Critical Review

Targeted siRNA Delivery to Diseased Microvascular Endothelial Cells—Cellular and Molecular Concepts

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Summary

Increased insight in the role of endothelial cells in the pathophysiology of cancer, inflammatory and cardiovascular diseases, has drawn great interest in pharmacological interventions aiming at the endothelium in diseased sites. Their location in the body makes them suitable targets for therapeutic approaches based on targeted drug delivery. Functional heterogeneity of the microvascular bed in normal organ homeostasis has been appreciated for a long time, and more recent studies have revealed heterogeneity in endothelial reactivity to inflammatory stimuli as well. Upon stimulation, each organ displays a vascular bed specific pattern of cell adhesion molecules providing challenging opportunities to deliver drugs or small RNAs to organ specific (micro)vascular endothelial subsets. In this review we introduce general concepts of endothelial heterogeneity in relation to disease state and its consequences for targeted therapeutic interventions. Furthermore, we will describe novel approaches to interfere with endothelial cell engagement in disease with a main focus on siRNA therapeutics and currently used nonviral lipid and polymer-based siRNA delivery systems. The last part of this review addresses some technical issues that are essential in proving the concept of target mRNA knock down in a vascular bed specific manner, and the further development of effective endothelial cell specific drug delivery devices. © 2011 IUBMB

Keywords drug delivery; siRNA; endothelial cells; liposomes; targeted delivery; intracellular release; nonviral delivery devices.

INTRODUCTION

The endothelium is the cell layer that forms the inner lining of blood vessels. It is a spatially distributed system that extends to all organs and tissues of the body. The endothelium is a key regulator of vascular homeostasis and functions not only as a barrier but also acts as an active signal transducer for metabolic, hemodynamic and inflammatory input that modifies the function and morphology of the vessel wall (1). Moreover, the smallest blood vessels engage in angiogenic processes that accompany wound healing, tissue repair, and solid tumor growth (2). Depending on the location in the body, endothelial cells (ECs) display their own molecular make up that drives basic behavior as well as responses to inciting stimuli (3). Along the vascular tree, major differences are observed in EC phenotype, permeability, endocytosis and transcytosis capacities, and responsiveness to activation. For example, brain microvasculature is an integral part of the impermeable blood brain barrier, whereas liver sinusoidal endothelial cells form a densely fenestrated sieve with support of a discontinuous basement membrane and engage in clearance of a variety of molecular entities from the circulation (4, 5). Furthermore, endothelial cells aligning the postcapillary venules are primarily responsible for mediating leukocyte trafficking, whereas arteriolar endothelial cells regulate vasomotor tone (2).

Being a keeper of internal homeostasis, ECs are continuously sensing and responding to changes in the extracellular environment. They are the first cells exposed to proinflammatory stimuli associated with systemic diseases such as atherosclerosis, sepsis, diabetes, vasculitis and other (chronic) inflammatory disorders. The proinflammatory stimulus leads to activation of ECs, the status of which varies according to the nature of the activating factor and the location of the vascular bed (6). For example, significant differences in response to proinflammatory stimuli such as tumour necrosis factor (TNF)-α are observed...
between venous and arteriolar endothelial cells (7). Activated endothelium also accompanies the outgrowth of many tumors, where sustained formation of new blood vessels is one of the key factors leading to progression of the disease (8).

A variety of pharmacological approaches to counteract endothelial activation are already applied in the clinic, tested in clinical trials, or in preclinical development, including the potent inhibitors that affect receptor tyrosine kinase activation as well as specific kinases involved in the various signal transduction cascades (9). Besides kinase inhibitors, drugs based on RNA interference (RNAi), i.e., small interfering RNAs (siRNAs), offer increased specificity and efficient gene silencing of disease-associated genes. Formulation of such drugs in a targeted delivery system would create potentially powerful gene silencing therapeutics with diminished side effects, and hold a great promise for successful treatment of chronic inflammatory diseases and cancer. In the current review, we will focus on recent developments in the design of siRNA delivery approaches with the aim to therapeutically affect abnormal endothelium. We will introduce general consideration of endothelial heterogeneity in relation to disease state and its consequences for targeted therapeutic interventions. Next we will focus on drugs based on RNA interference, on their mechanisms of action and obstacles limiting application of siRNA in the clinic. We will provide an overview of currently used siRNA delivery systems designed to interfere with endothelial cell engagement in disease, with emphasis on nonviral approaches including lipids and polymers. In the last part, we will discuss the in vivo complexity of endothelial cell behavior and the difficulties encountered when attempting to mimic this in an in vitro setting. This calls for the use of new technologies that allow for endothelial gene expression analysis and studying targeted drug delivery devices in the complex environment of an organ.

