# Inhibition of adhesion molecule expression on human venous endothelial cells by non-viral siRNA transfection

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## Abstract

**Objective**: Expression of adhesion molecule receptors on venous endothelial cells crucially influences the fate of venous grafts by mediating leukocyte-endothelium interactions. These interactions include adhesion of leukocytes to the endothelium, followed by transendothelial migration, leading to neointimal hyperplasia and finally graft occlusion. Therefore, inhibition of adhesion molecule expression may be a promising strategy to improve the quality of venous grafts. We tested the efficiency of non-viral transfection of human venous endothelial cells with short interfering RNA (siRNA) to specifically down-regulate adhesion molecule expression. **Methods**: Primary cultures of human venous endothelial cells (HVEC) were examined for expression of the adhesion molecules ICAM1, VCAM1 and E-selectin (SELE) after non viral siRNA transfection. Adhesion molecule expression was measured by flow cytometry, real-time PCR and immunoblotting after stimulation with TNF-alpha, an inflammatory cytokine. **Results**: Non-transfected cells showed a strong increase of adhesion molecule expression following cytokine stimulation (p<0.01). Upon transfection with specific siRNAs a six fold decrease in ICAM1 (p<0.001) and SELE expression and cell positivity (p<0.05) and a twofold decrease in VCAM1 expression and cell positivity (p<0.01) could be observed. SiRNA-mediated gene suppression of adhesion molecules was also reflected by corresponding decreases in adhesion protein and transcript levels. **Conclusions**: The expression of adhesion molecules on HVECs can be effectively inhibited by specific siRNAs using a safe, non-viral transfection approach. This is a promising tool to precondition venous bypass grafts in order to interfere with endothelium-leukocyte interactions and to prohibit neointima thickening or atherosclerosis, which are regarded to be the most important causes of venous graft failure.

**Keywords**: RNAi • venous graft disease • coronary bypass grafting • adhesion molecules

## Introduction

Despite the advances in conservative and catheter intervention treatment surgical bypass grafting remains an outstanding tool for therapy of coronary artery disease. The surgical success is limited by the patency rate of the grafts employed. Although the patency rate of venous conduits is reduced in comparison to arterial grafts, the saphenous vein remains the most commonly used conduit because of its easy disposability. However, the advantage of reduced surgical trauma will disappear, if the patient is at higher risk for redo operation associat-

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ed with improved perioperative morbidity and mortality [1] caused by a restenosed or occluded graft. Thus, there is a strong interest to develop strategies for improving the patency of venous grafts.

Several main causes are considered to be responsible for the limited patency of venous conduits. Graft thrombosis, which is mainly caused by technical factors such as narrowed anastomosis or improved comparative flow in the bypassed vessel contribute to an early occlusion of the graft. This appears within a few days up to several weeks after surgery. Further changes of venous grafts occur at later time points, which cannot be influenced by the surgeon. Four to six weeks after implantation, a significant alteration can be observed consisting of an of smooth muscle cells and deposition of extracellular matrix in the intimal compartment, called neointimal hyperplasia (NIH). This is commonly accepted as the major pathogenic process in venous grafts within the first year after implantation [2]. NIH leads to a graft alteration in two different ways. Excessive development of the neointima thickening may cause a significant stenosis or an occlusion by itself. Furthermore, it accelerates the development of graft atherosclerosis, superimposed on the previously thickened neointima.

Concerning the described pathomechanisms of NIH and venous graft atherosclerosis, adhesion molecule expression on human venous endothelial cells (HVEC) plays a crucial role [3]. Adhesion molecules represent a group of glycoproteins and carbohydrates expressed on the surface of a wide variety of cell types, including venous endothelial cells. They support an initial adhesive event resulting in the rolling of leukocytes along the endothelium followed by their subsequent firm adhesion [4–6]. Therefore, adhesion molecules enable transendothelial leukocyte migration from the intravascular space into the media of the vasculature, where leukocytes trigger smooth muscle cell proliferation by release of stimulating factors [7]. In conclusion, stress induced expression of adhesion molecules on endothelial cells can be d as a central key factor leading to a limited patency of venous bypass grafts.

