

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

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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 tumor 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 phar-

maceutical 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 molecular-1 (VCAM-1), (14)], myocardial injury [P-selectin, (15)], glomerulonephritis [E-selectin, (16)]; rheumatoid arthritis [$\alpha_v\beta_3$ integrin, E-selectin (17, 18)], and pulmonary inflammatory diseases [intercellular adhesion molecular-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-

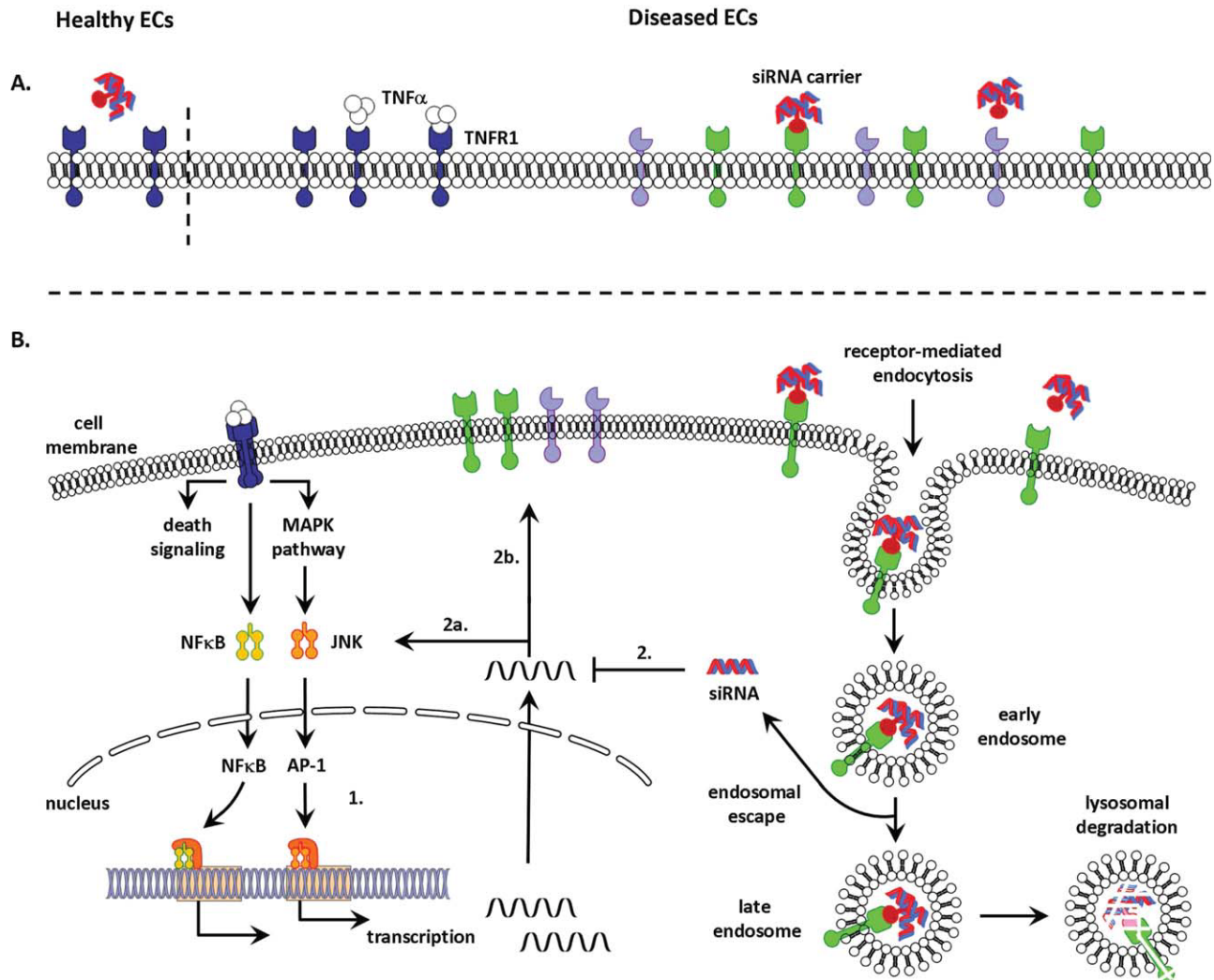


Figure 1. Simplified scheme of disease-associated cellular events that lead to endothelial cell activation and provide challenging opportunities for targeted siRNA delivery. (A) Microvascular endothelial cells (EC) are exposed to microenvironmental factors that differ from one vascular bed to the other, as a consequence of which their basic phenotype may vary. Exposure to proinflammatory conditions (e.g., $\text{TNF}\alpha$ or $\text{IL-1}\beta$) leads to activation and expression of a variety of cell adhesion molecules and other determinants associated with disease which may vary between endothelial subsets. (B) The differentially expressed determinants on the surface of disease-activated endothelium are excellent targets for drug delivery to abnormal endothelium. The targeted siRNA carriers (e.g., lipoplexes, polyplexes, or liposomes) interact with cell surface receptors and are taken up into the target cells via receptor-mediated endocytosis. The resulting endocytotic vesicles fuse to form early endosomes. These mature into late endosomes which ultimately become part of the lysosomes, where proteins and nucleic acids are degraded by acid hydrolases. To achieve target gene silencing, siRNAs need to be released from the carrier and escape from the endosomes into the cytoplasm, where it associates with the RNAi machinery and directs the cleavage of target mRNAs. **1** The activated intracellular signal transduction cascade controls transcription factor activation and proinflammatory gene expression. **2** Once escaped from the endosomes, the siRNA incorporates into the RNA-induced silencing complex, is subsequently unwound, and next guided to the complementary site in the target mRNA. This leads to specific gene silencing of, for example, (2a) transcription factors (TFs) $\text{NF}\kappa\text{B}$ or AP-1 to block downstream signaling pathways regulated by these TFs, or (2b) other determinants associated with disease activity. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

lial 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 $\alpha_v\beta_3$ and $\alpha_v\beta_5$ 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 $\alpha_v\beta_3$ -integrin, as do $\alpha_v\beta_3$ specific antibodies. During the past decade, RGD-peptides have become an established tool for the targeting of drugs and imaging agents to $\alpha_v\beta_3$ -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 messenger RNA (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 mRNAs, 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 proapoptotic 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 conventional drugs.

Unmodified and uncomplexed siRNAs (so-called naked siRNAs) 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 $t_{1/2} \sim 1$ h by serum RNases (42). Due to its relatively large molecular weight (~ 13 kDa), polyanionic nature (~ 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 system has the advantage that it does not affect the pharmacological potential of the siRNA, contrary to various forms of chemical modification.