**Endothelial Heterogeneity and Abnormal ECs as Therapeutic Target**

In recent years, the endothelium has become an attractive target for therapeutic intervention by virtue of its association with the pathophysiology of many diseases, its prevalence throughout the body, and its accessibility to intravenously administered agents (10). Much effort has been dedicated to the development of drugs that inhibit endothelial cell activation to treat chronic inflammatory diseases, to disrupt tumor vasculature, or to halt angiogenesis. However, most of the drugs lack specificity for the endothelium, giving rise to adverse effects in other cells in the body. Formulation of highly potent drugs in EC specific delivery devices will be essential to provide these drugs with a potential for future clinical application (9, 11).

Critical for success of these approaches is the identification of target epitopes on the diseased endothelial cells as well as choosing the proper drug target and concurrent molecular entity for therapeutic effects. This justifies an approach in which knowledge of microvascular endothelial cell biology and pharmaceutical sciences are combined, as we do in our own research as well as in outlining the content of this review.

Endothelial cells are differentially regulated in diverse (micro)vascular beds and in time, giving rise to the phenomenon of endothelial cell heterogeneity. Structural and functional heterogeneity of endothelial cells is evident between arteries and veins as well as between the capillaries in the different organs. Not only do different endothelial cell subsets in one organ have a different phenotype and function related to organ physiology, they also behave differently under pathophysiologic stress. As a consequence, endothelial cells in various (micro)vascular beds express different proteins at different moments in time during disease initiation and development (4). The differentially expressed determinants on the surface of disease-activated endothelium are excellent targets for drug delivery towards abnormal endothelium, and include molecules involved in leukocyte rolling and adhesion to the vascular wall during the inflammatory process, and in other disease–related processes (12). It should be noted, however, that many of these proteins are not homogeneously expressed by the endothelium but rather are (micro)vascular bed specific. Moreover, the kinetics of disease-induced target epitope expression may spatiotemporally differ between endothelial cell subsets in the diseased sites (13). This could have either an advantageous or a detrimental effect with regard to specificity and/or extent of local accumulation of the drug delivery formulation.

**Endothelial Adhesion Molecules as Targets for Inflamed Endothelium**

Their position in the body makes the ECs one of the first cells to be exposed to systemic proinflammatory stimuli such as bacterial endotoxin (lipopolysaccharide, LPS), or systemically released cytokines such as TNF-α, interleukin (IL)-1, and IL-6. Exposure to these proinflammatory conditions leads to EC activation and expression of cell adhesion molecules and various other molecules associated with proinflammatory activation. Several of them can serve as molecular targets for siRNA delivery in pathologies such as atherosclerosis [vascular cell adhesion molecule-1 (VCAM-1), (14)], myocardial injury [P-selectin, (15)], glomerulonephritis [E-selectin, (16)]; rheumatoid arthritis [z1β1 integrin, E-selectin (17, 18)], and pulmonary inflammatory diseases [intercellular adhesion molecule-1 (ICAM-1), (19)].

Ideally, target epitope expression should be restricted to diseased endothelium, thereby preventing accumulation of a drug in nondiseased endothelium. E-selectin is one of the few molecules that meet this criterion. Moreover, it is not present on nonendothelial cells and its expression is dramatically upregulated during inflammation in vivo. The endothelial adhesion molecule expression induced by systemic stimuli varies between organs according to the nature of the stimulus and the origin of the vascular bed. Each organ displays a unique pattern of molecules providing further challenging opportunities to deliver drugs or small RNAs to organ specific (micro)vascular endothe-
For example, van Meurs et al. observed E-selectin expression primarily in glomerular ECs in a study on microvascular activation following induction of systemic inflammation in a hemorrhagic shock mouse model. In contrast, VCAM-1 expression was induced in all vascular segments except in glomerular ECs (20).

