular adaptation to flow [193, 194]. These results obtained *in vitro* correlated with

in vivo results showing more effective internalization of anti-ICAM/nanocarriers in capillaries relative to arterioles [193]. In contrast, exposure to acute shear stress (which happens in reperfusion) accelerates endocytosis of PECAM-targeted nanocarriers, likely due to mechanical stimulation of the signaling mechanism [194]. Figure 4 illustrates this principle that should be taken into account in design of endothelial drug delivery and may operate with molecules other than PECAM.

4. Translational Targeting to Endothelial Adhesion Molecules

Almost two decades passed since initial prototype studies of drug targeting to endothelial adhesion molecules. Types of cargoes delivered to endothelial CAMs using diverse drug delivery systems included small chemical drugs [195], biotherapeutics, and imaging agents [145]. Drugs conjugated with anti-CAM exert therapeutic effects superior to untargeted drugs in cell cultures [57, 116, 117, 152], perfused organs [63, 116, 196], and animal models of human pathology [121, 196]. Furthermore, ICAM antibodies have recently been tested in model systems for targeting to pathological vascular wall of stem cells modified either by chemical conjugation [197] or by dual-targeted anti-ICAM/anti-CD34 immunoliposomes serving as the bridge between target and delivered cells [198]. This section overviews CAM-directed targeting of several types of therapeutic and imaging cargoes, with focus

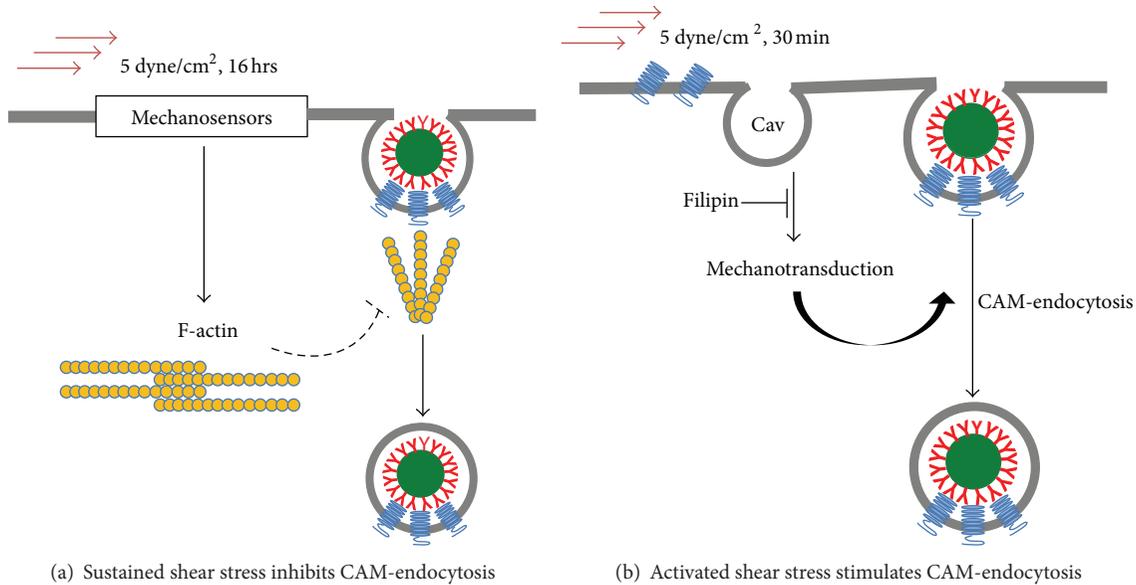


FIGURE 4: Chronic and acute flows differently regulate endocytosis of anti-PECAM/NC. Intracellular delivery of CAM-targeted nanocarriers (anti-CAM/NC) is controlled by their design and target cell phenotype, microenvironment, and functional status. Endothelial cells (EC) *in vivo* are constantly or intermittently (during ischemia-reperfusion) exposed to blood flow, which influences carrier-target interactions by changing NC transport properties and/or by direct mechanical effects upon the mechanisms involved in NC binding and uptake. EC adaptation to chronic flow, manifested by cellular alignment with flow direction and formation of actin stress fibers, inhibited anti-PECAM/NC endocytosis consistent with lower rates of anti-PECAM/NC endocytosis *in vivo* in arterial compared to capillary vessels (a). In contrast, acute flow without stress fiber formation, stimulated anti-PECAM/NC endocytosis (b). PECAM cytosolic tail deletion and disruption of cholesterol-rich plasmalemma domains abrogated anti-PECAM/NC endocytosis stimulation by acute flow, suggesting complex regulation of a flow-sensitive endocytic pathway in EC. Schema illustrates the tentative mechanism for this phenomenon: (a) sustained exposure of EC to flow induces formation of actin stress fibers involved in cellular alignment, which impairs recruitment of actin in the fibers needed for endocytosis of Ab/NC and (b) acute exposure of EC to flow stimulates endocytosis of Ab/NC likely via mechanisms involving cholesterol-rich domains of plasmalemma such as caveolae (cav). From Han et al. [194].

on the studies in animal models positioned for translation into the clinical domain.

4.1. Targeting Imaging Agents. Countless studies employed detection of inducible adhesion molecules in tissue samples (e.g., Western blotting and PCR) as markers of vascular inflammation [199]. Arguably, their detection on the luminal vascular surface in real time in intact organisms using noninvasive imaging techniques would be more clinically valuable. This is an area of active research and translational efforts (Figure 5). Using labeled ligands of endothelial adhesion molecules and ligand-directed nanocarriers enabled imaging of vascular inflammation in animals models of lung I/R, acid aspiration, systemic cytokine challenge [118, 120, 200], local cytokine insult, and atherosclerosis models [201–203].

