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Research Article

Sialyl Lewis^x-liposomes as vehicles for site-directed, E-selectin-mediated drug transfer into activated endothelial cells

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Abstract. E-selectin, exclusively expressed on activated endothelial cells, is a potential target for site-directed delivery of agents. We and others have shown that sialyl Lewis^x-liposomes (sLe^x-liposomes) are recognized by E-selectin. We now report an approach employing sLe^x-liposomes to deliver antisense oligonucleotides (AS-ODNs) directed against the adhesion molecule ICAM-1 to activated vascular endothelial cells. ICAM-1 expression was analyzed at the protein level by immunofluorescence and a cell surface ELISA, and at the RNA level by RT-PCR. We have investigated two different AS-ODNs complementary to the 3′ untranslated region and the AUG translation initiation codon of ICAM-1 mRNA. Both inhibited protein expression, but did not influence the mRNA level, pointing to a hybridization of AS-ODNs with the mRNA in the cytoplasm. Our results demonstrate the feasibility of a novel approach for the delivery of agents to activated endothelial cells by glycoliposomes targeted to E-selectin.

Key words. E-selectin; sLe^x-liposomes; site-directed delivery; antisense strategy; ICAM-1.

Interactions of leukocytes and endothelial cells play a crucial role in inflammatory diseases. They are regulated by several adhesion molecules and their ligands. E-selectin and P-selectin which are expressed on activated endothelial cells initiate leukocyte 'rolling.' This is a prerequisite for the subsequent firm adhesion of the leukocytes mediated by adhesion molecules like ICAM-1, a member of the immunoglobulin superfamily. ICAM-1 is expressed at low levels on surfaces of non-activated endothelial cells and is markedly upregulated by stimuli such as interleukin-1β (IL-1β) [1, 2]. Blockade of endothelial adhesion molecules or their down-regulation by specific antisense oligonucleotides (AS-ODNs) could be useful strategies to inhibit the adhesion process and reduce endothelial damage. Phosphorothioate oligonucleotides, which were designed to hybridize to ICAM-1 mRNA or pre-mRNA, can inhibit ICAM-1 expression on endothelial cells isolated from the vein of human umbilical cord (HUVECs) in the presence of cationic liposomes [3, 4]. Such liposomes are widely used to transport AS-ODNs into cells in vitro and in vivo [5, 6]. However, this approach lacks selectiv-

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ity. The encapsulation of ODNs into liposomes bearing ligands which are recognized by cell surface receptors would help to overcome this problem [7, 8].

E-selectin is a potential target for site-directed delivery of drugs or genes to vascular endothelial cells, because it is expressed exclusively at the cell surface in areas of activation [9]. E-selectin expression is induced by cytokines like IL-1β and tumor necrosis factor-α, or lipopolysaccharide [9–13]. In HUVECs, P-selectin expression is not initiated by these stimuli [10, 14]. It is constitutively synthesized and stored in the Weibel-Palade bodies of endothelial cells. Mediators such as thrombin, histamine, complement components, and oxygen-derived radicals cause a rapid presentation of P-selectin on cell surfaces. Selectins bind specifically carbohydrates of the Lewis type [15, 16]. Glycoliposomes bearing the tetrasaccharide sialyl Lewis^x (sLe^x) on their surfaces seem to bind effectively to E-selectin, because they are powerful inhibitors of E-selectin-mediated cell adhesion [17, 18]. Cell adhesion to immobilized E-selectin and to activated HUVECs is reduced by sLe^x-liposomes in a concentration-dependent manner. Murohara and colleagues [19] reported cardioprotection by sLe^x-liposomes in vivo.

The major purpose of the present study was to explore the feasibility of a selective transport of substances to activated endothelial cells with sLe^x-liposomes via E-selectin. We report on the delivery of AS-ODNs directed against the ICAM-1 mRNA by sLe^x-liposomes.