Furthermore, it is important to note that target epitopes should reside at the exterior of the cell membrane of the target subsets (Fig. 1A). For example, van Meurs et al., observed E-selectin expression primarily in glomerular ECs in a study on microvascular activation following induction of systemic inflammation in a hemorrhagic shock mouse model. In contrast, VCAM-1 expression was induced in all vascular segments except in glomerular ECs (20).

Furthermore, it is important to note that target epitopes should reside at the exterior of the cell membrane of the target...
cells, that they are not avidly shed, and that they become internalized upon ligand binding and intracellularly processed when intracellular drug release is a prerequisite. E-selectin is an internalizing receptor that routes its ligands including antibodies and antibody modified-liposomes to the lysosomal compartment (21–23). This feature substantially contributes to its outstanding quality as a target to be exploited for intracellular delivery of small RNAs. Also VCAM-1 and ICAM-1 are internalizing receptors (24) and although their expression is not restricted to endothelial cells in inflamed sites, they can still be considered attractive targets, provided that the therapeutic advantages outweigh the undesired side effects of delivery of the drug into nondiseased endothelium.

Integrins αvβ3 and αvβ5 are interesting molecules to consider for targeted drug delivery to ECs as they are upregulated in physiological as well as pathological angiogenic vessels, and possess proangiogenic functions (11, 25, 26). For example, peptides with the arginine glycine aspartic acid (RGD) amino acid sequence show high affinity for the αvβ3-integrin, as do αvβ3 specific antibodies. During the past decade, RGD-peptides have become an established tool for the targeting of drugs and imaging agents to αvβ3-integrin expressing ECs (27).

**Small Interfering RNAs, a New Class of Therapeutics**

Three decades ago, RNA was generally considered to be no more than just a passive intermediate transferring information from DNA to the protein-synthesizing machinery. The discovery of catalytic RNAs in the 1980s had a tremendous impact on this dogma. Small RNAs are key players in triggering post-transcriptional or translational gene silencing and nowadays they represent one of the most promising new classes of molecular target-specific therapeutics. There are two main groups of small RNAs, i.e., siRNA and micro RNA (miRNA), which are short double-stranded RNA (dsRNA) molecules generally composed of 21-23 (siRNA) or 18-25 (miRNA) nucleotides (nt). siRNA generally has perfect complementary sequence to its target mRNA, leading to gene-specific degradation of the mRNA, in contrast miRNA has predominantly imperfect sequence complementarity in the 3’ untranslated region of the target mRNA which leads to translational silencing without mRNA degradation (28, 29). Mostly chemically synthesized siRNA molecules are used to silence target gene expression, exploiting the cells’ endogenous RNAi processing machinery for further processing before hybridization with its target mRNA. Another method of mediating the RNAi effect involves exogenously administered vector-based short hairpin RNA (shRNA) which is transcribed in the nucleus, further processed and transported to the cytoplasm for silencing activity (30). Although a vector-based shRNA system may have advantages such as robust and long term gene silencing in the transfected cell, its expression is hard to control with regard to length of time and efficiency. Moreover, vector-based shRNA require nuclear entry, which represents an additional hurdle in the overall mechanism of action. The use of exogenous siRNA results in direct gene silencing since it does not require additional processing. Furthermore, its effect is transient, which may be preferred in a therapeutic setting.