Two different groups of adhesion molecules are essential for leukocyte adhesion and migration. Selectins are calcium-dependent transmembrane glycoproteins, which modulate a reversible and weak binding of leukocytes to the endothelium. They are important in the first contact, the so called tethering. Adhesion molecules belonging to the immunoglobulin superfamily are involved in the following step of firm leukocyte-endothel adhesion [5]. Both adhesion molecule classes are essential for the transendothelial migration of leukocytes, and the incurring NIH.

Several stimuli may lead to an enhanced expression of adhesion molecules in venous endothelial cells. Humoral stimulation by cytokines [8, 9]. siRNAs are small double-stranded oligoribonucleotides, which have been originally identified as intermediates of the RNA interference pathway. Upon transfection into cells, they are incorporated into a cytoplasmic ribonucleoprotein complex, the so called RNA-induced silencing complex (RISC). Upon hybridization to a complementary mRNA sequence, RISC cleaves this transcript leading to its degradation. Thus, despite ongoing transcription, no protein can be synthesized because of the RISC-mediated degradation of the mRNA. siRNA may be either transiently delivered by transfection, or can be endogenously expressed as small hairpin RNAs (shRNAs). For both types of delivery, *in vivo* efficacy has been demonstrated [16].

Concerning an improved protection of venous bypass material, we evaluated the potency of transfected siRNA in reducing adhesion molecule expression on venous endothelial cells in order to protect venous bypass material against NIH.

The aim of the study was to investigate, whether primary human venous endothelial cells can be transfected with siRNA, and whether specific siRNAs are able to silence the expression of E-selectin (SELE), intercellular adhesion molecule 1 (ICAM1) and vascular adhesion molecule 1 (VCAM1) on HVECs in order to inhibit leukocyte-endothelial interactions.

## Material and methods

### Patients

Endothelial cells were obtained from vein specimen of the saphenous vein which remained as remnants after elective CABG. All patients gave their written consent and the study was approved by the Ethical Committee of the University of Tuebingen, Faculty of Medicine.

## Isolation and cultivation of HVECs

Isolation and cultivation were done as previously described [17]. Briefly, all culture plates and flasks were coated overnight with collagen (40%) (Collagen G, Biochrom, Indiana, USA). After incubation in RPMI 1640 (containing 0.5%/ml gentamycin) the vein was rinsed with buffer solution (137 mM NaCl/ 5.4 mM KCL/ 4.2 mM NaHCO$_3$/ 5 mM D-Glucose in 500 ml H$_2$O, pH 7.3). HVECs were harvested by collagenase treatment (0.1% in PBS, PAA Laboratories GmbH, Cölbe, Germany) followed by culture in EGM-2 (+bullet kit, Cambrex Bio Science Verviers, S.p.r.l., Verviers, Belgium). Cells were splitted after reaching confluence. For all experiments, cells from the third or fourth passage were used. HVECs purity was controlled by staining with a FITC-labeled antibody for human CD31 (Immunotools, Friesothye, Germany).

## siRNA uptake studies

For siRNA uptake studies, cells were cultured in collagen coated plates without antibiotics. After reaching confluence, they were transfected with 100nM of FITC labeled non-sense siRNA using a cationic lipid medium (Cellfectin™, Invitrogen GmbH Karlsruhe). The amount of positive cells was determined by flow cytometry using a FACScan™ (Becton Dickinson GmbH).

## siRNA transfection of human endothelial cells

Before siRNA transfection, cells were cultured in collagen-coated 12-well plates for FACS analysis, in 6-well plates for real-time PCR or in culture flasks for immunoblotting. After reaching confluence (70%–80%), they were transfected with 100 nM of specific siRNA targeting ICAM1, VCAM1 or SELE, using 6.25 µg/ml Cellfectin (Invitrogen GmbH, Karlsruhe, Germany) for 2 h in serum-free medium, followed by the replacement of the supernatants with EGM-2 (without antibiotics).