Modalities for imaging of endothelial adhesion molecules include nuclear medicine, magnetic, ultrasound, and optical methods. Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are highly sensitive techniques that with spatial resolution of millimeters detect gamma-rays emitted from radionuclide probes. Magnetic resonance imaging (MRI, using nonionizing radiation generated from an electromagnetic field) is less sensitive, but offers submillimeter resolution. Ultrasound (US) imaging is widely available, inexpensive, and radiation-free, and new

contrast agents help to improve its sensitivity and spatial resolution. An analysis of these modalities in the context of vascular imaging was reviewed recently [45]. Carriers for the contrast include liposomes, echogenic liposomes, polymeric particles, antibody conjugates, and gold and magnetic particles [204–206].

4.1.1. Imaging of Selectins and VCAM-1. Inducible adhesion molecules have mostly been imaged using targeted nanoparticles providing contrasts for MRI and ultrasound (US). For example, ultrasmall superparamagnetic iron oxide (USPIO, diameter ~50 nm) targeted to E-selectin provided stable MRI contrast in a model of TNF-induced inflammation of the mouse ear [207] and in a rat model of traumatic brain injury [208]. MRI-contrast nanoparticles coated with the natural sLe^x ligand binding to E- and P-selectin provided fourfold increase in signal versus non-targeted particles in a rat model of brain inflammation induced by interleukin-1 β administration [209]. Magnetic nanoparticles coated with P-selectin binding peptide provided a similar enhancement of MRI signal in a mouse model of poststroke inflammation [210].

Intravital microscopy revealed accumulation in the site of vascular inflammation of anti-VCAM targeted magneto-optical particles injected in a mouse 24 h after induction of

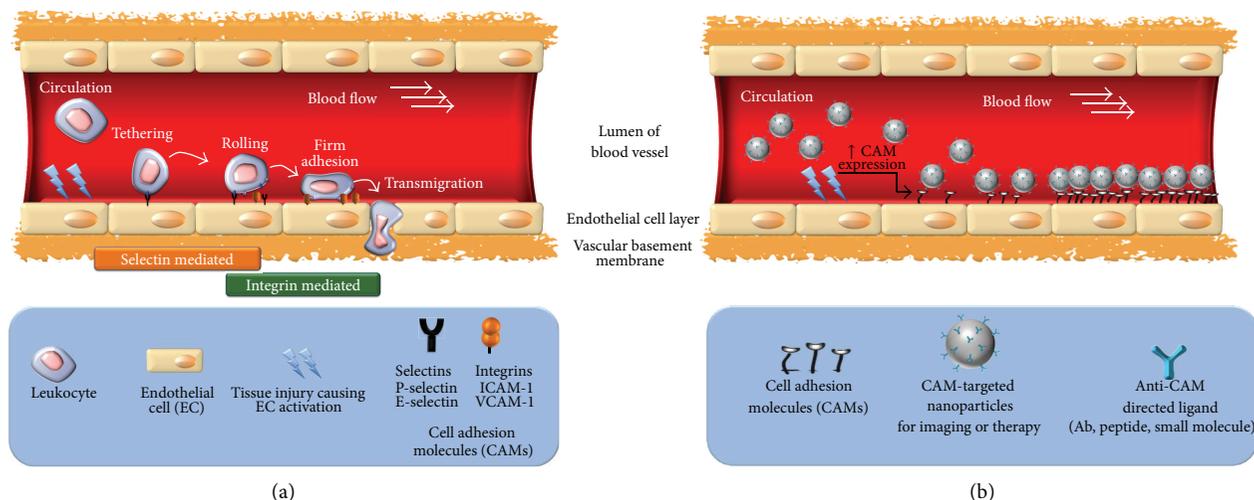


FIGURE 5: Cell adhesion molecule (CAM)-mediated approach to the delivery of diagnostic and/or therapeutic nanoparticles to injured vascular endothelium in early inflammation. (a) Leukocyte adhesion and transmigration mediated by CAMs resulting in propagation of inflammation; (b) imaging or therapy of vascular sites of early inflammation via delivery of CAM-targeted nanoparticles. From Chacko et al. [45].

focal inflammation in a ear with a subcutaneous injection of TNF [211]. Several generations of targeting peptides with homology to a natural ligand of VCAM-1 have been identified by phage display and coupled to the magneto-optical particles [85, 203]. The first peptide identified, termed VP, has 12-fold higher binding to VCAM-1 relative to anti-VCAM-1 and intravital microscopy in the previous mouse model showed accumulation of particles in the inflamed tissue relative to control particles and noninflamed tissue [85]. This and other VCAM-1 binding peptides provided enhancement of MRI signal from sites of vascular inflammation in the previous model and in the aortic arch lesions in a mouse model of atherosclerosis (apolipoprotein E (*apoE*^{-/-}) KO mouse on a high-cholesterol diet) [203].

Larger magnetic particles (1 μm diameter) targeted to VCAM-1 provided detectable enhancement of MRI signal in mouse models of acute cerebral inflammation induced by TNF [212] and ischemia/reperfusion [213]. Submicron VCAM-1 targeted perfluorocarbon nanoparticles provided a 4-fold increase of MR signal in the kidneys reflecting renal inflammation in *apoE*^{-/-} mice in good correlation with the increase in VCAM-1 expression in the organ [214]. In addition to MRI-based detection methods, VCAM-1 targeted microbubbles [215] and liposomes [17] are pursued for atherosclerotic plaque imaging using ultrasound.