RNAi has become a widely used approach for silencing gene expression in vitro and in vivo, to study gene functions and elucidate molecular mechanisms in mammalian cells. Both endogenously produced siRNAs and chemically synthesized siRNAs become assembled within a multisubunit ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). Subsequently, the sense strand of the siRNA is removed, leaving the antisense strand to guide the ‘activated’ RISC to its site of action where it hybridizes with its target mRNA resulting in mRNA cleavage by the RNAi endonuclease Argonaute 2 (31). This type of silencing occurs when siRNA molecules perfectly match their complementary target mRNA. The RISC complex can also guide and incorporate partially homologous siRNA strands to target miRNAs, causing translational repression of the particular mRNA present in the cytoplasm. In this case the siRNA acquires miRNA activity, and can control the expression of numerous target mRNAs at the translational level, which remains a critical issue for therapeutic applications of RNAi (32, 33). The siRNA-loaded RISC is recycled for additional rounds of gene silencing activity. The rate of target cell divisions determines the persistence of siRNA, mediated gene silencing not of shRNA when stably incorporated in the DNA, since the siRNAs will be diluted after each cell division.

Apart from siRNA, gene silencing involving RNAi can also be achieved using so called DNA enzymes (deoxyribozymes) which can be perceived as molecular scissors containing a catalytic core of 15 deoxyribonucleotides that binds to and cleaves its target RNA [reviewed by (34)]. Moreover, interference with miRNA pathways is possible by means of antisense oligonucleotides, which are complementary to the sense strand of the target miRNA duplex and block its processing [reviewed by (35)]. RNAi based drugs may allow for specific silencing of a gene involved in downstream signaling of proinflammatory and pro-angiogenic stimuli, whereas interference with miRNA pathways may result in downregulation of multiple proinflammatory genes, both theoretically leading to suppression of inflammation. The recent discovery of miRNA involvement in tumor angiogenesis (36) furthermore paves the road for inhibitory miRNAs to be further developed for endothelium-specific therapy of cancer. For reasons of space limitations we will restrict ourselves in the next paragraphs to siRNA, although several of the concepts discussed may be applicable to other types of inhibitory RNAs as well.

**siRNA Delivery Into Endothelial Cells**

The first decade of targeted endothelial drug delivery research focused on the identification of molecular targets on the endothelial cells that are selectively expressed during disease development (27, 37). Initially employed drugs include the
cell death inducing molecules doxorubicin (38) and the pro-
apoptotic heptapeptide dimer D(KLAKLAK)2 (39). Moreover, 
anti-inflammatory enzymes that provide anti-oxidant protection 
(40) and corticosteroids that inhibit intracellular signaling and 
concomitant proinflammatory gene expression (16, 22) have 
been formulated and were shown to improve disease status. 
Also, targeted delivery of antisense oligonucleotides has been 
investigated in detail in the last 15 years (41). The design of 
new siRNA delivery devices for future therapeutic application 
has benefitted and will continue to benefit from the knowledge 
gained by advancements in targeted delivery of these conven-
tional drugs.

Unmodified and uncomplexed siRNAs (so-called naked si-
RNAs) have a half-life in the blood of only a few minutes 
which limits their usefulness as a drug per se. They are rapidly 
eliminated by renal excretion and are also degraded with a $t_{1/2}$ 
$\approx$1 h by serum RNases (42). Due to its relatively large molecu-
lar weight ($\sim$13 kDa), polyanionic nature ($\sim$40 negatively 
charged phosphate groups), and high hydrophilicity, naked 
siRNA will furthermore not passively cross the membrane of 
unperturbed cells. To apply siRNA for in vivo gene silencing, 
it either needs to be chemically modified or formulated and 
delivered to protect it from rapid clearance and degradation by 
serum RNases, to prevent activation of the immune system and 
interactions with other nontarget cells, and to allow cellular 
uptake, finally leading to participation in the RNAi pathway 
(43). Formulation of siRNA in an advanced drug delivery sys-
tem has the advantage that it does not affect the pharmacologi-
cal potential of the siRNA, contrary to various forms of chemi-
cal modification.