Ideally, for *in vivo* application the siRNA needs to be efficiently formulated in carrier systems to contain sufficient amounts of siRNA and be stable to resist degradation or disassembly 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 surface 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 liver (45). To limit these interactions and prolong circulation half-life, the surface charge can be masked by covering the carrier with a hydrophilic polymer such as (poly)ethylene glycol (PEG) (46). Recent work revealed that lipid-based particles containing 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 variants 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). Furthermore, 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 microsized particles in targeting microcapillary 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 vessels due to RBC-hindered margination which may decrease particle 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 navigate through the circulatory system bringing along minimal risk of vessel occlusion (10). Furthermore, Muro et al., reported that *in vitro* endothelial ICAM-1-targeted $0.1 \times 1 \times 3 \mu\text{m}$ elliptical polystyrene disks exhibited four times slower uptake rates when compared to 0.1 and 5 μm diameter spheres (55).

To achieve specific delivery of siRNA to the desired endothelial subsets, carriers can be surface-modified with monoclonal antibodies, peptides, small-molecule ligands, or for example, aptamers, to recognize determinants on the cell surface. Antibodies 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 endothelial cells can also be achieved by employing their basic functional 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 polyacetylated human serum albumin, a ligand for scavenger receptors (57). Moreover, pulmonary endothelial cells which function as a nonthrombogenic semipermeable barrier and provide a vast surface for gas exchange, readily express high levels of thrombomodulin and angiotensin-converting enzyme at the cell surface as compared to other ECs, allowing effective preferential targeting (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 lipoplex, 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 endocytotic 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 natu-

rally 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 nitrogens 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 Felgner *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 polycation-DNA nanoparticles (LPD) and lipid like molecules called lipidoids [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 $\alpha_v\beta_3$ using RGD based homing peptides has first been reported by Schiffelers *et al.* (74). In this study, the siRNA that

Table 1
Examples of nonviral systems suitable for siRNA delivery to endothelial cells

Validated	Delivery system	Chemical composition	Target epitope	Pharmacological Target	Endothelial subset	Ref.
In vivo	Polyplex	PEGylated branched PEI	Integrin $\alpha_v\beta_3$	VEGFR2	Tumor vasculature	[74]
	Lipoplex	Chitosan nanoparticles AtuPlex (PEG-DSPE: AtuFECT01: DPhyPE)	Integrin $\alpha_v\beta_3$ –	PLXDC1 PECAM-1, PKN3	Tumor vasculature Tumor vasculature	[79] [80, 60]
In vitro	Liposome	DDAB:chol SAINT-O-Somes (SAINT: POPC:chol: PEG-DSPE)	– E-selectin	Caveolin-1 VE-cadherin	Lung vasculature HUVEC	[81] [65]
	Lipoplex	POPC:DAP: PEG-DMA SAINTarg (SAINT:DOPE)	– E-selectin	GAPDH VE cadherin	HUVEC HUVEC	[82] [76]

PEI, polyethyleneimine; PEG, (poly)ethylene glycol; DPhyPE, 1,2-diphytanyl-sn-glycero-3-phosphoethanolamine; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; DDAB, dimethyldioctadecylammonium Bromide; SAINT, (1-methyl-4-(cis-9-dioleoyl)methyl-pyridiniumchloride); DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DAP, 1,2-dioleoyl-3-dimethylammonium-propylpane; DMA, 2-dimethylamino ethyl methacrylate; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; Chol, cholesterol.

