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Investigation of the cellular uptake of E-Selectin-targeted immunoliposomes by activated human endothelial cells

Stephan Kessner [a], Annett Krause [a], Ulrich Rothe [b], Gerd Bendas [a,*]

[a] *Department of Pharmacy, Martin-Luther-University Halle, Wolfgang-Langenbeck-Strasse 4, D-06120 Halle, Germany*
[b] *Institute of Physiological Chemistry, Martin-Luther-University Halle, Hollystrasse 1, D-06097 Halle, Germany*

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Abstract

In the present study the cellular uptake of targeted immunoliposomes by interleukin-1 activated human endothelial cells has been analysed by several spectroscopical and microscopical fluorescence techniques. Previous in vitro experiments demonstrated that the targeting of immunoliposomes to vascular selectins is a potential way for a selective drug delivery at inflammatory sites. In attempts to further adapt the targeting experiments to physiological conditions, we demonstrate that E-Selectin-directed immunoliposomes are able to bind their target cells under the simulated shear force conditions of capillary blood flow cumulatively for up to 18 h. In order to consequently follow the fate of liposomes after target binding, we analysed the route and degree of liposome internalization of the cells concentrating on cell activation state or various liposomal parameters, e.g., sterical stabilization, type of antibody or antibody coupling strategy. The use of NBD-labelled liposomes and subsequent fluorescence quenching outside the cells with dithionite show that circa 25% of the targeted immunoliposomes were internalized. According to inhibition experiments with agents that interfered with the endocytotic pathway, we found out that the major mechanism of liposome entry is endocytic. The entry involves, at least in part, receptor-mediated endocytosis via E-Selectin, a liposome accumulation in the endosomes and their acidification was proved by pyranine spectroscopic results. Furthermore, microscopical investigations demonstrate that also a fusion of liposomes with the cell membrane occurs followed by a release of entrapped calcein into the cytoplasm. These observations gain insight into the behaviour of E-Selectin-targeted immunoliposomes and indicate that these immunoliposomes have great potential to be used as drug carriers for intracellular drug delivery at inflammatory sites.

Keywords: Immunoliposome; Drug targeting; Selectin; Endocytosis; Fusion

1. Introduction

The site-specific drug delivery to cells or organs is a potentially attractive mode of therapy for increasing the therapeutic efficiency of drugs and reducing their toxicity. Liposomes, especially antibody-targeted liposomes, also referred to as immunoliposomes (IL) have been regarded as very promising drug targeting vehicles [1–3]. A variety of techniques

Abbreviations: Chol, cholesterol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HPTS, 8-hydroxypyrene-1,3,6-trisulfonate; HUVEC, human umbilical vein endothelial cells; IL, immunoliposomes; mAb, monoclonal antibody; NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl); N-Glut-PE, *N*-glutaryl-phosphatidylethanolamine; PEG, poly(ethylene glycol); PBS, phosphate-buffered saline; SPC, soy phosphatidyl choline

* Corresponding author. Fax: +49-345-552-7022.
E-mail address: bendas@pharmazie.uni-halle.de (G. Bendas).

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exist for the preparation of IL which differ in their strategies for the antibody coupling onto the liposomes. It could be shown that the sterical stabilization of liposomes by polyethylene glycol (PEG), in order to avoid rapid removal from the circulation, can be combined effectively with antibody coupling [4,5]. Principally, two types of sterically stabilized IL exist: (i) conventional IL with antibodies directly coupled onto the liposomal surface in the presence of PEG [6,7] and (ii) IL with antibodies coupled onto the terminal ends of the grafted PEG-chains [8–10]. The latter could be demonstrated to be more advantageous for targeting because of its exposed accessible and flexible antibody position.

The most attractive therapeutic IL application has been in the field on chemotherapeutic cancer treatment [11]. Furthermore, the site-specific treatment of inflammatory diseases by targeting liposomes to vascular adhesion receptors that normally mediate the recruitment and extravasation of leukocytes to inflamed tissues is of growing interest [12]. Among these adhesion receptors, E-Selectin seems to be the most attractive candidate for targeting since its expression is strictly time and spatial in relationship to the inflammation. E-Selectin is a transmembrane glycoprotein which is expressed by de novo synthesis in response to inflammatory mediators like interleukin-1 or tumour necrosis factor α in a time course of 4–24 h after activation [13]. Various studies demonstrated the involvement of an E-Selectin expression in several pathological inflammatory diseases such as rheumatoid arthritis, myocardial ischaemia or atherosclerosis [14,15]. A few studies reported on the successful targeting of liposomes or IL to E-Selectin in order to create anti-inflammatory directed drug targeting vehicles [16,17].

An accumulation of liposomes at the target site is a prerequisite, but does not in itself mediate a therapeutical effect of the encapsulated drugs. Therefore, the specific mode of liposome cell interaction has to be considered. In principle, these kinds of interactions are not to be generalized, but depend on various factors such as the kind and activation of the target cells and on several liposomal parameters like particle size, charge, sterical stabilization and specific characteristics of the homing devices. A cellular uptake of the liposomes is mostly required since most drugs act intracellularly, and liposomal gene therapy with the desired delivery of oligonucleotides or genes is of growing importance. The cellular incorporation of liposomal content can occur in different ways, namely (i) as the uptake of the soluble content which was released from the liposomes outside the cell, (ii) as a result of liposomal fusion within the cell membrane followed by an intracellular release of the liposomal content and (iii) by an active uptake of the liposomes on an endocytotic pathway.