Ideally, for in vivo application the siRNA needs to be effi-
ciently formulated in carrier systems to contain sufficient 
amounts of siRNA and be stable to resist degradation or disas-
sembly in the circulation. At the same time, carrier systems 
should allow efficient release of the cargo once the carrier 
arrives in the endocytotic vesicles and/or in the cytoplasm of 
the cells [discussed in more details by (44)]. Physicochemical 
characteristics of the carrier such as composition, size and sur-
face charge can handicap pharmacokinetic behavior by engaging 
in interactions with serum proteins including serum albumin, 
lipoproteins and immunoglobulins, which leads to clearance by 
cells of the reticulo-endothelial system (RES) in the spleen and 
and liver (45). To limit these interactions and prolong circulation 
half-life, the surface charge can be masked by covering the car-
rrier with a hydrophilic polymer such as (poly)ethylene glycol 
(PEG) (46). Recent work revealed that lipid-based particles con-
taining high levels of PEG (10 mol%) are not taken up by the 
liver after systemic administration (47). Moreover, different 
types of polymers, such as pH-sensitive or diffusible PEG var-
iants are available for shielding off the vesicle surface. Such 
alterations provide increased carrier responsiveness to low pH 
and enhanced cytoplasmic release of its cargo (48). Further-
more, it is possible to control the circulation time of particles 
by time dependent diffusion-mediated release of PEG shielding 
which can reduce side effects of formulations containing toxic 
Drugs (49, 50).

Size and shape of the carrier also determine its fate in vivo. 
Most of the systems used for targeting endothelial cells have 
size ranging between 100–200 nm to minimize clearance from 
the circulation by renal filtration and liver uptake (51, 52). 
Moreover, several groups reported that nano-sized particles are 
more effective than micr osized particles in targeting microcapill-
ary sized vessels, where red blood cells (RBCs) preferentially 
line up in the center of the blood stream, thereby increasing 
nanoparticle contact with the vessel wall. They may, however, 
not be adequate for targeting to medium-to-large size blood 
vesels due to RBC-hindered margination which may decrease par-
ticle contact with the vessel wall (53, 54). The shape of the carrier 
may also determine the extent and mode of interaction with the 
vascular wall and affect the rate of cellular uptake. Spherical 
nanoparticles are typically proposed for vasculature-targeted drug 
delivery by virtue of their relatively unrestricted capacity to navi-
gate through the circulatory system bringing along minimal risk 
of vessel occlusion (10). Furthermore, Muro et al., reported that 
in vitro endothelial ICAM-1-targeted 0.1 $\times$ 1 $\times$ 3 $\mu$m elliptical 
polystyrene disks exhibited four times slower uptake rates when 
compared to 0.1 and 5 $\mu$m diameter spheres (55).

To achieve specific delivery of siRNA to the desired endo-
thalial subsets, carriers can be surface-modified with monoclo-
nal antibodies, peptides, small-molecule ligands, or for example, 
aptamers, to recognize determinants on the cell surface. Anti-
odies and small antigen binding fragments have been studied 
most extensively for this purpose. A good ligand needs to be 
specific for a target expressed on the surface of ECs and should 
bind with sufficient capacity to promote internalization of drug 
loaded carrier to deliver therapeutically effective amounts of 
drug into the cells’ interior. Furthermore, ligand-target molecule 
binding should avoid (prolonged) disruption or interference with 
normal functions of the target epitope (56). Targeting endo-
thelial cells can also be achieved by employing their basic func-
tional heterogeneity without concomitant diseases activity. For 
example, liver sinusoidal endothelial cells, acting as scavengers 
specialized in the uptake of polyanionic macromolecules, could 
be efficiently reached by carriers conjugated with polyacidity-
lated human serum albumin, a ligand for scavenger receptors 
(57). Moreover, pulmonary endothelial cells which function as a 
nonthrombogenic semipermeable barrier and provide a vast sur-
face for gas exchange, readily express high levels of thrombo-
modulin and angiotensin-converting enzyme at the cell surface 
as compared to other ECs, allowing effective preferential target-
g (58, 59). In addition, delivery to endothelium does not 
always require harnessing the carrier with a targeting ligand. 
Santel et al. reported that systemic administration of an siRNA 
complexed with a lipid-based carrier, creating a so called lipo-
plex, led to significant uptake of siRNA by endothelial cells in 
different organs. siRNA-lipoplexes were extensively taken up
by the vasculature of the heart, lung, and liver resulting in RNAi mediated silencing of endothelial cell restricted genes CD31 and Tie2 (60).