Materials and methods

Preparation of liposomes. Liposomes were prepared by a detergent method [20]. Briefly, solutions of egg yolk phosphatidylcholine (PC; Sigma) and dimyristoylphosphatidylethanolamine (PE; Sigma), or sLe^x-PE [18] were mixed, dried, and equilibrated in 0.1 M Tris-buffer/0.15 M sodium chloride/100 mM sodium cholate solution. An aliquot of oligodeoxynucleotides, solubilized in physiologic sodium chloride solution (0.06 nmol/µl), was added, except in the case of ODN-free preparations. Oligonucleotides were synthesized as phosphorothioates (BioTez, Berlin, Germany). Two previously described AS-ODNs were used: AS-ODN1 (5′ CCCC-CACCACTTCCCCTCTC 3′), which targets the human 3′ untranslated region (3′UTR), and AS-ODN2 (5′ TGGGAGCCATAGCGAGGC 3′), which hybridizes to the AUG translation initiation codon [3, 4]. Control sense oligonucleotides (S-ODNs) were used with the reverse sequences of the AS-ODN.

Lipid/ODN/detergent mixtures were chromatographed on Sephadex G75 (Pharmacia) with 0.1 M Tris-buffer pH 7.2, supplemented with 0.15 M sodium chloride, and 2 mM calcium chloride. Liposomes or glycoliposomes were eluted at the void volume of the column. Liposome-associated ODNs amounted to 9.5 %, as quantified by a radioiodinated ODN tracer provided by Dr. E. Schmidt (BioTez). Size distributions of the liposome preparations were analyzed by laser light scattering. The mean diameter of most preparations was about 40 nm, the polydispersity index was measured to 0.37. Some preparations were very polydisperse with PI > 1.5. However, no influence was observed on the feasibility of these liposomes.

Cells. HUVECs were isolated from human umbilical veins by α-chymotrypsin treatment as described by Haller et al. [21]. Cultures were grown in 1 % gelatine precoated tissue flasks with medium 199 supplemented with 20% v/v fetal calf serum, 2 mM L-glutamine, non-essential amino acids (1:100), 100 U/ml penicillin/streptomycin, 10 mM Hepes, and 1 mM sodium pyruvate. All components were purchased from Life Technologies. HUVECs were used within three passages.

Antisense protocol. HUVECs were grown for the antisense experiments on multiwell slides for immunofluorescence, in 96-well plates for cell surface ELISA, or in 24-well plates for RT-PCR. Two days after seeding, cells were incubated with medium containing sLe^x/AS-ODN-liposomes (20–150 nM ODN) or with controls for 4 h at 37 °C. Medium was removed and fresh cell growth medium was added. Cells were then cultivated for additional hours as indicated. Cells were activated simultaneously with the ODN incubation by addition of IL-1β (2 ng/ml; Boehringer). ICAM-1 protein expression was analyzed by immunofluorescence and cell surface ELISA after about 24 h. ICAM-1 mRNA levels were analyzed by RT-PCR at 4–24 h after treatment.

Lipofectin (Life Technologies) was sometimes used as an alternative vector for the ODN transport. In these cases, lipofectin (10 µg/ml) in Opti-MEM (Life Technologies) was added to the cells, immediately followed by the addition of ODNs (0.1–1 µM). This incubation medium was replaced by fresh medium after 4 h, and IL-1β was added for HUVEC activation [3]. Some experiments were performed using the same incubation procedure as used with sLe^x/ODN-liposomes.

Cells were treated with IL-1β but without ODNs to determine the 100% value of ICAM-1 expression. Basal expression of ICAM-1 on HUVECs was measured without IL-1β treatment.

Immunofluorescence. After incubation according to the antisense protocol, cells were fixed on slides with 5% formaldehyde for 5 min and incubated with a 1:5 dilution of the FITC-labelled anti-ICAM-1 monoclonal antibody BBA20 (R&D Systems) for 1 h at room temperature. Expression of ICAM-1 was viewed in an Axiophot photo microscope (Zeiss). Micrographs were taken with a CF20DXC digital camera.