### Sequences for ICAM1 siRNA
Sense: 5'-GCCUCAGCACGUACCUCUAdTdT-3'
Antisense: 5'-UAGAGGUACGUGCUGAGGCdTdT-3'

### Sequences for VCAM1 siRNA
Sense: 5'-AAUGCAACUCUCACCUUAAdTdT-3'
Antisense: 5'-UUAAGGUGAGAGUUGCAUUdTdT-3'

### Sequences for SELE siRNA
Sense: 5'-GGUUGAAUGCACCACUCAAdTdT-3'
Antisense: 3'-UUGAGUGGUGCAUUCAACCdTdG-3'

24 hrs later, HVECs were stimulated with 2.5 ng/ml TNF (Immunotools, Friesoythe, Germany) for 14 hrs in case of ICAM1 stimulation, 14 hrs with 10 ng/ml TNF for VCAM1 and 14 hrs with 2.5 ng/ml for SELE detection. The cells were splitted in three groups for FACS analysis, immunoblotting and real time RT-PCR.

## Flow cytometry

Cells were washed using EGM-2 media followed by incubation in 0.5% FCS. HVECs were stained for specific adhesion molecules with a PE labeled human ICAM1 antibody (Becton Dickinson, GmbH), a FITC labeled VCAM1 antibody (Becton Dickinson, GmbH) and a PE-Cy-5 labeled E-selectin antibody (Becton Dickinson, GmbH). After washing and detaching, cells were fixed with 2.5% paraformaldehyde in PBS. FACS analyses (5000 cells/measurement) were performed with the same device described above and evaluated with the CellQuestPro-software (Becton Dickinson, GmbH). The results shown represent the averages of three independent experiments. All experiments were performed with primary cells obtained from the same patient.

## Immunoblotting

Total cellular protein was extracted from HVECs with the CelLytic MEM protein extraction kit (Sigma-Aldrich, Munich, Germany), containing a protease inhibitor cocktail. Lysates were mixed with two volumes of reducing (ICAM1, VCAM1) or non-reducing (SELE) loading buffer and incubated for 5 min at 95°C. Protein equivalent to 17,000 cells (ICAM1, SELE) or 60,000 cells (VCAM1) was separated on 8% SDS-polyacrylamide gels and then transferred by semi-dry blotting (15 V, 30 min) to nitrocellulose membranes (Bio-Rad Laboratories, Munich). Immunodetection was performed using mouse monoclonal antibodies against human ICAM1 (clone 28, BD Biosciences, Heidelberg, Germany), VCAM1 (clone 1.G11B1, Chemicon, Hampshire, UK) and SELE (clone P2H3, Hölzel Diagnostika, Cologne, Germany). After incubation with the primary antibody, blots were washed three times with TBS and incubated with goat anti-mouse IgG anti-

body conjugated with alkaline-phosphatase (Coulter-Immunotech, Krefeld, Germany). Bands were visualized using Sigma Fast ™ 5-bromo-4-chloro-3-indolyl phosphatase (BCIP)/nitro blue tetrazolium chloride (NBT) tablets (Sigma-Aldrich, Munich, Germany), dissolved in distilled water.

## Quantitative real-time RT-PCR

Total RNA from cultured human venous endothelial cells was purified by AurumTM total RNA mini kit (BIO-RAD Laboratories, Inc., Hercules, CA, USA). The OD260/OD280 absorption ratio was >1.95. Consecutively, 200 ng of every RNA sample was reverse transcribed by iScriptTM cDNA Synthesis Kit (BIO-RAD Laboratories, Inc., Hercules, CA, USA) according to the manufacturer`s instructions. Standard curves were established for the primer pairs as described previously [18]. Primer design was done with the software "Primer3" [19] and Primer Premier 5 (PREMIER Biosoft International). All primers were synthesized by Operon Biotechnologies GmbH (Köln, Germany) and MWG-BIOTEC AG (Ebersberg, Germany). PCR was performed as described previously [20]. All PCR reactions contained IQ™SYBR®Green Supermix (BIO-RAD Laboratories, Inc. (Hercules, CA, USA), 400 nM forward and reverse primer and 2 ng of reverse transcribed RNA in a total volume of 15 µl. All PCR reactions were performed in triplicates. Normalized gene expression was calculated by the $\Delta C_t$ method using GAPDH as a reference.