In previous studies, we demonstrated the target binding of IL at E-Selectin-expressing cells [17] and we have continued doing these experiments with human umbilical vein endothelial cells (HUVEC). As we were able to demonstrate in this study that IL bind E-Selectin under simulated shear force conditions of capillary blood flow for a circulation and therapy relevant time of up to 18 h, we chose as our main intention of this study to analyse a possible cellular uptake of the targeted IL. There can be no prediction as to whether or not the liposomes will be taken up at all and which way will be the preferred one, because only few studies exist that dealt with liposome uptake by endothelial cells, which could rarely be compared due to the different origin of the endothelial cells [18–21]. Furthermore, various experimental techniques exist to prove an uptake of liposomes or liposomal compartments, which in most cases detect only one possible mode of internalization. Research indicates that to date no direct comparison of all these techniques applied to a certain liposome cell system has been done.

In the present study, we applied different spectroscopical and microscopical fluorescence techniques to investigate the way and the degree of internalization of E-Selectin-targeted IL by HUVEC. Since we detected that certain amounts of liposomes are taken up by different ways, our results are discussed in the context of targeted drug delivery focusing on E-Selectin's active role in internalization and to liposomal parameters. In addition, the parallel application of four different fluorescence techniques should further allow a characterization of analytical values and limitations of the individual techniques.

2. Materials and methods

2.1. Materials

Soy phosphatidylcholine (SPC) was obtained from Lucas Meyer (Hamburg, Germany). Antimycin A, cholesterol, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), sodium hydrosulfite (dithionite), calcein, cytochalasin D and 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) were purchased from Sigma (Deisenhofen, Germany). Polyethylene glycol-PE (PEG-PE 2000), Lissamine Rhodamine-PE and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The substances were used without further purification. The membrane anchors for antibody coupling Cyanur-PEG-PE and N-Glut-PE were synthesized in our group as previously described [22]. All salts and buffers were of analytical grade.

The murine anti (human) E-Selectin mAb (BBA 26) was purchased from R&D Systems (Wiesbaden, Germany).

2.2. Preparation of IL

Large unilamellar vesicles (LUV) were prepared by extruding multilamellar vesicles. For this purpose, a lipid film (10 μmol) was suspended in 1 ml aqueous solution (buffer, NaCl or 100 mM calcein) at 60°C. The resulting multilamellar vesicles were extruded six times (Extruder, Lipex Biomembrane, Vancouver, Canada) subsequently through a 100-nm and 50-nm polycarbonate membrane (Costar, Bodenheim, Germany). Vesicle size of around 70 nm was determined by dynamic light scattering using a Malvern Autosizer II c (Malvern, UK) in mass distribution mode. The lipid composition of vesicles was modified according to the coupling procedure. The basic lipid composition was SPC/Chol 2:1 (molar ratio), whereby either 5 mol% N-Glut-PE or 5 mol% Cyanur-PEG-PE were incorporated to prepare IL. The addition of PEG-PE 2000 diminished the fraction of SPC. Likewise, for fluorescence detection 2 mol% NBD-PE were incorporated in all vesicle preparations.

For all coupling procedures, an initial phospholipid/Ab molar ratio of 1000:1 was chosen, as previously optimized by Hansen et al. [7]. The coupling yield of all reactions was quantified with a protein determination assay according to Peterson and Lowry [23] and correlated to the actual liposome concentration detected by phosphate assay [24].

Liposomes containing Cyanur-PEG-PE were prepared in 0.15 M NaCl. The indicated amounts of antibodies, which were dissolved in borate buffer to adjust to a pH of 8.8 were added to this preparation and incubated at room temperature for about 16 h. Unbound antibodies were separated by passing the liposomes over a Sepharose 4B (Sigma) column and eluting them with PBS, pH 7.4.

To form a protein linkage to liposomal N-Glut-PE, 6 mg EDC was added to 10 μmol liposomes in PBS (pH 7.4) followed by an incubation period of at best 6 h at RT and by gel permeation chromatography (Sephadex G50, Pharmacia, Sweden) to remove excess EDC. Antibodies were added and incubated overnight at room temperature. The IL were separated from unbound antibodies by gel permeation chromatography by using Sepharose 4B.

2.3. Cell cultivation and cell-related materials

Human endothelial cells from umbilical cords (HUVEC) were used as target cells. HUVEC were isolated and cultured in medium 199 with Earle's salts (Gibco BRL) supplemented with 20% foetal calf serum, 1% penicillin/streptomycin, 1% essential amino acids (all c.c.pro GmbH, Germany) and 1% 1mM HEPES (pH 7.4).

Flasks seeded with freshly isolated HUVEC were incubated at 37°C in 5% CO_2 for approx. 4 days until cells were grown nearly confluent. Passage 2 or 3 cells were used for the experiments.

For comparison, HPTS endocytosis assay was performed with two further cell lines. Keratinocytes (HaCat), which were used as negative control were a kind gift from Professor Wohlrab, University of Halle. Cells were cultivated in Keratinocyte SFM (Gibco BRL) at 37°C in 5% CO_2 for 4–5 days. A murine macrophage cell line (J 774, ATCC) was a much appreciated gift from Professor Schröder, De-

partment of Pharmacy, University of Halle. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% PBS and 1% penicillin/streptomycin at 37°C in 5% CO_2 until the cells were nearly confluent.

2.4. Cell-binding studies

2.4.1. Liposome binding under static conditions

For binding studies, 96-well plates (BMG Labsystems) coated with 1% gelatine were seeded with nearly 40 000 HUVEC per well and incubated at 37°C in 5% CO_2 for 3 h until the cells formed a stable monolayer at the well bottom. Afterwards, 3 h before liposome addition HUVEC were activated with recombinant human interleukin-1β (10 ng/ml medium, E-coli, Boehringer Mannheim, Germany) at 37°C. After an exchange of the medium, liposomes containing NBD-PE were added (150 nmol/well) followed by an incubation of over 60 min at 4°C before replacing unbound liposomes, this was done by washing three times with PBS and fluorimetric detection (Fluostar Galaxy, BMG).