Cell surface ELISA. HUVECs, grown in 96-well plates, were fixed with 3% formalin solution (Sigma) and blocked with 1% bovine serum albumin solution (Sigma) about 24 h after incubation and activation. Cells were incubated with anti-ICAM-1 monoclonal antibody BBA4 (0.5 μg/ml; R&D Systems) for 1 h at 4 °C and washed three times with phosphate-buffered saline containing 0.3% bovine serum albumin. Antibody bound to the cells was measured by incubation with a 1:200 dilution of a peroxidase conjugate of goat anti-mouse IgG (Sigma) for 1 h at 4 °C. Wells were washed again and developed with o-phenylenediamine (Sigma) according to the manufacturer's protocol. The optical density (OD) was measured at 492 nm. Relative ICAM-1 expression was calculated as follows: [(OD of treated activated cells − basal OD of treated cells)/(OD of activated cells − basal OD)] × 100.

RT-PCR. Total cellular RNA was prepared from treated and control HUVEC monolayers using the RNeasy Total RNA kit (Qiagen). For reverse transcription, 200 ng RNA was denatured at 70 °C for 5 min and cooled on ice. RNA was mixed in a final volume of 20 μl containing 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies), 9 pmol oligo-dT primer, 500 μM dNTP mix (Boehringer), 20 U recombinant RNase inhibitor RNAsin (Promega), 10 mM dithiothreitol, 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. The mixture was incubated at 42 °C for 30 min.

For RT-PCR, probes were added to a final 50 μl reaction mixture containing 2.5 U Taq DNA polymerase (Life Technologies), 200 μM dNTP mix (Boehringer), 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 15 pmol oligonucleotide primers for ICAM-1 (BioTez), and 1.5 pmol oligonucleotide primers for the housekeeping gene glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) as internal standard. The following primer sequences were used: ICAM-1: F, 5′-AAC CGG AAG GTG TAT GAA CTG-3′; R, 5′-CGA GGT GTT CTC AAA CAG CTC-3′; GAPDH: F, 5′-ATG ACA ACA GCC TCA AGA TCA TCA G-3′; R, 5′-CTG GTG GTC CAG GGG TCT TAC TCC T-3′ [22]. Thermal cycling (23 cycles) was carried out in a Personal Cycler (Biometra) under the following conditions: 95 °C for 30 s (denaturation), 58 °C for 1 min (annealing), and 72 °C for 1 min (extension).

The PCR products (4 μl) were separated on 10 % polyacrylamide gels (25 W, 1 h). Gels were silver-stained. Densitometric quantification of the bands was performed with NIH Image 1.6 software (National Institutes of Health). ICAM-1 mRNA levels were determined from the ratios of the mRNA levels of ICAM-1 to GAPDH.

Results

Effects of ODN-treatment on ICAM-1 protein expression on cell surfaces of HUVECs. Using sLe^x/ODN-liposomes, both antisense ODNs (AS-ODN1, AS-ODN2) specifically reduced protein expression as analyzed by the immunofluorescence technique. Figure 1 shows typical micrographs obtained from one HUVEC population. ICAM-1 expression was visualized by a FITC-labelled monoclonal antibody. IL-1β-activated HUVECs showed strong ICAM-1 fluorescence (fig. 1A). The fluorescence intensity of untreated cells was rather low, indicating a basal level of expression (fig. 1B). Incubation of cells with sLe^x/AS-ODN2-liposomes simultaneously with IL-1β activation reduced the fluorescence signal (fig. 1C). In contrast, sLe^x/S-ODN2-liposome-treated cells showed a high fluorescence intensity (fig. 1D), comparable to that of activated cells. Control treatments of cells with free AS-ODNs, or with AS-ODNs encapsulated in liposomes without any sLe^x decoration, or empty liposomes without sLe^x decoration had no effect on the fluorescence intensity (results not shown).