## Statistical procedure

The results are expressed as mean ± standard deviation. Statistical significance of differences between the groups was examined by two sided student's t-test assuming unequal variances. A p-value of less than 0.05 was considered to be significant.

# Results

## siRNA uptake tested by flow cytometry

FITC-labeled non-sense siRNAs (so called scrambled-siRNA) were delivered to HVECs by liposomal trans-fection using Cellfectin™ (Invitrogen GmbH Karlsruhe). In comparison to other transfection agents, Cellfectin™ showed a high transfection efficiency combined with a low cell toxicity. Nearly 85% of the cells harbored FITC-labeled siRNA two hours after transfection as judged by FACS-analysis (data not shown).

## Screening of different siRNA sequences

First steps within the silencing experiments were to find out from several siRNA sequences those with the highest knockdown potency. For SELE we used four and for VCAM1 five different sequences respectively (Fig. 1). Sequence 1 (Fig. 1A) showed the highest knockdown for SELE and sequence 5 (Fig. 1B) the best results for VCAM1. Therefore, these siRNA sequences were used for all further experiments. For ICAM1 we used a previously tested sequence [17].

## Basal receptor expression and increase after TNF stimulation.

HVEC showed a low basal expression of each adhesion molecule receptor in culture.

In the uninduced state, 17% of the cells were positive for VCAM1, followed by 15% for ICAM1 and 2.1% for SELE (Fig. 2A, B).

## Cytokine treatment and adhesion molecule expression

Incubation with TNF induced a substantial increase of the examined adhesion molecules. Cytokine stimulation of untransfected cells induced a fivefold, threefold and fourtyfold increase of ICAM1, VCAM1 and SELE positive cells, and a tenfold, threefold and thirtyfold increase of the surface expression of the corresponding adhesion molecules, respectively (p < 0.01; Fig. 2).

## siRNA-transfected cells

Transfection with siRNA strongly reduced the expression of the corresponding adhesion molecules to levels comparable to the uninduced state (Fig. 2A). Transfection with ICAM1 siRNA (siICAM1) decreased the fraction of ICAM1-positive cells seven-

**Fig. 1A** Effects of 4 different sequences (S1-S4) siRNA targeting SELE.
S1: sense: (GGU UGA AUG CAC CAC UCA A)dTdT
    antisense: (UUG AGU GGU GCA UUC AAC C)dTdG
S2: sense: (UGG UAG AAU UGG AGA GUA A) dTdT
    antisense: (UUA CUC UCC AAU UCU ACC A) dTdG
S3: sense: (CAG UGU GGU UUG UGU UUG A)dTdT
    antisense: (UCA AAC ACA AAC CAC ACU G) dGdT
S4: sense: (CGG AAG CUA UGA CUU AUG A)dTdT
    antisense: (UCA UAA GUC AUA GCU UCC G)dTdG
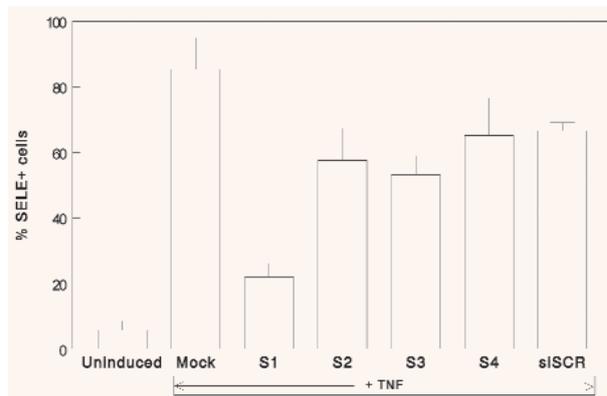