2.4.2. Liposome binding under simulated shear force conditions

The dynamic liposome-binding experiments were performed in a laminar flow chamber as previously described [17] with slight modifications. The polyacrylic device contains two flow chambers one beneath the other at the bottom. The chambers can be closed by round microscope slides (18 mm diameter), which were coated with ca. 500 000 HUVEC (incubation 3 h at 37°C, gelatine-coated 0.5%). The chambers allow the HUVEC layers to be rinsed with a buffer containing the liposomes (corresponding to 2 μmol/10^6 cells) for several hours at shear force conditions that simulate the capillary blood flow (shear rate of about 200 s^{-1}) by connecting a peristaltic pump. To detect the liposome-binding events at the HUVEC layer, the laminar flow chamber was mounted on the stage of an inverted laser scanning microscope (LSM 410 invert, Carl Zeiss) in combination with a fluorescent microscope Axiovert 135. In order to get an internal negative control for the binding experiments, HUVEC on one slide were always activated by interleukin-1β (3 h) while the HUVEC on the other slide were not.

2.5. Detection of an immunoliposome uptake by HUVEC

2.5.1. Dithionite technique

The chemical basis of the dithionite technique is the quenching of liposomal NBD-fluorescence, achieved by chemically reducing NBD into the corresponding amino derivative which in turn is caused by dithionite. A prerequisite for this is the use of targeted liposomes which contain NBD-PE only in the outer monolayer. Therefore, liposomes were prepared as described (see Section 2.2) and NBD-PE (2 mol%) was added to the liposomes afterwards as an ethanolic solution (about 1 vol.%) on vortexing followed by the antibody coupling techniques. The asymmetric distribution of the NBD-PE in these liposomes could be proven with quenching experiments adding dithionite solution (1 M Tris, pH 10) in a molar ratio NBD/dithionite 1/25 000.

The internalization studies were performed at 37°C for over 90 min following the binding experiments in the wells described in Section 2.4.1. Afterwards, 75 μl of a 1.5 M dithionite solution was added to the wells for 3 min. Following the washings, the remaining NBD fluorescence was analysed representing the internalized fraction.

For endocytosis blocking experiments, cells were pretreated with either antimycin A (1 μg/ml), 0.1% NaN_3 or cytochalasin D (20 μg/ml dissolved in DMSO and diluted with medium to final concentration $<1\%$). Depletion of intracellular K^+ was performed as previously described [25] using the hypotonic shock procedure. Medium was removed and cells were washed three times with buffer A (50 mM HEPES, 100 mM NaCl, pH 7.4) and incubated in hypotonic medium (medium/water 1:1) followed by a 10-min incubation in buffer A.

For dithionite experiments with microscopic detection, 500 000 HUVEC were cultivated onto slides for 3 h followed by an activation. Liposomes were added for 90 min at 37°C and images were taken after removing unbound liposomes and after dithionite reduction.

2.5.2. Pyranine assay

The absorbance characteristics of a 60 mM solution of HPTS were first analysed between 200 and 500 nm (emission 512 nm) in a pH range from 5.5 to

8.5 (Fluorimeter Hitachi Fluoroscan), in order to detect the spectroscopical changes in HPTS during acidification within the endosomes/lysosomes. For the endocytosis assay, liposomes were prepared in a 35 mM HPTS solution, whereas HPTS outside the liposomes was replaced by gel chromatography (Sephadex G50) eluted with PBS followed by an antibody conjugation. For the cell experiments, about 10^6 HUVEC (activation afterwards), keratinocytes as negative control and J774 as positive control were transferred into dishes. After cell adherence, 2 μmol of liposomes were added and incubation occurred at either 4°C or 37°C. After several washings, cells were removed from the dishes and placed in cuvettes for absorbance measurements (405, 416 and 460 nm). An acidification due to endocytotic uptake was evaluated because of an increase in the 405/416 nm quotient with a simultaneous decrease of the 460/416 nm quotient.

2.5.3. Microscopic investigation of double-labelled liposomes

In order to follow the path of liposomes within the HUVEC and to detect a liposome fragmentation, the liposomes were labelled twice. Therefore, liposomes were prepared with incorporated 100 mM calcein as a soluble label and with 2 mol% Rhodamine-PE as a membrane marker. HUVEC on glass slides and liposome incubation were handled as described in Section 2.5.1. In order to completely remove all liposomes outside the cell (bound and unbound), the cells were washed several times with a buffer followed by an incubation period with citric buffer (pH 3.0) for 3 min (acid wash) before washing again with buffer twice. Microscopic detection was performed with the confocal LSM at excitations of both 488 nm to illustrate the localization of calcein and 543 nm to detect rhodamine.

2.5.4. Fusion assay using calcein

Liposomes for these experiments were prepared in 100 mM calcein with the subsequent removal of calcein outside the liposomes by gel chromatography. HUVEC adherence and activation on glass slides were performed as described above. Liposomes were incubated at both 4°C and 37°C for 90 min. After washing and an 'acid wash', microscopic images were taken at excitation of 488 nm.

3. Results and discussion

3.1. Immunoliposome targeting under dynamic conditions

In a previous study, we demonstrated that targeting IL to E-Selectin is a promising way to achieve drug delivery at inflammatory sites [17]. Furthermore, it was evident that liposomes with antibodies coupled to the terminal ends of PEG as with our newly established 'Cyanur-PEG-PE IL' had a much higher targetability [22]. These in vitro experiments were performed under static conditions, which do not adequately simulate the physiological situation. In order to follow the fate of targeted liposomes and to obtain therapeutical advancements from this behaviour, physiological relevant information about the ability and duration of the binding are necessary. Therefore, we applied a flow chamber system, allowing IL to interact with a HUVEC monolayer out of the streaming medium at a physiological shear rate of 200 s^{-1}. The binding of NBD-labelled liposomes could continuously be documented by microscopical images in a period of about 18 h, which is relevant for immunoliposome circulation in vivo.

To evaluate these results with respect to HUVEC activation (E-Selectin expression) and type of IL, we either prepared conventional IL containing 5 mol% PEG-PE 2000 coupled with the anti-E-Selectin mAb BBA 26 at N-Glut-PE, or terminally coupled Cyanur-PEG-PE (5 mol%) IL and analysed their bindings at both activated and non-activated HUVEC.