A cell surface ELISA was used to quantify ICAM-1 protein expression after treatment with sLe^x/AS-ODN-liposomes. Figure 2 shows that both AS-ODNs investigated reduced protein expression in a concentration-dependent manner. In contrast, S-ODNs encapsulated in sLe^x-liposomes reduced ICAM-1 expression only by about 10%, and independently of their concentration. Figure 3 illustrates the specificity of antisense activity of sLe^x/AS-ODN-liposomes. Compared to activated HUVECs, a significant reduction in protein expression was achieved exclusively by sLe^x/AS-ODN-liposomes. Experiments were performed at least three times in triplicate. Differences of treated and untreated cells were compared within one series using Student's t-test. SLe^x-decoration of liposomes was essential for the suppression of ICAM-1. Treatment of cells with sLe^x/AS-ODN1-liposomes (60 nM AS-ODN) reduced ICAM-1 expression to 55 % ($p < 0.01$). A comparable level of inhibition of ICAM-1 expression (47 %, $p < 0.05$) was measured for sLe^x/AS-ODN2-liposomes (78 nM AS-ODN2). Simultaneous treatment of cells with IL-1β and AS-ODN2 reduced ICAM-1 expression significantly more than incubation of cells first with AS-ODN2 and then with IL-1β (41 versus 32 %, $p < 0.01$). Controls were without significant effects. Preincubation of HUVECs with the monoclonal antibody BBA2, which blocks the lectin domain of E-selectin, reduced the antisense effect on ICAM-1 expression caused by treatment with sLe^x/AS-ODN-liposomes. A concentration of 5 μg BBA2 per well diminished the effect by 10%; lower antibody concentrations were without effect.

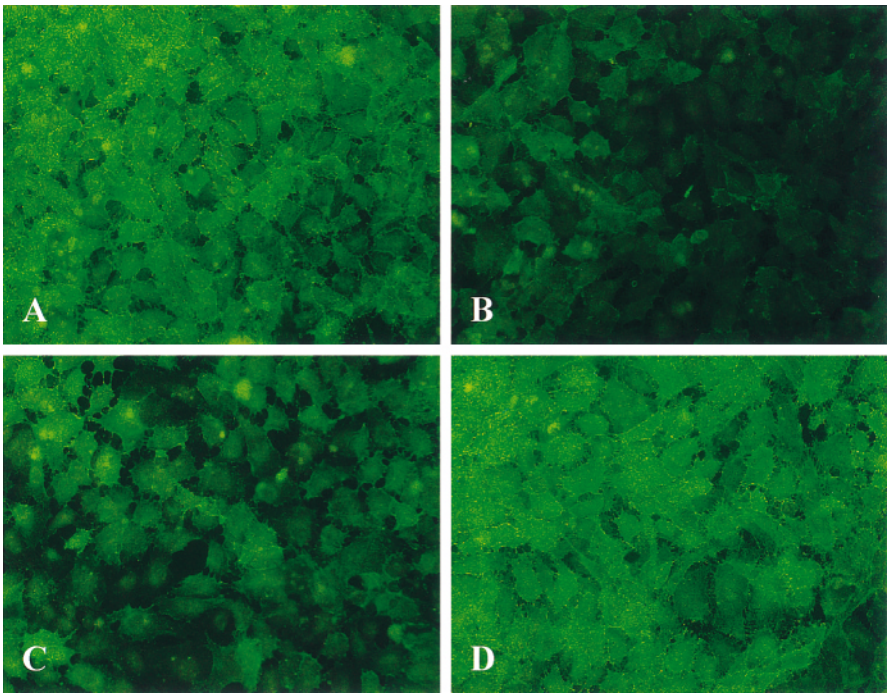

Figure 1. Immunofluorescence of ICAM-1 protein expression on HUVECs. All micrographs were taken with identical sensitivity settings. (*A*) IL-1β-activated cells. (*B*) Basal expression of cells. (*C*) Simultaneous treatment of cells with sLe^x/AS-ODN2-liposomes (AS-ODN2 156 nM) and IL-1β. (*D*) Simultaneous treatment of cells with sLe^x/S-ODN2-liposomes (S-ODN2 156 nM) and IL-1β.