**Intracellular Fate of Endothelium Targeted Delivery Systems and Their Cargo**

When a delivery system has reached the target endothelial cells, the carrier has to be internalized by the cell and release its content into the cytoplasm. Many carriers conjugated with ligands for extracellular receptors are internalized via endocytic pathways leading to degradation, transcytosis, or sorting of internalized material to different cell compartments (58). The type of pathway utilized depends on the target receptor, and the size and the nature of the drug carrier (44). Clathrin-mediated uptake and caveolae-mediated uptake are two main mechanisms involved in endocytosis in ECs. The clathrin-mediated pathway mainly guides the delivery system to the endosomes with subsequent degradation in the lysosomes, whereas the caveolae-mediated pathway predominantly serves as an entry for transcytosis through endothelial monolayers that usually avoids lysosomal compartments (58). Carriers targeting plasma membrane proteins like selectins are mainly taken up via clathrin-mediated endocytosis guiding the internalized content to the lysosomal degradation pathway within 2–4 h (22, 61). On the other hand, ligands that bind to ICAM-1 were shown to enter ECs by a nonclassical endocytic mechanism named cell adhesion molecule (CAM) mediated endocytosis. It requires formation of small multimeric complexes of the receptors and depends on target molecule clustering and size of the conjugates (100–300 nm). This mechanism also delivers materials to lysosomal compartments within ~3 h (40, 62). If the vesicular cargo enters the lysosomal degradation pathway, the initially formed early endosomes mature to more acidified (pH ~5.0–6.0) late endosomes and eventually merge with lysosomes, rendering their content for degradation by lysosomal enzymes and low pH (63). For siRNA delivery, however, endocytosis via the nondegradative route (e.g., caveolae-mediated pathway) likely leads to entrapment of the cargo in the endosomes (64).

We have shown that E-selectin targeted, lipid-based, conventional liposomes are extensively taken up by TNF-α activated HUVEC but also that they are degraded to a minor extent inside the endocytic vesicles of the endothelial cells (65). Regardless of the entry pathway, a lack of endosomal escape generally leads to poor siRNA efficacy, thus carriers have to be able to release their siRNA before entering lysosomes and enable escape of intact siRNA from the endosomal compartment into the cytoplasm (Fig. 1B). To aid siRNA delivery, several mechanisms allowing penetration of the endosomal membrane before transfer to the lysosomal compartment have been proposed. Vesicle type carriers can be modified with pore forming peptides that are able to disturb the continuity of the bilayer by introducing a pore in the membrane, thereby facilitating release of endosomal contents. Those peptides are often based on naturally occurring toxins or venoms like diphtheria toxin or melittin, a major component of bee venom (64). Cationic lipids can destabilize the endosomal membrane by inducing ‘flipping’ of anionic lipids in the endosomal bilayer, leading to formation of ion pairs which facilitates vesicle fusion with the endosomal membrane and release of the cargo into the cytoplasm (66, 67). Moreover, addition of a helper lipid with fusogenic properties [e.g., 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)] to a carrier formulation can significantly improve content release and escape from the endosomes (68). Furthermore, so called pH-sensitive carriers were developed by formulating DOPE with a pH-titrable lipid (displaying a pH-dependent charge) such as cholesteryl hemisuccinate (CHEMS) or by combination of cationic and anionic lipids in one lipid membrane. These formulations promoted content release by increased destabilization of the carrier in the endosomal compartment upon low pH (69). In contrast, polymers like polyethylenimine (PEI) and polyamidoamine (PAM) can induce endosomolysis by the so called proton sponge effect. These polymers have a strong buffering capacity due to protonation of amino nitrogen upon endosome acidification. This invokes a high chloride ion influx into the endosome, causing osmotic swelling of the endosome and eventually endosome lysis (70).