4.1.2. Imaging ICAM-1. ⁶⁴Cu-labeled nanoparticles (diameter ~100 nm) coated with anti-ICAM provided specific (versus control IgG coated particles) PET imaging of pulmonary vasculature in normal rats, and the signal was further elevated few hours after LPS injection [200]. Quantum dots (QD) conjugated with anti-ICAM or anti-VCAM-1 provided a 5-fold increase in vascular retinal fluorescence versus control IgG-coated nanoparticles one hour after injection in a rat model of diabetes [216]. ICAM targeted MRI contrast agents

have been used for imaging stroke in animals [217], and US-contrast liposomes targeted to ICAM or VCAM-1 injected in pigs with a chronic model of atherosclerosis enhanced contrast in the vascular lesions by ~40% compared to control untargeted particles [17].

As discussed previously, controlled reduction of anti-ICAM surface density on the nanocarrier helps to suppress binding to normal endothelium to the basal level, while retaining significant binding to pathologically activated endothelium expressing high surface density of ICAM. The utility of this approach to boost vascular selectivity for drug delivery remains to be appraised (reduced level of binding may impede dosing) but seems an attractive avenue for imaging purposes, where selectivity is a higher priority than the dosing.

Coupling of a contrast agent to the ligands or ligand-carrier compound with preserved functionalities is a challenging task. One reason for the artifacts, for example, is conjugation of isotopes and other tracers to the components of the ligand-carrier complex that get easily detached *in vivo*. In contrast, a direct labeling of stable polymeric backbone of anti-ICAM/nanocarriers permits their PET imaging and visualization of pulmonary inflammation in animals free of this artifact [218].

4.2. Targeting Antiinflammatory Agents. Endothelial CAMs are logical target for delivery anti-inflammatory agents (AIA). This section briefly discusses this translational direction of research with focus on AIAs whose effects either require or can be drastically improved by targeting. For example, improved delivery reducing systemic dose of glucocorticoids may help alleviate their side effects including hypertension, hyperglycemia, osteoporosis, and adrenal insufficiency. Currently, steroids are used mainly as a bridging therapy for the acute phase of chronic conditions such as rheumatoid

arthritis. They have a complex mechanism of action involving interaction with diverse targets in both the cytosol and nucleus, and improving their delivery into the endothelial cells may lead to more potent and specific effects.

Dexamethasone- (Dex-) loaded liposomes conjugated with RGD peptide accumulated in LPS-induced inflammatory sites and provided protective effects superior to non-targeted Dex liposomes in a rat adjuvant-induced arthritis model [219]. In a glomerular inflammation model, (Dex-) loaded liposomes coated with E-selectin antibody exerted ~4-fold greater uptake in inflamed kidneys versus non-targeted liposomes and alleviated inflammatory markers by 60–70% relative to controls, with negligible side effects typically associated with bolus Dex [220]. In a mouse model of autoimmune eye inflammation, Dex-loaded liposomes coated with a natural selectin ligand, sLe^x, displayed selective accumulation at the inflamed eye within 5 min of iv injection and suppressed expression of pro-inflammatory genes in the tissue, whereas non-targeted liposomes showed negligible accumulation and effect [221]. Targeting to E-selectin improved delivery of Dex-liposomes to activated dermal and renal endothelium in animal models of inflammation of skin [222] and kidneys [223]. In the latter model, E-selectin targeted Dex-liposomes were shown to reduce glomerular expression of pro-inflammatory genes and proteins and renal injury without affecting blood glucose level [223].

Liposomes carrying anti-VCAM and loaded with an anti-inflammatory prostaglandin, PGE₂, were administered daily for 2 weeks in genetically modified mice susceptible to atherosclerotic showed ~50% higher uptake in inflamed sites versus untargeted liposomal formulations, and, quite astonishingly, reversed atherosclerotic lesions to the extent that mutant mice survived to old age despite being fed a high-fat diet [224].

RGD-targeted liposomal delivery of anti-inflammatory siRNA to the endothelium was also studied in mice [225]. E-selectin- and ICAM-targeted nanoparticles carrying siRNA silencing inflammatory mediators suppressed their expression in cell culture [226]. Double stranded small interfering RNA (siRNA) silences gene expression via sequence-specific destruction of complementary message RNA, but to achieve therapeutic knockout of pro-inflammatory proteins, effective siRNA delivery into the cytosol of target cells is necessary [227, 228]. Several siRNA-loaded lipid and cyclodextrin-based NCs have reached clinical trials, mostly for oncological purposes [229].

Cationic lipid-based formulations of siRNA targeted to E-selectin silenced VE-cadherin in activated endothelial cells *in vitro* [230]. Furthermore, adenovirus targeted to E-selectin homed to the glomerular microvasculature and suppressed expression of adhesion molecules in a mouse model of glomerulonephritis [226]. Targeting to selectins favors endocytosis, whereas using membrane permeating moieties and pH-dependent disruption of intracellular vacuoles may enhance the efficacy of siRNA transfer from endocytic vacuoles to the cytosol, the major challenge for siRNA delivery. However, the toxic effects of endosomal disruption may complicate management of inflammation. Design of NCs for safe and effective delivery of siRNA and other nucleic acid agents

is a rapidly evolving area and the subject of large investments, providing hope for their utility not only in inflammatory conditions but also in other areas of biomedicine [231, 232].

4.3. Targeting Antioxidant Agents. Excessive levels of reactive oxygen species (ROS) superoxide anion O₂^{•-} and H₂O₂ cause vascular oxidative stress [11–13, 15]. Activated leukocytes release ROS causing tissue damage. Endothelial cells also produce ROS via enzymatic systems including NADPH oxidases (Nox) [233]. Cytokines activate endothelial Nox that releases O₂^{•-} in the endosomes, where this ROS is implicated in inflammatory signaling [199, 234–237]. Angiotensin II also activates Nox to produce excessive O₂^{•-} in the vasculature, where it quenches a vasodilatory agent NO, producing toxic species peroxynitrite ONOO⁻ and causing hypertension [238, 239]. Detoxification of endothelial ROS is an important goal [39, 42].