Effect of ODN treatment on ICAM-1 mRNA. Treatment of HUVECs with lipofectin/AS-ODN complexes, targeting the 3′UTR of the RNA reduced the ICAM-1 mRNA level by an RNase H-sensitive mechanism which includes destabilization and cleavage of the double-strand mRNA in the nucleus [3]. The sequence of our AS-ODN1 was identical to this AS-ODN sequence. We analyzed ICAM-1 mRNA expression by RT-PCR, performed with RNA samples isolated from HUVECs, treated either with lipofectin/AS-ODN1 complexes or with sLe^x/AS-ODN1-liposomes. Samples of untreated, IL-1β-activated, lipofectin/S-ODN1-, or sLe^x/S-ODN1-liposome-incubated cells were examined in parallel experiments. Because the time course of ODN transport and/or ODN hybridization to ICAM-1 mRNA were not known, total RNA was isolated from HUVECs at different times after treatment and activation. Figure 4 shows the densitometric analysis of three to five individual experiments at indicated times after activation of HUVECs. Lipofectin/AS-ODN1 complexes caused a time-dependent decrease in ICAM-1 mRNA after IL-1β activation, which was already significantly different ($p < 0.05$) after 8 h of incubation. This is in agreement with published results. Two different experimental schemes were used to incubate HUVECs with lipofectin/ODN complexes. HUVECs were either incubated with these complexes and activated with IL-1β in a second step, or were simultaneous incubated with AS-ODN and activated with IL-1β (comparable to the procedure with sLe^x-liposomes).

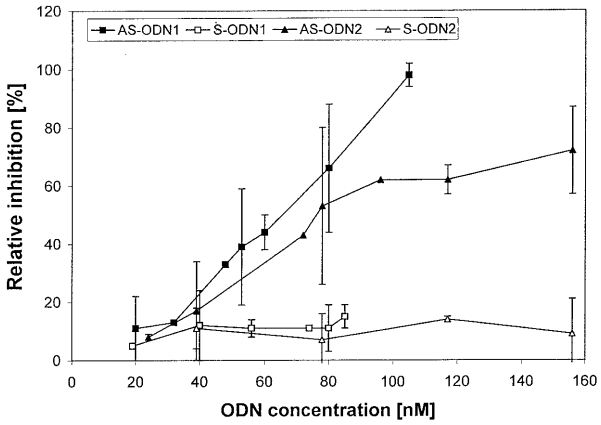

Figure 2. Dose-dependent inhibition of ICAM-1 protein expression on activated HUVECs after treatment with sLe^x-liposomes encapsulating different ODNs. Cells were treated according to the antisense protocol and ICAM-1 expression was analyzed in a cell surface ELISA. Each value is the mean of two to eight individual experiments.

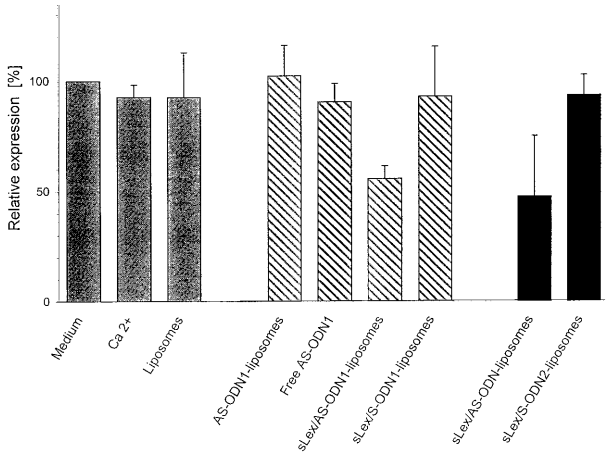

Figure 3. Effect of sLe^x/AS-ODN-liposome treatment on ICAM-1 protein expression of activated HUVECs. Protein expression was analyzed about 24 h after activation by a cell surface ELISA. Cells were treated for 6 h at 37 °C with sLe^x-liposomes containing antisense or sense oligonucleotides (60–80 nM). Incubations with free AS-ODN1 (1 μM), empty liposomes without sLe^x decoration, and AS-ODN1-liposomes without sLe^x-decoration were used as controls. Values represent the mean ± SD of at least three individual experiments.