As a control experiment, plain liposomes do not bind HUVEC under flow, neither with activated nor non-activated cells. On the other hand, both types of IL are able to bind the HUVEC out of the streaming medium, and with time the binding intensity increases cumulative. Despite the Cyanur-PEG-PE IL have a slightly lower degree of coupled antibodies (about 35 μg/μmol phospholipid corresponding to about 25 antibodies/liposome versus 49 μg/μmol or 30 antibodies/liposome for the N-Glut-PE preparations); however, they have a slightly higher targetability which is attributed to the better accessibility of the mAb. As illustrated in Fig. 1, nearly no binding occurs at inactivated HUVEC, demonstrating the specificity of binding via the E-Selectin.

These binding studies prove that both types of IL

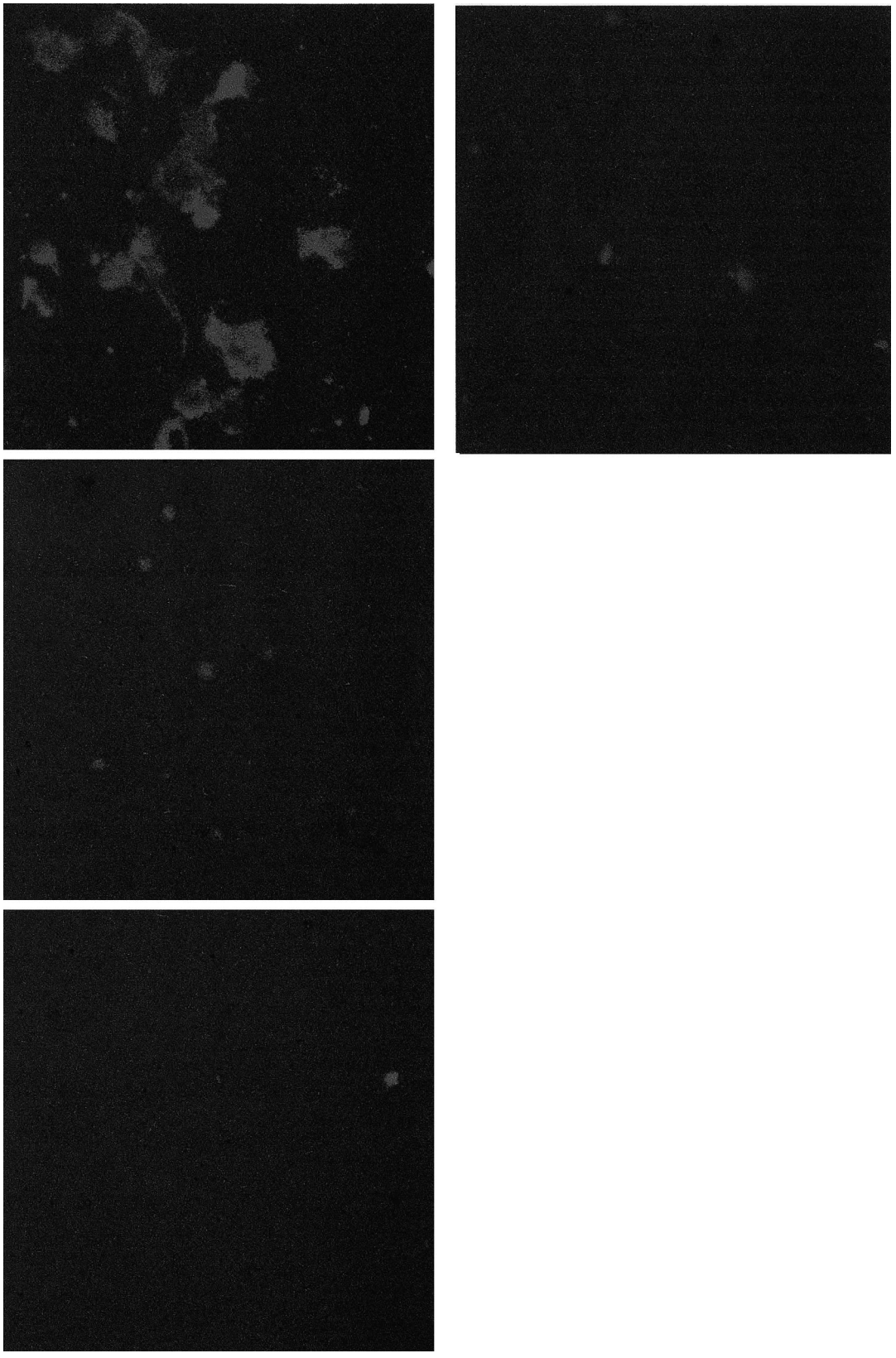

Fig. 1. Binding of NBD-labeled Cyanur-PEG-PE IL with E-Selectin mAb BBA26 onto stimulated HUVEC under shear force conditions after 10 min, 5 h and 18 h (upper line from left) compared to the binding toward non-stimulated HUVEC after 18 h (right, low).

are able to target E-Selectin under shear stress in a circulation- and therapy-relevant time range. Because binding resists the shear forces for a sufficiently long time, the fate of liposomes should be analysed further for achieving a therapeutical drug effect.

3.2. Determination of the cellular uptake of liposomes by HUVEC

Following the fate of liposomes after targeting the HUVEC, a cellular uptake of the liposomes would be desirable since this offers the most potential for drug or gene therapy. Several ways of liposome uptake exist which can be analysed by different spectroscopical or microscopical means. We first wanted to select an analytical technique which illustrates a general uptake of intact liposomes, which clearly distinguishes between surface bound and incorporated liposomes by fluorescence spectroscopy for quantification and microscopy for qualitative evaluation. We therefore chose the so-called dithionite technique where the fluorescence of NBD-labelled liposomes were quenched by chemical reduction induced by dithionite [26]. Since dithionite cannot permeate the cell membrane, liposome quenching occurs only outside the cells. Therefore, this technique offers excellent prerequisites for correlating the cellular uptake of liposomes with their cell-binding ability when considering the type of IL (conventional versus terminally coupled), sterical stabilization or the role of the coupled antibodies.