Antioxidant enzymes (AOE) superoxide dismutase (SOD) and catalase are good candidates for alleviation of acute oxidative stress, on the condition that their delivery can be improved [38, 39]. Covalent modification by PEG, loading in liposomes and experimental gene therapy improved AOE delivery and effects in animal models of vascular oxidative stress [240–246]. Thus, PEG-based “stealth” technology improves bioavailability of biotherapeutics [247]. PEG chains coupled to a protein or a carrier enhance aqueous solubility and form hydrated shell inhibiting interactions with cells and defensive proteins [248]. Conjugation with PEG [249] or PEG-based pluronic [250] and loading into PEG-nanocarriers [251, 252] prolong AOE circulation [253], enhancing their systemic bioavailability and protective effects in animal models of stroke [254], chronic noninfectious inflammation [255–259] and radiation lung injury [260].

To optimize cellular binding, formulations including SOD mimetics [261–263], mutant SOD binding to the endothelial glycocalyx [264, 265], AOE fused with membrane permeating peptides [266], and cell transfection by AOE genes [267] have been designed and reported to alleviate oxidative stress in cell cultures and, at more limited extent, in animal models [268, 269]. Lecithin-modified SOD (PC-SOD) binds to some cell types including endothelial cells *in vitro* and was protective in several animal models of human pathologies including myocardial infarction, colitis, and tumor growth [270–272]. A recombinant fusion of mitochondrial MnSOD (SOD2) and heparin-binding domain of EC-SOD (SOD3) has been synthesized; this SOD2/3 chimera binds to cellular glycocalyx and alleviates vascular dysfunction in models of myocardial ischemia [257, 273].

However, the endothelial delivery of these agents remains suboptimal and did not provide controlled delivery needed for interception of intracellular ROS [274]. The endothelial uptake of PEG-AOE is similar to that of naked AOE [199]. Intratracheal delivery of AOE, PEG-AOE and transgenic expression of AOE alleviated oxidative stress in the airways but not in the lung vasculature [241, 275]. AOE location is the key: ROS activities in tissues occur on the nanometer scale and precise delivery of AOE into desirable cells and

their compartments is needed. Non-targeted delivery systems simply cannot achieve such a precision.

Targeting antioxidants to endothelial CAMs offers an avenue to achieve this challenging goal. Initial studies in endothelial cell cultures supported this notion [276, 277]. Little was known about endothelial adhesion molecules thirty years ago, and those prototype studies employed admittedly primitive polyclonal “anti-endothelial” antibodies, in audacious anticipation that “targeting must be achieved using highly specific monoclonal antibodies, capable, like white blood cells, to identify abnormal endothelium” [276].

Fulfilling these expectations, SOD and catalase conjugated with anti-CAM, but not naked AOE or PEG-AOE, bind to and enter endothelial cells and quench corresponding ROS [116, 278], conferring immediate protective effect that lasts for several hours after a single dose delivery [152]. Anti-CAM/AOE conjugates provided antioxidant effects superior to non-targeted AOE formulations including PEG-AOE in models of acute pulmonary vascular oxidative stress caused by infusion of ROS or ischemia-reperfusion [32, 63, 122, 196, 279].

Anti-PECAM/SOD alleviated toxicity of extracellular and intracellular $O_2^{\bullet-}$ in cell culture [278, 280], alleviated angiotensin-II-induced vasoconstriction in mice [279], and inhibited cytokine-induced endothelial ROS flux and VCAM-1 expression in cells and mice via quenching of $O_2^{\bullet-}$ signaling in endothelial endosomes (Figure 6) [199]. Anti-PECAM/catalase normalized elevation of endothelial permeability caused by H_2O_2 [281], while anti-PECAM/SOD attenuated VEGF-induced endothelial barrier dysfunction, implicating $O_2^{\bullet-}$ in this type of pathological redox signaling [281]. Ab/catalase alleviates lung ischemia-reperfusion [196, 279] and vascular oxidative stress [121]. Initial success in protecting lungs against oxidative stress in transplantation achieved in lab rodents using anti-ICAM/AOE [60] and anti-PECAM/AOE [196, 279] has been translated from rats to larger animal species and more realistic models including warm ischemic period [64, 68]. Non-targeted AOE formulations including PEG-AOE provided no effect in these studies even in cell cultures, due to lack of delivery to the site of ROS influx and effect.

Targeting AOE to endothelial endosomes via CAM-endocytosis enables protective mechanisms unavailable to non-targeted AOE, including interception of the endosomal superoxide [199, 281, 282]. Yet, the subsequent lysosomal delivery that leads to degradation of AOE [283] terminates the protective effect within few hours after internalization [117]. Drugs affecting lysosomal trafficking and degradation prolong the protective effects of anti-ICAM/catalase [117, 283]. As an alternative bioengineering approach, catalase has been encapsulated in polymer nanocarriers permeable for ROS but not to proteases [284–286]. PECAM antibody conjugated to AOE-loaded nanocarriers delivered catalase into endothelial endosomes and lysosomes, where polymeric shell protected catalase from proteolysis, allowing detoxification of ROS diffusing through the polymer shell and prolonged antioxidant protection *in vitro* and in animal models (Figure 7) [148].

Targeted delivery of inhibitors of enzymes producing ROS may provide an interesting alternative or additive strategy for ROS detoxification by antioxidant enzymes. This notion has been tested using anti-PECAM/liposomes loaded with a small lipophilic agent MJ33, an indirect inhibitor of Nox: studies in cell cultures and in mice showed that PECAM-directed targeting of MJ33 markedly enhances its endothelial delivery and antioxidant effects *in vitro* and *in vivo*, providing effective alleviation of LPS-induced acute pulmonary inflammation in animal studies [287].