**Endothelial Cell Targeted siRNA Delivery Systems**

Lipid-based systems have been used for the delivery of nucleic acids for over 20 years, starting with studies by Feltigner et al. (71). Liposomes and lipoplexes are the two main categories of lipid-based systems, although novel types of carriers such as stabilized nucleic acid-lipid particles (SNALP), lipid polyacation-DNA nanoparticles (LPD) and lipid like molecules called lipoidods [extensively reviewed by (72)] have entered the stage in recent years. For siRNA delivery, liposomes and lipoplexes are usually composed of a cationic lipid, helper lipid (e.g., DOPE and/or cholesterol) and a (poly)ethylene glycol-lipid (44). Liposomes consist of an aqueous core enclosed in a phospholipid bilayer with nucleic acids mainly entrapped in the central aqueous compartment. Liposomes have generally stable physicochemical characteristics, while lipoplexes are spontaneously formed via interaction of positively charged lipids and negatively charged nucleic acids which makes them more unstable (73). The advantages of lipid-based systems are their low toxicity (several liposomal formulations are FDA approved), easy sizing to below 200 nm, and great flexibility in tailoring them on demand with targeting ligands. Lipid structures can be easily modified by coupling targeting ligands to improve their delivery potential or by adding pH-sensitive or fusogenic moieties to aid intracellular release of siRNA (41).

So far only a few types of carriers suitable for systemic siRNA delivery into endothelial cells have been developed (Table 1). Successful siRNA delivery to tumor endothelial cells expressing integrin αvβ3 using RGD based homing peptides has first been reported by Schiffelers et al. (74). In this study, the siRNA that
inhibited vascular endothelial growth factor receptor 1 (VEGFR2) expression was incorporated into self-assembling nanoparticles constructed with RGD-harnessed PEGylated polyethyleneimine, after it was shown that formulation into conventional liposomes did not lead to target gene silencing. We recently developed two novel lipid-based systems which show potential for systemic siRNA delivery to activated endothelial cells. The first system is based on a lipoplex composed of the cationic amphiphilic lipid SAINT (1-methyl-4-(cis-9-dioleyl)methyl-pyridiniumchloride), a well established delivery agent of nucleotides and proteins, and the helper lipid DOPE (75). To achieve specificity towards activated endothelium, SAINT was covalently coupled to a monoclonal anti-E-selectin antibody forming a construct referred to as SAINTarg. With this anti-E-selectin-SAINTarg we were able to substantially enhance siRNA uptake, transfection specificity and efficacy of VE-cadherin down regulation in activated endothelial cells, as compared to transfection with a nontargeted SAINT formulation (76).

In addition, we have developed a novel generation of liposomes called SAINT-O-Somes, based on formulation of conventional long circulating liposomes by the addition of the cationic amphiphilic lipid SAINT. These liposomes harnessed with anti-E-selectin antibody showed specific uptake by activated endothelial cells, and displayed good size stability (∼100 nm in diameter) in the presence of serum, but were destabilized at lower pH as occurs in the endosomes of endothelial cells, thereby showing superior intracellular release of their content. We were able to efficiently encapsulate low molecular weight compounds such as doxorubicin and siRNA in these carrier systems, rendering this formulation an interesting candidate for systemic application (65).

### Table 1

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**Issues to be Addressed for Further Development of EC Specific Delivery Devices**

Significant progress has been made in the last decade with regard to the development of endothelial cell specific drug delivery devices. Increased knowledge of the molecular changes within these cells during the onset and progression of disease has spurred identification of new potential targets on the cell membranes, while new molecular entities give rise to the design of novel carriers with important added value over conventional ones. Knowledge of the molecular control and pharmacology of microvascular endothelial cells remains, however, scarce. Basic heterogeneity in the control of EC behavior makes it highly likely that their responsiveness to ‘drugs’ is also microvascular bed dependent, although data to support this are only slowly emerging. Moreover, the loss of microenvironment driven EC behavior upon culturing the cells in vitro requires solid validation of in vitro molecular control or responsiveness to stimuli and pharmacological observations in the in vivo context (Fig. 2). This is only occasionally pursued, which is mostly due to the limited availability of methods to assess kinase activity, gene and protein expression in tissue biopsies, and to locate them specifically in the endothelium. As such, the choice of molecular tar-
get(s) allowing successful therapeutic interference by means of targeted siRNA approaches remains somewhat elusive.