Since dithionite cannot permeate bilayers, the liposomes have to be only labelled in the outer monolayer. We performed this by adding an ethanolic solution of NBD-PE to the preformed liposomes before coupling the antibodies. In order to prove the NBD-orientation and to quantify the NBD quenching, we added a dithionite solution to these liposomes. As illustrated in Fig. 2, nearly 75% of NBD-fluorescence was quenched within the first 10 s increased to about 90% within the next 5 min, which confirms an asymmetric NBD distribution in the liposomes.

In order to compare liposome binding and cellular uptake in one experiment, we added liposomes to activated HUVEC for 60 min at 4°C (no active internalization) and after several washings, their internalization at 37°C for 90 min was studied. The amount of liposomes added was previously optimized and kept constant at 150 nmol/40 000 HUVEC per well, representing about 2×10^{12} liposomes or 5×10^{7} liposomes per cell.

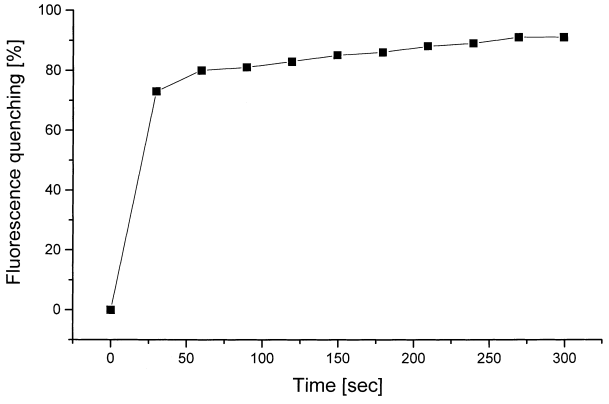

Fig. 2. Time course of fluorescence quenching after adding dithionite solution to liposomes containing 2 mol% NBD-PE in the outer monolayer (molecular ratio NBD/dithionite 1:25 000).

To demonstrate the specificity of binding via E-Selectin and cooperation with the internalizing factors, we prepared conventional (N-Glut-PE) IL with and without sterical stabilization by 5 mol% PEG 2000 and terminal-coupled Cyanur-PEG-PE IL, each as plain liposomes, coupled with irrelevant IgG or with selectin-specific IgG (BBA 26). As shown in Fig. 3A, the PEG-free N-Glut-PE liposomes with BBA 26 bind strongly to activated HUVEC, regarded as relative 100% fluorescence corresponding to about 5×10^{8} liposomes per well or 12 500 per HUVEC. This value should in part be attributed to unspecific liposome adherence, since the addition of PEG drastically reduces the amount of bound liposomes. Both data represent the specificity of binding via E-Selectin, whereas the coupled irrelevant antibodies are without effect since these liposomes behave like plain ones. Despite the same sterical stabilization, the Cyanur-PEG-PE liposomes have a much higher targetability, attributed to their exposed antibody position. Non-stimulated HUVEC were used for the control, the binding of all three BBA 26 IL was depressed to blank levels (not shown).

When evaluating the corresponding liposome internalization in Fig. 3B, the PEG-free N-Glut-PE preparation with the specific BBA 26 antibody was again regarded as relative 100%. The absolute fluorescence and the corresponding number of liposomes

(1.25×10^8 or 3100 per HUVEC) demonstrate that about 25% of targeted liposomes were internalized. This ratio is nearly true for all of the investigated preparations, regardless of the presence or the specificity of the coupled antibodies. When considering the non-complete dithionite quenching of NBD (Fig. 2) outside the cell which yielded slightly increased internalization data, it can be concluded that in all cases about 20–25% of bound liposomes were internalized. This fact speaks against an active, internalizing role of E-Selectin, otherwise all three BBA 26 preparations would have a much more distinct deviation from the others. Furthermore, the presentation of IgG is also not an internalizing factor, which could be explained due to a postulated internalizing IgG-receptor [27]. The slightly increased internalization of the Cyanur-PEG-PE liposomes is not easy to interpret.

Also in relationship to other studies, the internalization capacity seems to be low, since Mastrobattista et al. report about a sixty percent uptake of ICAM-targeted IL by epithelial cells [18].

To look for the uptake mechanisms, we modified the experimental conditions. Firstly by fixing the HUVEC with formaldehyde, active internalization was prevented. We obtained identical binding data of the liposomes at both living and formaldehyde-fixed HUVEC (data not shown), therefore, the corresponding internalization data are well to compare. The passive internalization capacity (Fig. 3C) is approximately 40–70% of the total internalization. The degree of reduction indicated no correlation to liposomal features. In general one can conclude that the liposomal uptake is mediated by active and passive processes, both are comparable in their intensities. Dithionite addition immediately after the 4°C period shows a non-complete fluorescence quenching which indicates that a part of liposomes has already been taken up by the cells under these conditions, necessarily on a passive way.