4.4. Targeting Enzyme Replacement Therapies (ERT). In case of antioxidant enzymes (and many other biotherapeutics), lysosomal destination is a problem, unless the cargo is protected from degradation. In contrast, lysosomal destination is necessary for drugs that are either activated in this organelle or act upon lysosomal targets. From this standpoint, CAM-mediated endocytosis offers a natural pathway for delivery of such drugs into endosomes and subsequently lysosomes. Capitalizing on this discovery, Dr. Muro had pioneered a new targeting strategy for improved delivery of clinically used recombinant enzyme replacement therapies (ERT) for lysosomal storage diseases, LSD [138].

The LSDs represent pathological manifestations of dysfunction of lysosomal hydrolases, in most cases due to genetically transferred mutations, leading to accumulation of the enzyme substrate in the lysosomes and subsequently in other compartments. This metabolic disorder results in cellular abnormalities throughout the body (nervous system usually is the major pathological site), in severe cases associated with high morbidity and premature mortality [288–290]. Vascular endothelium also suffers damage and dysfunction in LSDs, which aggravates inflammation and the injury to other tissues [138].

The enzyme replacement therapy relies on repetitive injections of recombinant form of dysfunctional enzyme [291–293]. Cells bind and take the ERT into the endosomes-lysosomes via mannose and/or mannose-6-phosphate receptors [294–296]. In the absence of gene therapy, the ERT provides the only treatment of lysosomal diseases, more successful in the conditions with modest neurological and marked peripheral components [293, 297], such as type B Niemann-Pick disease (NPD), caused by a mutation of acid sphingomyelinase (ASM), resulting in pathological accumulation of sphingomyelin and cholesterol in the cellular vesicles and membranes [298].

However, delivery of ERT to certain cell types including the endothelium is not efficient for several reasons including low affinity of the enzyme binding to these cells. As a result, management of vascular and pulmonary pathological changes in NPD-B and other LSDs involving endothelium is less effective than in some other organs including liver, heart, and spleen [297, 299, 300]. In addition, proper glycosylation of recombinant ERT represents still not fully resolved biotechnological challenge, whereas expression and functions of mannose and mannose-6-phosphate receptors are generally suppressed in cells suffering LSD, which further impedes intracellular delivery of ERT. In order to overcome

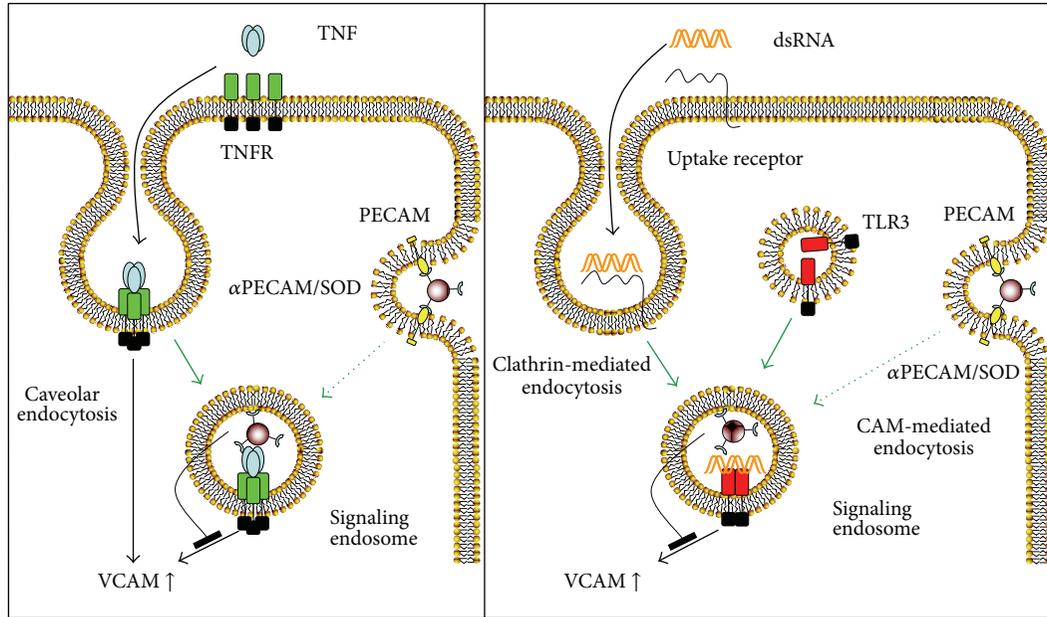


FIGURE 6: Intracellular delivery of anti-PECAM/SOD inhibits proinflammatory signaling of superoxide anion produced in endothelial endosomes in response to inflammatory mediators. Elevated generation of superoxide by endothelial enzymes including NADPH-oxidase is implicated in vascular oxidative stress and endothelial pro-inflammatory activation leading to exposure of VCAM-1. Anti-PECAM conjugated SOD and catalase bind specifically to endothelium and inhibit effects of their ROS substrates. Anti-PECAM/SOD, but not anti-PECAM/catalase or non-targeted enzymes including PEG-SOD, inhibited VCAM expression caused by tumor necrosis factor (TNF), interleukin- 1β , and LPS. Anti-PECAM/SOD, but not non-targeted counterparts, accumulated in vascular endothelium after intravenous injection, localized in endothelial endosomes and inhibited LPS-caused VCAM-1 expression in mice. Anti-PECAM/SOD colocalized with EEA-1 positive endosomes and quenched ROS produced in response to TNF. Anti-PECAM/SOD even more effectively abolished VCAM expression caused by poly(I:C)-induced activation of toll-like receptor 3 localized in intracellular vesicles. Site-specific interception of endosomal superoxide attained by targeted delivery of anti-PECAM/SOD into endothelial endosomes may have anti-inflammatory effects. Schematic representation of proposed action of anti-PECAM/SOD entering endothelial cells via CAM-endoctyosis on pro-inflammatory activation via plasmalemma TNF receptor internalized via caveolar endocytosis (left) and intracellular TLR3 (right). Binding of cytokine to cell surface receptors induces a cascade of events including endocytosis of receptor-ligand complexes via caveolar pathway and inflammatory activation. TLR3 involved in anti-viral defense localized in intracellular vesicles is activated upon the ligand endocytosis via clathrin-mediated endocytosis. Anti-PECAM/SOD enters cells via CAM-mediated endocytosis, and apparently traffics to these specific types of signaling endosomes. For details and explanations, please see Shuvaev et al. [199].