**Fig. 1B** Effects of 5 different sequences (S1-S5) siRNA targeting VCAM1.
S1: sense: (GGA GGA UAC GGA UAU GAA A)dTdT
    antisense: (UUU CAU AUC CGU AUC CUC C)dAdA
S2: sense: (GAG CUA AAU UAC ACA UUG A)dTdT
    antisense: (UCA AUG UGU AAU UUA GCU C)dGdG
S3: sense: (CAU CUA CGC UGA CAA UGA A)dTdT
    antisense: (UUC AUU GCU AGC GUA GAU G)dTdG
S4: sense: (CUC UAU AUU UAG AUU GUU A)dTdT
    antisense: (UAA CAA UCU AAA UAU AGA G)dTdG
S5: sense: (AAU GCA ACU CUC ACC  UUA A)dTdT
    antisense: (UUA AGG UGA GAG UUG CAU U)dTdT
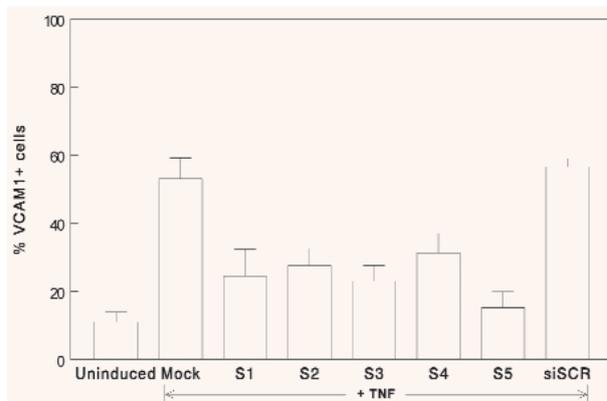


**Fig. 1** Effects of several siRNA sequences and TNF treatment on the fraction of cells positive for VCAM1 and SELE. . Each bar represents the mean of three independent experiments, error bars show standard deviations.

fold (p < 0.001; fig. 2B), and the total ICAM1 surface expression sixfold (p < 0.001; Fig. 2C). In the case of VCAM1, the corresponding siRNA (siVCAM1) diminished the fraction of positive cells twofold (p < 0.01; Fig. 2b) and VCAM1 expression threefold (p < 0.01; Fig. 2C). Furthermore, application of the SELE siRNA (siSELE) reduced fraction of SELE-positive cells tenfold and global SELE expression ninefold. Transfection with scrambled siRNA (siSCR) hardly affected the fractions of cells positive for ICAM1, VCAM1 or SELE. Furthermore, siSCR treatment interfered only marginally with total adhesion molecule expression, when compared to the active siRNAs siICAM1, siVCAM1 and siSELE (p < 0.05; Fig. 2B, C).

## Western blotting

The Western blot confirmed the FACS results for total adhesion molecule expression (Fig. 3). In all three groups (ICAM1, VCAM1 and SELE) only a weak staining could be observed in the untransfected and non-stimulated cells. After TNF treatment the protein detection was strong. The group of specific siRNA treated cells showed only slight bands after TNF-treatment comparable to the group of untransfected and unstimulated cells. In contrast, application of the scramble control siRNA siSCR did not substantially affect adhesion protein levels, when compared to TNF-stimulated untransfected cells.