ICAM-1 mRNA was measured 12 h after activation. The reduced levels of ICAM-1 mRNA were comparable for both incubation/activation procedures. In contrast, AS-ODN1 transported by sLe^x-liposomes did not change the level of ICAM-1 mRNA at any time investigated.

Discussion

We studied the feasibility of using sLe^x-liposomes, which bind selectively to E-selectin, for specific targeting and delivery of encapsulated material to activated vascular endothelial cells. We used HUVECs as an in vitro model. E-selectin, but not P-selectin, is expressed after activation under the conditions used [10, 14]. E-selectin expression is restricted to activated vascular endothelium and recognizes the tetrasaccharide sLe^x via its lectin domain [11, 23]. We encapsulated antisense oligonucleotides directed against ICAM-1 RNA into sLe^x-liposomes. AS-ODNs specifically block the expression of the respective gene. Despite the many reports that have appeared during the last 10 years, the precise mechanism(s) by which translation of targeted RNA is inhibited is not fully understood. Three different effects are discussed for the action of AS-ODNs: sequence-specific antisense activity, sequence-specific non-antisense activity, and non-sequence-specific activity [24]. Liposomes are potential carriers for the transport and delivery of ODNs into cells [25]. Suppression of the human ICAM-1 gene has been tested with various AS-ODNs using the cationic liposome formulation lipofectin as a vector for transport and delivery in vitro and in vivo [3, 4, 26, 27]. The most effective sequences targeted the 3′UTR and the AUG translation initiation codon of ICAM-1 mRNA. Both AS-ODNs inhibited protein synthesis by at least two different mechanisms. Hybridization to the 3′UTR creates an RNase H-sensitive complex. As a result, the ICAM-1 mRNA is decomposed, and decreased amounts of mRNA were quantified. Hybridization of AS-ODNs to the coding region of the mRNA blocks the translation process. In this case, the level of mRNA is unchanged [3].

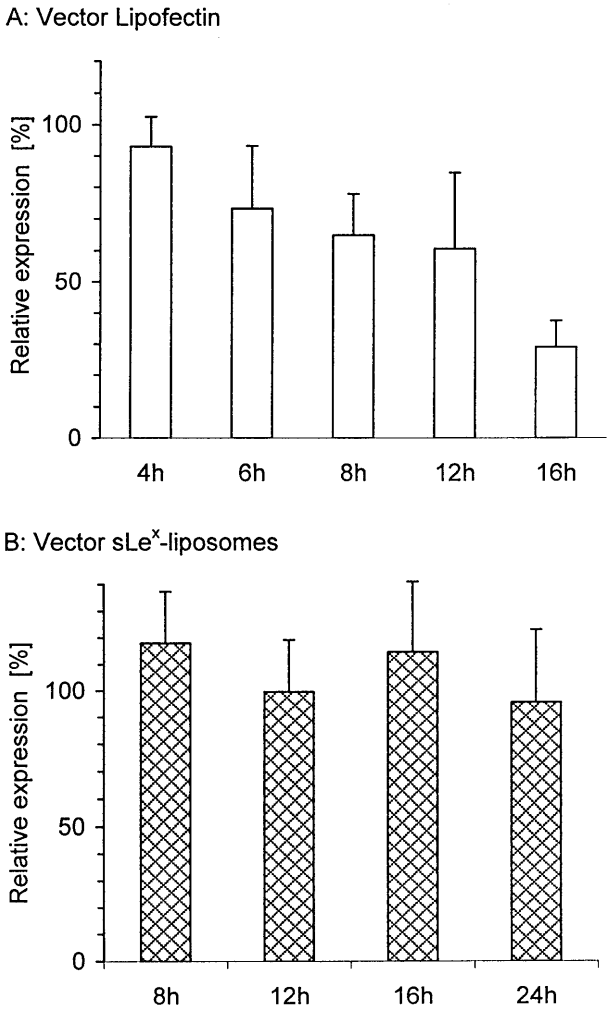

Figure 4. Effect of AS-ODN1 on ICAM-1 mRNA expression. Semiquantitative densitometric analysis of the PCR products after gel electrophoresis. Bars represent the ratio of ICAM-1 to the coamplified GAPDH mRNA levels at indicated times after IL-1β activation of HUVECs. (*A*) Vector lipofectin, AS-ODN1 1 μM. (*B*) Vector sLe^x-liposomes, AS-ODN1 100 nM. Values represent the mean ± SD of three to five individual experiments.