After a drug delivery system carrying siRNA has been developed and properly optimized and sufficient siRNA can be delivered inside the target cell leading to the desired down regulation of the disease-associated target gene, novel technologies should be used to bring siRNA as therapeutic tool closer toward clinical application. Laser microdissection (LMD) of endothelial cells from specific microvascular segments in tissues allows compartmentalized analysis of gene expression and hence the examination of local effects of the targeted drug treatment (77). LMD can be applied to both animal and human tissues and it allows for enrichment of endothelial cells from (micro)vascular segments which can be prior assessed histologically for disease activity. Combining targeted drug delivery systems carrying siRNA to selectively down regulate disease-associated genes in restricted microvascular segments with LMD-based validation of gene silencing in the target endothelial compartment represents not just a powerful, but rather an essential strategy to provide proof of concept of in vivo siRNA delivery studies (Fig. 2).

Another technology that can significantly assist in further development of novel targeted delivery systems toward clinical application is precision-cut tissue slices. They closely resemble the organ from which it is prepared, with all cell types present in their original tissue-matrix configuration (78). The circumstance that in tissue slices the architecture of the original organ is retained makes them an attractive tool for drug delivery studies. Using this system, we showed that anti-E-selectin-SAINT-arg specifically associated with activated endothelial cells in human kidney tissue slices subjected to inflammatory conditions, exactly following the expression pattern of E-selectin (76).

Furthermore, one should take into account the differences in behavior between primary endothelial cells and endothelial cell lines. Endothelial cell lines, for example, are easier to transfected...
and hence are often chosen for protein overexpression studies, yet they display quite some differences in phenotype and responsiveness compared to primary cells. Moreover, their internalization machinery is often more tumor cell like and less relevant to the uptake features of primary cells. Since primary endothelial cells are closest, though not identical, to endothelial cells in vivo, they should at one point in the design of targeted drug delivery systems be used to further validate binding, internalization and intracellular drug release characteristics.

CONCLUSIONS

Increased insight in the role of endothelial cells in the pathophysiology of cancer, inflammatory and cardiovascular diseases, has drawn great interest in the design of pharmacological interventions aiming at the endothelium in diseased sites. The effectiveness of drugs intended to affect diseased endothelium is however limited which is likely partly due to the existence of endothelial subset specific responsiveness to proinflammatory cytokines (6). This heterogeneity on the other hand provides an opportunity for identification of disease-associated target epitopes expressed by vascular segment restricted endothelial cells as well as for selection of the proper drug target and concurrent drugs for therapeutic effects. Emerging therapeutic strategies based on RNAi have a great potential for therapeutic application. siRNAs can, however, not be directly applied for in vivo treatment of diseased endothelium due to a short half-life of the molecules, in the circulation, inability to pass the cellular membrane, high toxicity, and low cell selectivity. Formulation of these therapeutic molecules into delivery devices such as lipid-based or polymer-based systems targeted to diseased endothelial cells can provide them with a potential for further clinical application. Target determinants like E-selectin, VCAM-1, ICAM-1, and integrins with expression restricted to endothelial cells and upregulated during inflammation or angiogenesis may help to achieve specific and safe delivery of drugs into EC subsets involved in diseases, thereby improving pharmacological efficacy. Widespread use of RNAi therapeutics for endothelial diseases requires a clinically suitable, safe and effective delivery vehicle. This can be achieved by developing new formulations and bio-materials (e.g., novel cationic lipids, polymers or pH-sensitive PEG) to avoid detection by cells of the RES and improve intracellular siRNA release properties of existing lipid or polymer-based systems. Combined research efforts in the field of microvascular endothelial cell biology and pharmaceutical sciences are crucial to achieve the final goal, that is, the development of an efficient and cell-specific siRNA drug delivery system that can be applied in the clinic to effectively silence endothelial cell engagement in the patho(physio)logy of disease.

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