## Real Time PCR

To assess the effects of siRNA transfection onto ICAM1, VCAM1 and SELE mRNAs, transcript levels were analyzed by semi-quantitative real-time RT-PCR. In general, the observed effects of siICAM1, siVCAM1 and siSELE on target protein expression were reflected by changes in the corresponding transcript levels (Fig. 4). All three siRNAs siICAM1, siVCAM1 and siSELE reduced the levels of their cor-
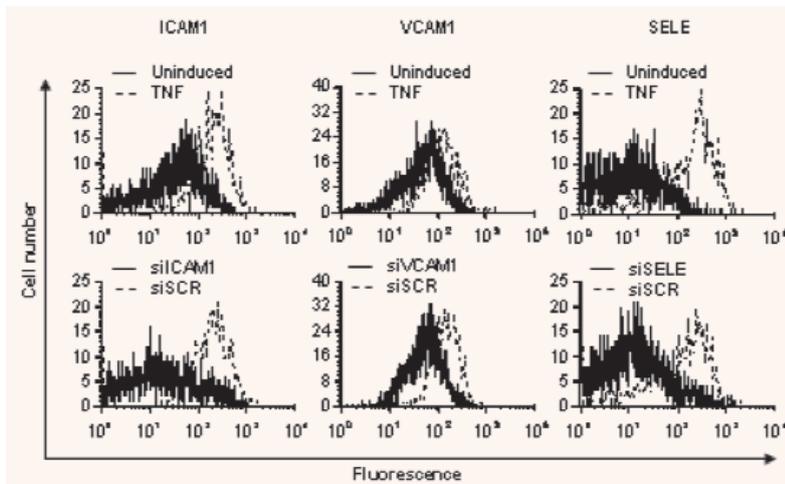
**Fig. 2A** Histograms showing TNF stimulation and siRNA-mediated suppression of adhesion molecule expression. Top row, unstimulated and TNF stimulated cells; bottom row, TNF stimulated cells treated beforehand either with the indicated adhesion molecule siRNA or a scrambled control siRNA. Analyzed adhesion molecules are indicated on the top.
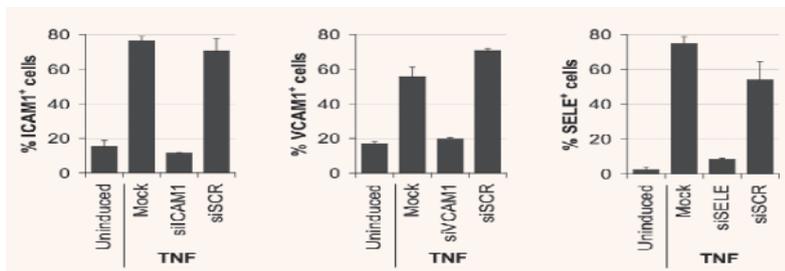
**Fig. 2B** Effects of siRNA and TNF treatment on the fraction of cells positive for ICAM1, VCAM1 and SELE. Each bar represents the mean of three independent experiments, error bars show standard deviations.
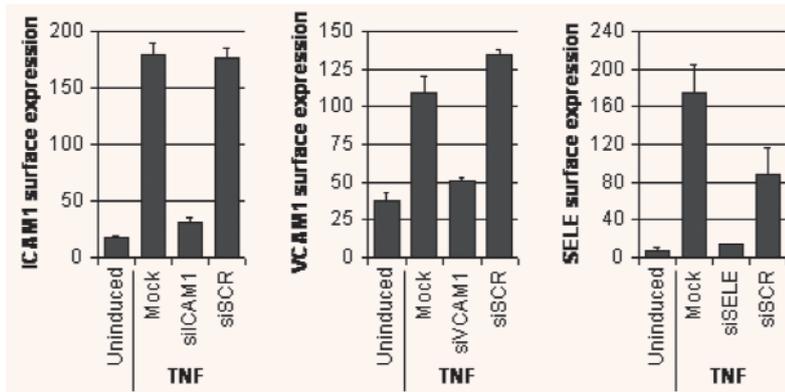
**Fig. 2C** Effects of siRNA and TNF treatment on the total surface expression of ICAM1, VCAM1 and SELE. The y-axis reflects the geometrical mean of the adhesion molecule expression. Each bar represents the mean of three independent experiments, error bars show standard deviations. Uninduced, untreated cells; TNF, TNF-stimulated cells; Mock, TNF-stimulated untransfected cells; siICAM1, ICAM1 siRNA; siVCAM1, VCAM1 siRNA; siSELE, E-selectin siRNA.

**Fig. 2** siRNA-mediated interference with adhesion molecule expression

responding target mRNA tenfold. In contrast, TNF stimulation of the other two adhesion molecule transcripts was affected only two- to threefold. Furthermore, the control siRNA siSCR caused a twofold drop in SELE transcript levels and did not affect ICAM1 and VCAM1 levels.

## Discussion

The 2006 Nobel Prize in Physiology/Medicine was awarded to Andrew Fire and Craig Mello for their discovery of RNA interference, a mechanism for controlling the flow of genetic information. RNAi, which occurs naturally in plants and animals, allows a gene to be specifically "silenced". This process can also be induced experimentally by injecting tailor-made short double-stranded RNA (siRNA) into cells to effect protein knockdown, giving scientists a method for specific silencing a target gene.