In conclusion, we can state that about 25% of the targeted liposomes were internalized by stimulated

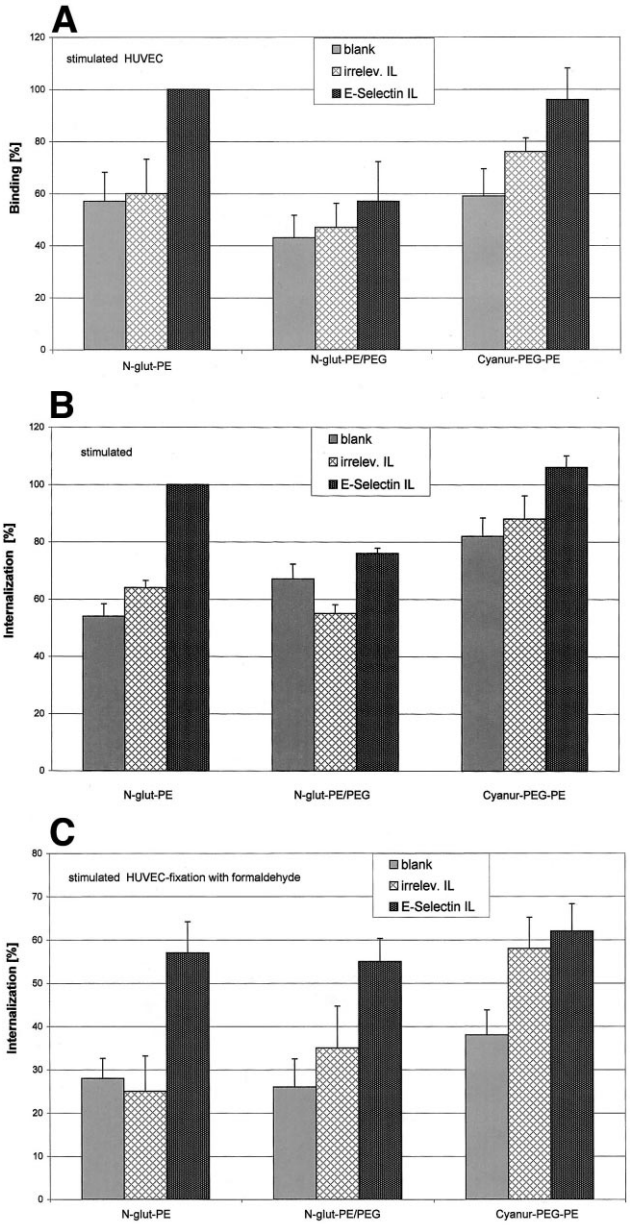

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Fig. 3. Binding and internalization of NBD-labeled immunoliposomes by stimulated HUVEC. Immunoliposomes with the E-Selectin mAb BBA26 were N-Glut-PE-coupled (conventional IL) without PEG-PE 2000 (left group), with 5 mol% PEG-PE 2000 (middle group) and Cyanur-PEG-PE-(terminally coupled) IL (right group), each compared to plain liposomes and IL coupled with irrelevant mAb. Data are means of at least four experiments ± S.D. (A) Liposome binding onto stimulated HUVEC after 60 min at 4°C. Binding data are expressed as relative fluorescence, regarding the conventional PEG-free preparation with BBA 26 as 100%. (B) Internalization of the bound liposomes (A) by stimulated HUVEC after 90 min at 37°C detected by NBD-quenching with dithionite. Data are expressed as relative fluorescence related to conventional PEG-free preparation with BBA 26 as 100%. (C) Corresponding liposome internalization data after fixing the HUVEC with formaldehyde.

endothelial cells. Active as well as passive mechanisms are involved in internalization, and a correlation between liposome functionalization and internalization is not evident.

3.3. Characterization of active liposome internalization

To define the mechanisms involved in the active internalization of the liposomes, HUVEC were pretreated with different chemical agents that interfere with various aspects of the uptake process. Cytochalasin D is known to disrupt the microfilament network by inhibiting actin polymerization and thereby blocking phagocytosis and pinocytosis, but not receptor-mediated endocytosis [28]. Antimycin A as well as a mixture of formaldehyde and NaN_3 restrict the metabolic activity of cells and thus inhibit both receptor- and non receptor-mediated endocytosis [29,30]. Cell depletion of K^+ inhibits the receptor-mediated endocytosis by removing clathrin from the plasma membrane [25,31]. The blocking experiments were performed with the PEG-free conventional IL as representatives, again using plain liposomes and irrelevant IL for control.

Internalization data in the presence of different blocking agents are illustrated in Fig. 4 which clearly demonstrate that phagocytosis is important for blank liposomes but plays only a minor role in the internalization process of the specific IL. Cytocalasin reduces the uptake of the previous ones by about 45%, whereas specific IL are reduced by about 15%. The metabolic inhibitors antimycin or formaldehyde/NaN_3 considerably decrease the liposome uptake, which in general confirms the data from Fig. 3C and the fact that internalization results, in part, from endocytosis. The most interesting data came from cell depletion of K^+. Whereas the control liposome uptake is not affected by this treatment, the entry of the selectin-directed IL is drastically reduced to the level of the blanks which indicates that only specific liposomes were taken up by a receptor-mediated process. Despite the fact that the percentage of internalization versus the binding is comparable between blanks and selectin-directed IL, these findings clearly indicate the different mechanisms of uptake. Whereas when the specific IL were taken up by a receptor-mediated process, blank liposomes were most likely internalized on other endocytic routes like phagocytosis or by caveolae. Furthermore, these data confirm that the internalizing role of selectins seems to be relatively low as outlined in Fig. 3B.

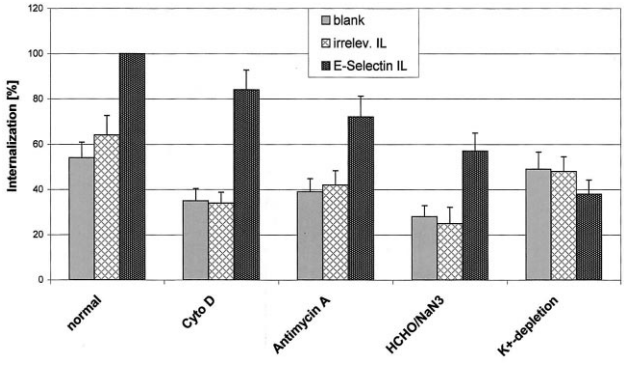

Fig. 4. Internalization of bound conventional IL without PEG-PE 2000 and their blank analogues after 90 min at 37°C by stimulated HUVEC, which have been pretreated with several agents that interfere with different routes of an endocytotic pathway. Data represent mean ± S.D. ($n = 4$).