We encapsulated two different AS-ODNs (AS-ODN1 which targets the 3′UTR, and AS-ODN2 which targets the translation initiation codon) in sLe^x-liposomes and investigated their potency to deliver the oligonucleotides to activated HUVECs. We measured ICAM-1 protein expression by two independent experimental methods: immunofluorescence and cell surface ELISA. Both demonstrated specific inhibition of ICAM-1 protein expression. Compared to lipofectin, tenfold lower AS-ODN concentrations were needed to inhibit ICAM-1 protein expression if sLe^x-liposomes were used as a vector. However, the mechanism of action of sLe^x/AS-ODN-liposomes is still unknown. No downregulation of ICAM-1 mRNA was observed with AS-ODN1 which targets the 3′UTR, as we demonstrated by RT-PCR. In contrast to this observation and in good agreement with data published recently [3], a decreased level of ICAM-1 mRNA was measured if lipofectin was used as a vector. The decrease in ICAM-1 was independent of the incubation procedure with AS-ODN and IL-1β, which were added either simultaneously or sequentially. We conclude that different modes of action were responsible for these results. Lipofectin is a cationic liposome formulation. Its potency to transport and deliver ODNs into various cell types is based on electrostatic interactions between the lipids and the ODNs on the one hand, and between the resulting complexes and cells on the other. The mechanism of transport and delivery is not known in detail [25]. Moreover, kinetic aspects could be of importance. Lipofectin delivers ODNs very quickly to the nucleus [25, 28]. As a result, hybridization to the target occurs immediately on upregulation of ICAM-1 mRNA and its destabilization and decomposition can take place by an RNase H-mediated mechanism in the nucleus.

Uptake of sLe^x-liposomes should work mainly by an endocytotic mechanism, because Spragg and coworkers [29] demonstrated an endocytotic pathway for the uptake of immunoliposomes by activated HUVECs after specific E-selectin recognition. The mechanism of subsequent release of ODNs into the cytoplasm is unknown. We assume that hybridization to the target mRNA proceeds in the cytoplasm, and thus that protein synthesis is interrupted. In this context, kinetic aspects are important. We measured comparable time courses of E-selectin protein, and ICAM-1 mRNA expression after stimulation of HUVECs with IL-1β (data not shown). Therefore, E-selectin-mediated transport and the delivery of AS-ODNs take place simultaneously with the occurrence of ICAM-1 mRNA in the cytoplasm. As a consequence, AS-ODNs could reach their target in the cytoplasm. RNase H, which is able to decompose the mRNA/AS-ODN hybrid, occurs predominantly in the nucleus. The level of ICAM-1 mRNA remains unchanged if hybridization happens in the cytoplasm. We speculate that the hybrid hinders the subsequent steps in translation. Several regions of mRNA can regulate translation, and 3′UTR-mediated regulation is also possible [for a review see ref. 30]. Protein expression is downregulated, whereas mRNA expression remains unaffected.

In summary, our results demonstrate the feasibility of the sLe^x-liposome approach for site-selective delivery of agents to areas of activated cells within the vascular system. Treatment of activated HUVECs with AS-ODNs encapsulated in liposomes inhibited ICAM-1 protein expression in a concentration-dependent manner. sLe^x-decoration of liposomes was essential for a significant reduction in ICAM-1 protein expression. Targeting of activated endothelium via the cell type-specific receptor E-selectin offers a new strategy for systemic and possibly also for local application.

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