The method is now widely used as a basic genetic tool and is worldwide under most intensive research regarding his potential as a therapeutic entity against numerous diseases itself, including the number one killer in the western countries: Cardiovascular disease
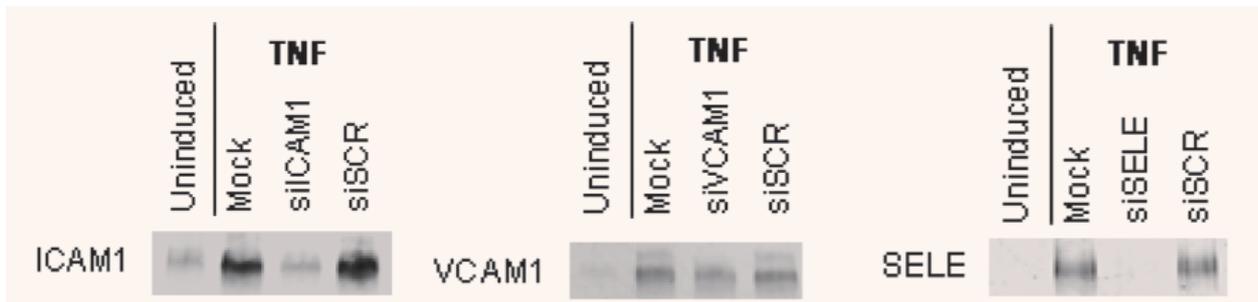
**Fig. 3**  siRNA-dependent effects on adhesion protein levels. Adhesion protein levels were analyzed by immunoblotting as described in Material and methods. Cell treatment is shown on top of each blot, the analyzed adhesion proteins are indicated on the left. Uninduced, untreated cells; TNF, TNF-stimulated cells; Mock, TNF-stimulated untransfected cells; siICAM1, ICAM1 siRNA; siVCAM1, VCAM1 siRNA; siSELE, E-selectin siRNA.

[21]. Despite medical and interventional procedures like PTCA, coronary artery bypass grafting remains a powerful tool to combat myocardial infarction and disease related symptoms in patients suffering from coronary artery disease. A long term patency rate of the grafts is one of the preconditions for a definite success of surgery and is enormously affected by the material employed. The saphenous vein remains the most commonly used conduit for CABG. However, follow up investigations demonstrated a reduced patency rate of venous grafts in comparison to arterial conduits. Up to 50% of the venous conduits are occluded within ten years after implantation [22]. Arterial conduits are accompanied by a prolonged patency rate. However, their availability is reduced and harvesting is commonly associated with an increased operative trauma. Furthermore vessel length is limited. Concerning these aspects cardiac surgeons are frequently faced with the problem to decide for the best therapy, especially for the best conduit. An excellent graft would combine the advantages of both graft materials: the long term patency of the arterial conduit and the relatively easy availability of venous material.
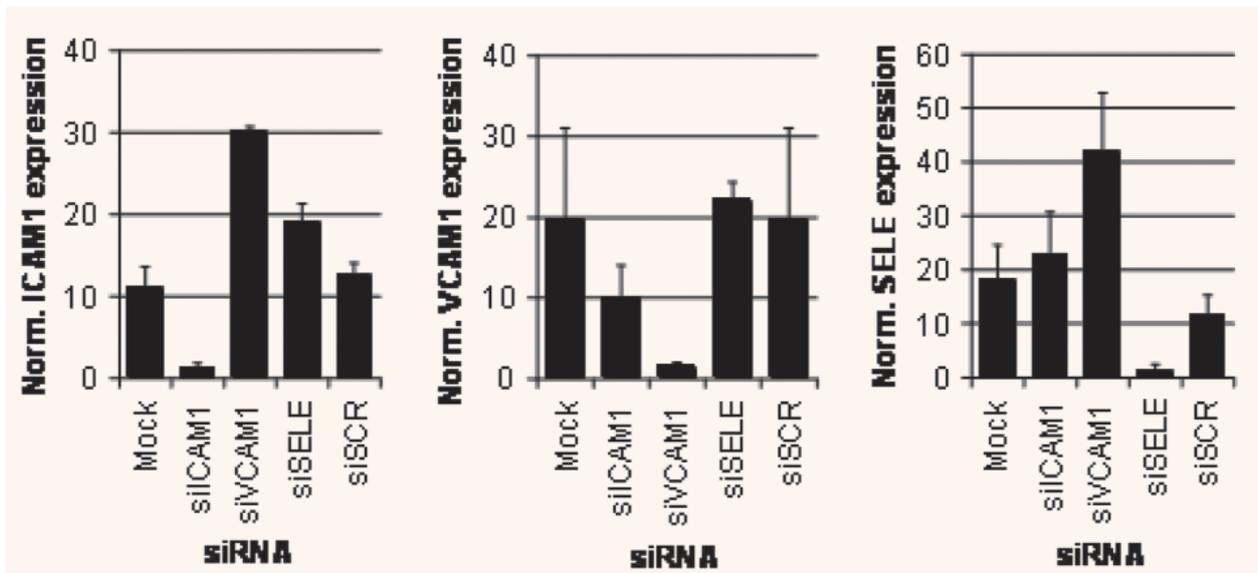