In order to procure optical evidence for cellular liposome incorporation, we studied targeted stimulated HUVEC after dithionite addition by confocal fluorescence microscopy. A *z*-scan through the cell in Fig. 5 provides clear evidence for an intracellular localization of the liposomes. They appear as fluorescent spots in discrete areas which might be endosomal vesicles.

3.4. Evaluation of the intracellular route of liposome using HPTS

In order to follow the intracellular fate of liposomes we applied an endocytosis assay based on the pyranine dye HPTS. HPTS is a well-established, membrane-impermeant and pH-dependent fluorophore, that emits maximally at 512 nm after excitation at 405 nm in acidic medium or at 460 nm in neutral environment [32]. Therefore, the endocytotic uptake of HPTS-loaded liposomes with subsequent acidification of the dye in the endosomes can be characterized by spectral changes. The overlay of HPTS spectra at different pH displays a pH-independent (isosbestic) point at 416 nm. Consequently, increasing quotients of 405/416 nm and decreasing 460/416 nm absorbance detect a drop in pH during liposome incorporation in a concentration independ-

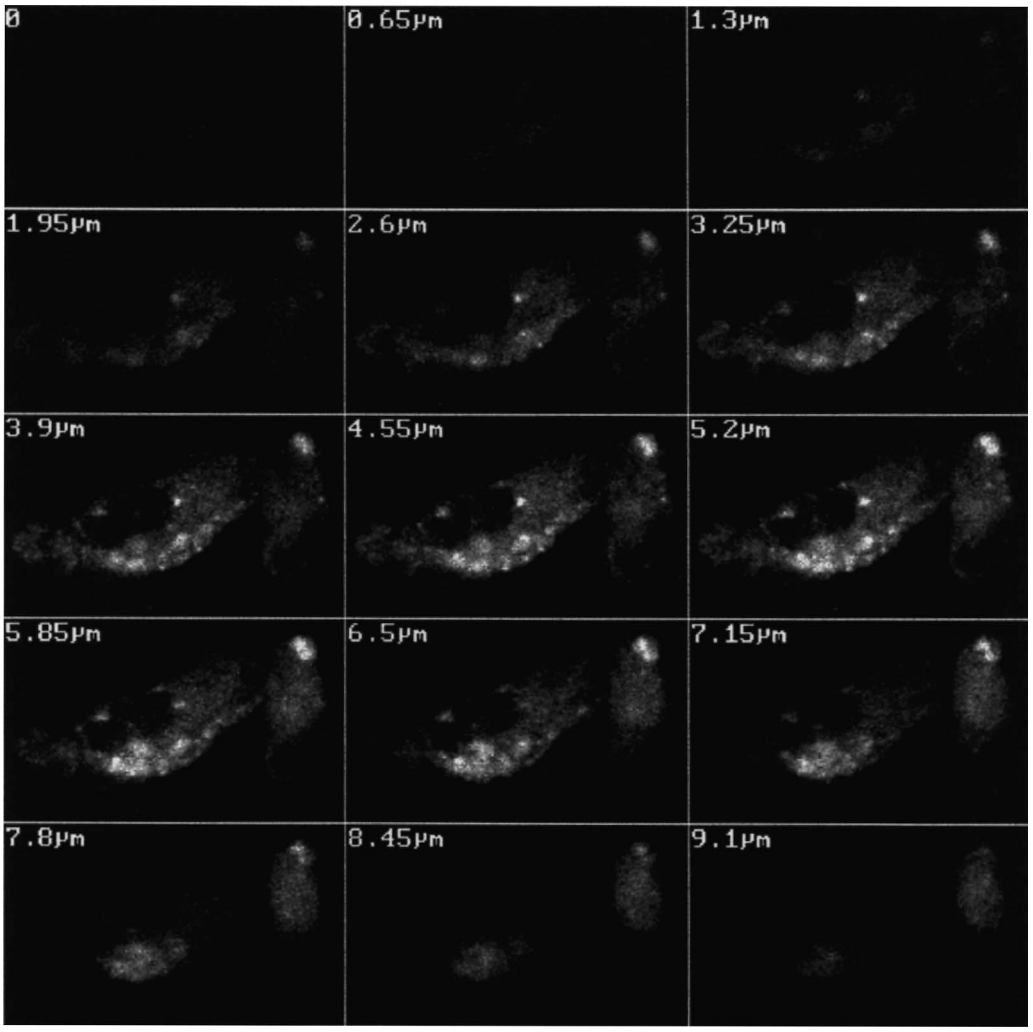

Fig. 5. Confocal laser scanning microscopic images (*z*-scan) through a stimulated single HUVEC targeted with NBD-PE-labeled conventional BBA 26-IL after fluorescence quenching (dithionite) outside the cell.

ent manner and confirm an endocytotic liposome uptake.

In order to illustrate the applicability of this technique, we studied the interaction of plain HPTS-loaded liposomes with control cells that either displayed a high degree or no ability for endocytosis. Incubation the liposomes with the highly endocytic macrophage cell line J774 at 37°C for 2 h led to a dramatic increase in the 405/416 (Fig. 6A) and a strong decrease in the 460/416 nm ratio (Fig. 6B), which is attributed to a strong endocytotic liposome uptake. In contrast, keratinocytes as a negative control showed absolutely no change in the 405/416 nm ratio and only a marginal shift in the 460/416 nm ratio within 5 h, attributed to no endocytotic activity.

Incubating the HUVEC with either the blank or the specific IL for up to 5 h at 37°C demonstrates that neither the plain liposomes nor the IL with the irrelevant antibodies provide data that can be interpreted for endocytosis. Only in the case of the selectin-directed IL, by slightly increasing 405/416 nm (Fig. 6A) and by decreasing 460/416 nm absorbance (Fig. 6B), a low but clearly detectable liposomal appearance in the endosomes could be demonstrated. These findings might confirm the differences in the uptake mechanisms.