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-dioleoyl)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-SAINArg 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).

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-

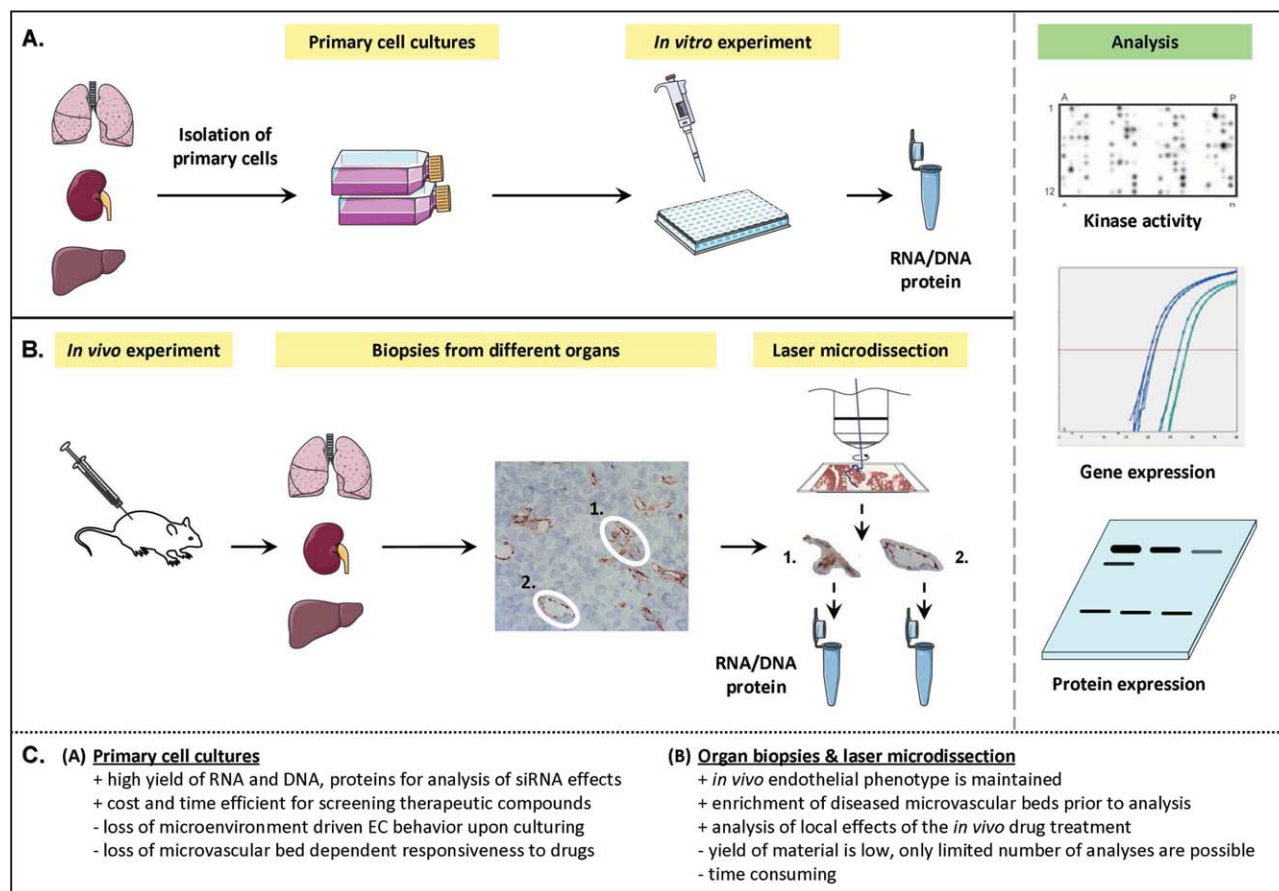


Figure 2. Experimental approaches to study the selectivity, intracellular delivery capacity and efficacy of endothelial targeted siRNA delivery systems *in vitro* and *in vivo*. Studying endothelial cell association, internalization and siRNA delivery using primary endothelial cell cultures (A) may be inadequate due to high rate of endothelial cell dedifferentiation upon *in vitro* culturing. This leads among others to loss of heterogenic behavior of ECs associated with the location of the microvascular bed *in vivo*. Laser microdissection (B) of ECs from specific microvascular segments in tissues is an essential technique to assist in the development of endothelial specific siRNA delivery devices, as it allows validation of local target gene knock down and studying downstream molecular consequences of gene knock down. The advantages and disadvantages of both approaches are summarized in (C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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 transfect

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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