**Fig. 4**  Effects of siRNA and TNF treatment on ICAM1, VCAM1 and SELE mRNA levels. Transcript levels were examined by real-time RT-PCR. Each bar represents the mean of at least two independent experiments, error bars show the range of variation in the case of two experiments and standard deviations in the case of three or more experiments. Mock, TNF-stimulated untransfected cells; siICAM1, ICAM1 siRNA; siVCAM1, VCAM1 siRNA; siSELE, E-selectin siRNA.

Considering the main pathomechanisms leading to graft occlusion in venous bypass material the neointimal hyperplasia remains to be the most important alteration after implantation. It alters the newly implanted graft in two ways. The neointimal hyperplasia narrows the intraluminal space finally leading to a significant stenosis and occlusion. Furthermore, it facilitates the way for atherosclerotic changes resulting in severe graft alterations within months to years after surgery. One of the initial key factors for neointimal hyperplasia is determined by adhesion molecules expression on the endothelial cells of the venous graft. They are modulating leukocyte-endothelium interactions leading to transendothelial migration of leukocytes, thereby stimulating smooth muscle cells for proliferation by a release of paracrine molecules as well as toxic radicals [2]. Regrettably, many different stimuli lead to adhesion molecule expression during CABG: Cytokines or the mechanical trauma while harvesting the conduit [23, 10].

Using fluorescently labeled siRNA in combination with a cationic lipid formulation, we showed that HVEC can be efficiently transfected with siRNA with low concomitant toxicity. Transfection with siRNAs homologous to ICAM1, VCAM1 and SELE mRNA caused a substantial decrease in both target mRNA and protein levels. The minor effects exerted by the scrambled siRNA emphasizes the sequence specificity of this effect. However, one limitation of siRNA-application could be an interference with other, unintended mRNA sequences in addition to the targeted one. In particular, siVCAM1 caused a further two- to threefold increase in ICAM1 and SELE transcripts levels. The reasons for this off-target effect are currently unclear, but may be related to the sequence of this siRNA. For a potential clinical application of siRNAs targeting the family of adhesion molecules off-target effects within this family are rather welcome than frightened. Probably, future therapeutics containing siRNA will include application of a cocktail of many different siRNAs to knock down several targets of interest. Nevertheless, we are aware that comprehensive investigations including DNA-chip analyses are obligatory to screen possible off-target effects before clinical application of this new technology.

The transfection of bypass conduits represents an ideal field of therapeutic siRNA application. The conduits can be transfected ex vivo, so that no systemic application of siRNA is required. This is a great advantage because efficient siRNA delivery *in vivo* in a clinically acceptable fashion is still a major challenge for therapeutic siRNA applications. Furthermore, gene suppression is restricted to the conduit and does not affect other tissues. Finally, with this liposomal technique the graft can be easily transfected inside the operating room without any further biological and genetical security requirements.

In conclusion, our results strongly suggest that the application of siRNA is a promising evolving technique probably resulting in a quality improvement of venous bypass grafts. Applying this novel tool in venous bypass material would result in a conduit, which combines the advantages of venous and arterial graft material, namely the prolonged patency rate comparable to arterial grafts and the unproblematic availability of venous material.

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