this hurdle, Dr. Muro and coworkers used ICAM as a target for nanoparticles carrying ERTs [301]. ICAM expression by endothelial and many other cell types relevant to NPD-B and other LSDs is upregulated in inflammation typical of many lysosomal disorders [302–305].

Coupling ERT to anti-ICAM polymer nanocarriers (~100 nm diameter spheres) markedly enhanced enzyme delivery and effects in several preclinical models. In initial studies, cellular uptake of ASM was an order of magnitude higher for ICAM-targeted versus ASM-coated particles, in good agreement with qualitatively more effective utilization of fluorescent sphingomyelin to background levels in ASM-deficient cells in comparison with effect of “non-targeted” ASM formulations offering only a partial substrate utilization [301].

Next, investigators demonstrated that lysosomal delivery of ASM by ICAM targeted carriers can be optimized by carrier’s geometry: small spherical (100–200 nm) carriers

offered more effective lysosomal delivery of ASM than discoid or large micron-size spherical particles: whereas micron-size anti-ICAM/ASM carriers provided <10% degradation of sphingomyelin two hours after internalization, small 100 nm counterpart degraded ~50% of the cellular substrate, returning levels of the lipid back to baseline [149].

In order to boost the translational potential of this approach, anti-ICAM/ASM formulation using 100 nm nanoparticles made of biodegradable polymer of poly (lactic-co-glycolic acid) (PLGA) were developed. In ASM knockout mouse model, anti-ICAM/ASM PLGA nanocarriers within 30 min after IV injection accumulated in the lungs and other target organs (spleen, liver, and lung are main targets for type B NPD) at an order of magnitude higher level than free ASM [306].

This approach was adapted for delivering of other lysosomal ERTs including α -galactosidase, or α -Gal. Its dysfunction in Fabry disease causes accumulation of galabiosylceramide

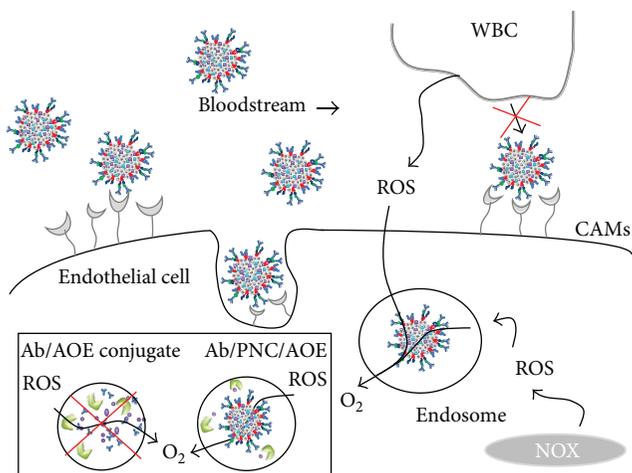


FIGURE 7: Endothelial targeting of antioxidant enzymes (AOE) encapsulated into protective semipermeable polymeric nanocarriers. AOE encapsulated in anti-CAM coated carriers bind to endothelial cells. Depending on the selection of target epitope and size and shape of the carrier, it will either preferentially enter into endosomes (left), or remain on the surface (right), thereby protecting endothelial cells against ROS released by activated white blood cells (WBC). Internalized AOE quenches ROS fluxed by activated NOX into the endosomes, as well as ROS diffusing across the membranes. Upon delivery into lysosome, AOE encapsulated in the carriers permeable to ROS but not proteases retain their enzymatic activity and antioxidant potency for a longer duration than protease-susceptible anti-CAM/AOE conjugates (inset).

and globotriaosylceramide (Gb3) in body fluids and lysosomes, leading to cerebrovascular, cardiac, vascular, pulmonary, and renal impairment. Anti-ICAM/ α -Gal nanocarriers similar in design to ASM delivery system improved delivery of the cargo to lungs and liver in animals by ~30 and 3 times, respectively, versus free enzyme [307]. In a Fabry disease cell model, about 75% of anti-ICAM/ α -Gal nanocarriers were found in the endothelial lysosomes within 3 h and extent of degradation of Gb3 more than doubled versus cells treated with untargeted α -Gal [307]. Finally, this delivery system has been applied to recombinant ERT α -glucosidase, or GAA, the deficient enzyme in Pompe disease in which glycogen storage in lysosomes leads primarily to hepatic and muscular dysfunction. Again, in comparison with non-targeted GAA formulations, anti-ICAM/GAA nanocarriers showed greatly elevated binding, uptake, lysosomal delivery and degradation of glycogen in a Pompe disease cell model, as well as accumulation in mouse organs including lungs, heart, spleen and brain, achieving unprecedented level of ~600-fold increase in the pulmonary uptake versus non-targeted GAA formulations [308].

Very recent studies from Muro's lab further expand basic and applied aspects of this intriguing approach. Thus, ASM delivery via ICAM has been compared *in vivo* with that via transferrin receptor, entering cells via clathrin endocytosis. Interestingly, the latter target was more amenable to delivery by free antibodies than antibody-coated nanocarrier system, whereas ICAM served antibody-coated nanocarrier system

more effectively than free antibody [309]. Furthermore, the double-targeting approach (briefly discussed previously) has been explored for ASM delivery in animal studies. Nanocarriers carrying ASM and targeted by antibodies to both ICAM and transferrin receptor showed different organ distribution versus non-targeted ASM and either of monotargeted carriers [143]. In addition, somewhat unexpectedly, this group revealed that engaging ICAM by multivalent nanocarriers induces ASM activity in target cells, facilitating membrane turnover and endocytosis. Potential utility and significance of these findings are worth further investigations [138].

4.5. Targeting Antithrombotic Agents. Pathologically altered vasculature is predisposed for thrombosis, in part due to suppression of natural anti-thrombotic mechanisms in endothelium [310]. Anchoring of recombinant anti-thrombotic proteins such as thrombomodulin and plasminogen activators (tissue type, tPA, or urokinase, uPA) on the endothelial lumen may help to compensate for this dysfunction. Vascular gene transduction of these proteins in animal models supports this notion [311]. Immunotargeting of anti-thrombotic proteins to the endothelial surface would provide a more practical approach in acute settings, providing thromboprophylaxis in patients with a high propensity for thrombosis, particularly in settings where the risk of bleeding prohibits use of systemic anticoagulation.

In prototype studies, anticoagulant hirudin cross-linked to anti-E-selectin antibody bound to cytokine-activated endothelial cells and inhibited thrombin *in vitro* [312]. In animal studies, tPA and uPA conjugated with anti-ACE and other antibodies to endothelial determinants preferentially accumulated in the pulmonary vasculature after intravenous injection in rats [74, 313]. However, constitutive endocytosis of ligands of these determinants removes the drugs from vascular lumen, where they need to exert their activity, thereby limiting therapeutic effect *in vivo* [314].

PECAM and ICAM, which do not internalize their antibodies, provide good targets for anchoring anti-thrombotic drugs. In support of this notion, after IV injection in rats, pulmonary uptake of anti-ICAM/tPA conjugate was two orders of magnitude higher than that of control IgG/tPA, which resulted in enhanced fibrinolysis of subsequent pulmonary emboli [120]. Thrombin upregulates the expression of ICAM, which provides an additional rationale for its use as a target for delivering anti-thrombotic agents.

Chemical conjugation of proteins to antibodies is a challenging proposition from the translational standpoint. In contrast, recombinant fusion of enzymes with genetically engineered antibody fragments, single-chain Fv (scFv, comprising variable domains of heavy chain V_H and light chain V_L) yields a monovalent, homogeneous, and relatively small bifunctional biotherapeutics. As a proof of principle, an anti-PECAM scFv was fused with urokinase (uPA), and resultant scFv/uPA construct accumulated in the pulmonary vasculature after IV injection in mice, resided in the pulmonary lumen for hours in active form and augmented pulmonary fibrinolysis [116, 127, 196]. Compared with non-targeted uPA, anti-PECAM scFv/uPA more effectively augmented local

lysis of pulmonary emboli in a mouse pulmonary thrombotic model [108]. Further, scFv/uPA accumulated in the cerebral vasculature after intra-arterial and IV injection, dissolved cerebral clots and improved blood reperfusion without hemorrhagic complications, thereby mitigating postthrombotic brain edema in a mouse model of cerebral embolism [123].

Urokinase is produced naturally as an inactive precursor scuPA [315]. 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 uPA. However, the scuPA prourokinase can be activated by trace amounts of plasmin over time, which may cause adverse effects. Further, thrombin inactivates uPA by cleaving Arg156-Phe157, negating its effect at sites of active thrombolysis [316]. These problems might be solved by deleting Phe157 and Lys158, which yields a plasmin-resistant mutant activated by thrombin (uPA-T) [317]. This proenzyme will not be activated by plasmin *in vivo* (thus avoiding systemic effects and premature inactivation by inhibitors), while thrombin will activate it locally at sites of nascent thrombolysis within seconds of clotting.

Replacing the native plasmin activation site in the uPA moiety of scFv/uPA with a thrombin activation site provided thrombin-activated anti-PECAM scFv/uPA-T [318]. This construct was also 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 scFv/uPA-T is latent and resists the PA inhibitor PAI-1 until activated by thrombin (Figure 8). After IV injection in mice, scFv/uPA-T did not consume plasma fibrinogen, in contrast with scFv/uPA that has this liability. However, scFv/uPA-T is bound to the endothelium and accumulated in the vascularized organs, particularly the lungs. In a mouse model of thrombin-induced pulmonary thrombolysis, scFv/uPA-T provided more potent and durable thrombolysis than both plasmin-sensitive scFv/uPA and lmw-scuPA. Further, injection of mice with scFv/uPA-T prior to unilateral lung ischemia/reperfusion attenuated pulmonary fibrin deposition and restored arterial oxygen tension, to a significantly greater extent than plasmin-sensitive scFv/uPA [318].

The suppression of endothelial thrombomodulin (TM, a transmembrane glycoprotein that switches procoagulant and pro-inflammatory effects of thrombin to opposing effects via activation of Protein C) is characteristic of many vascular pathologies including sepsis. Some success has been found with a replacement therapy using soluble recombinant TM and activated protein C (APC) [319]. TM fused with a tissue factor antibody has potent antithrombotic activity in a rat model [320]. Yet, utility of these biotherapeutics is limited by fast disappearance from the vascular lumen. In order to solve this problem, a new anti-PECAM scFv/TM fusion has been produced and shown to bind and reside on endothelial surface, accumulate in the pulmonary vasculature and attenuate thrombolysis and tissue damage in mouse models of lung ischemia-reperfusion and endotoxin-induced acute inflammatory lung injury to a greater extent than non-targeted soluble TM, without causing bleeding (known liability of APC treatment) [321].

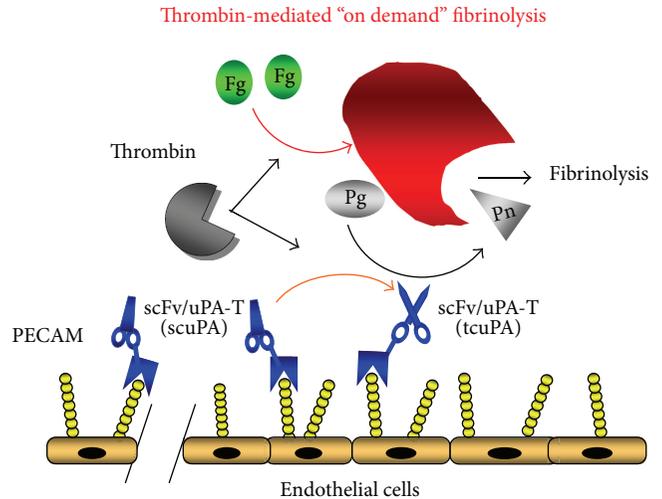


FIGURE 8: "On demand" fibrinolysis by vascular targeting of thrombin-activated anti-PECAM scFv/uPA (scFv/uPA-T). scFv-uPA/T circulates in a prodrug form, single-chain uPA (scuPA), binds to PECAM-1 and remains anchored on the endothelial luminal surface for at least several hours. Upon environmental stress or tissue injury, thrombin is generated to cleave fibrinogen (Fg) and form fibrin clots. While this causes *in situ* thrombolysis, 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 that restores the blood flow and mitigates the tissue ischemia and injury. From Simone et al. [322].

In summary, endothelium-targeted thrombolysis triggered by a pro-thrombotic enzyme illustrates a novel approach to time- and site-specific regulation of "on demand" reactions that can be modulated for therapeutic benefit. In clinical settings, this strategy of targeting anti-thrombotic drugs to the endothelial surface may provide local thrombolysis in patients with an acute risk of developing new or recurrent thrombi and prevent clot extension.

5. Conclusion: Challenges, Opportunities, and Translational Perspectives

Endothelial targeting of drugs directed to cell adhesion molecules has been achieved in intact animals and animal models of human pathologies. ICAM and PECAM are especially versatile anchors for targeting biotherapeutics and probes to diverse endothelial compartments, especially in the context of oxidative stress, inflammation, and thrombolysis. Their functions are fairly well understood, which helps avoid unintentional side effects and achieve secondary benefits in a given pathological context, such as inhibition of leukocyte transmigration in inflammation. Careful selection of targets and modulation of features of the carriers, such as valence and surface density of ligands and carrier geometry, prodrug activation features, and encapsulation into protective polymeric carriers, provide powerful tools for the control of

subcellular addressing, activation, and duration of the effects of the cargoes.

Scaling-up synthesis and quality control of targeted drug delivery systems with a standard, FDA-acceptable level of homogeneity is a challenge for translation of these delivery systems in the clinical domain. Recombinant mutant prodrugs fused with anti-PECAM or anti-ICAM scFv may be more amenable this development. Advantages of using scFv and other affinity peptides include (i) lack of side effects mediated by Fc-fragment including activation of complement and Fc-receptor bearing cells; (ii) lack of endothelial activation and internalization induced by CAM cross-linking; (iii) established techniques for humanization and reduction of immunogenicity of scFv even further minimize the likelihood of eliciting immune reactions; (iv) modular recombinant format for design of targeted variant cargo protein mutants lacking unnecessary domains and insertion of mutations endowing products with novel, favorable pharmacokinetics and/or functional features. Established expression systems enable large-scale, GMP-production of homogeneous monovalent scFv/PA fusions [323, 324].

Avoidance of adverse effects is the top priority in drug delivery to endothelium. From this standpoint, prophylactic application of CAM-targeted drug delivery is well suited for treatment ischemia injury in the organ transplantation or cardiopulmonary bypass. Anti-PECAM scFv achieves peak pulmonary targeting 5 min of IV delivery [127], permitting injection into a donor prior to organ removal; hence, a recipient will receive only the drug bound and eventually metabolized within the graft, which further boosts safety. Efforts invested in devising inhibitors for adhesion molecules so far did not yield effective anti-inflammatory interventions, in part because adhesive pathways are redundant [325, 326]. However, these studies showed that adhesion-blocking peptides and antibodies have relatively benign, if any, side effects in homologous species [326, 327]. It is tempting to hope that in the next decade interventions into endothelial cells targeted via adhesion molecules will be translated from initial successes reported in laboratory animals to medical practice.

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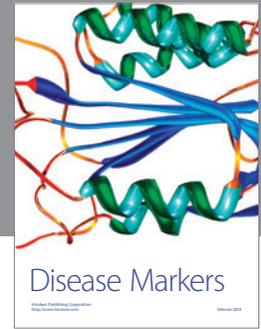
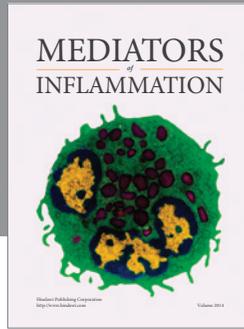
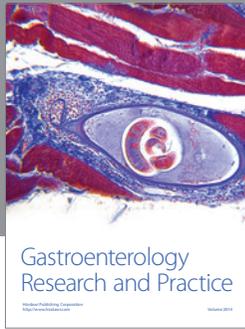
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