These differences may also be a result from the

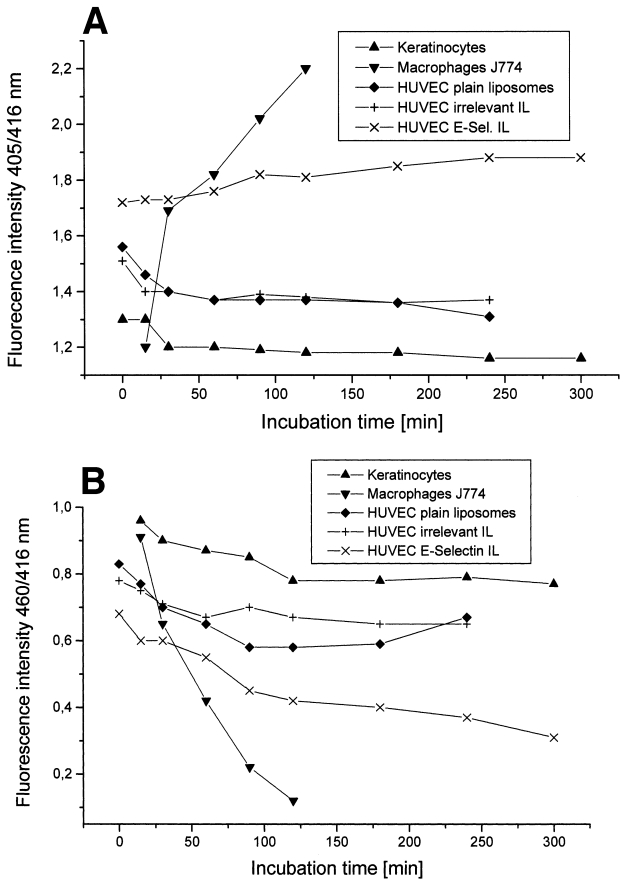

Fig. 6. HPTS-assay to detect the endocytotic uptake of conventional IL by stimulated HUVEC compared to the endocytotic activity of keratinocytes or a macrophage cell line ($n = 3$). Both the increase of the fluorescence ratio 405/416 nm (A) and the decrease of the fluorescence ratio 460/416 nm (B) are indicative as signal for an endocytotic uptake of HPTS-loaded liposomes.

sensitivity of the HPTS assay. The specific IL have the highest binding rate and consequently the highest absolute internalization, which might be detectable by HPTS while not for the other liposomes.

In general, these experiments confirm that the specific IL were taken up by endocytosis and appear in the endosomes. The HPTS assay does not allow an exact quantification of this process.

3.5. *Detection of liposomal fusion with the cells by calcein release*

In order to characterize passive liposome cell interactions we searched for liposomal fusion with the cell membrane. A common technique to follow this process is analysing the release of entrapped fluorescent dyes such as calcein. We again chose the PEG-free conventional IL with incorporated calcein at self-quenching concentrations (100 mM) as representatives and studied their cell interaction by fluorescence microscopy. Intracellular fluorescence might either be caused by liposomal fusion with the cell membrane and calcein release into the cytosol or by intact uptake of the liposomes with subsequent

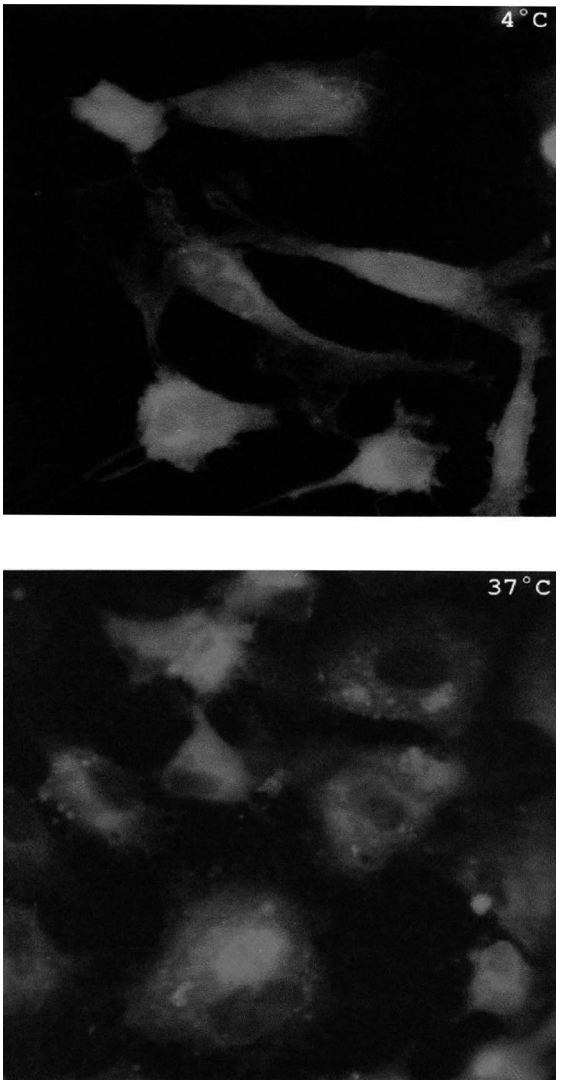

Fig. 7. Interaction of calcein-loaded conventional IL without PEG-PE 2000 with stimulated HUVEC for 90 min. At 4°C (upper image), calcein fluorescence is homogeneously distributed within the cell, attributed to liposomal fusion with the cell membrane. The fluorescence spots in the 37°C image (lower image) demonstrate an endocytotic liposome uptake in addition to fusion.

degradation in the endosomal compartments. Both processes may be differentiated by temperature, at 4°C only the passive fusion process occurs, whereas studies at 37°C detect both fusion and active liposomal uptake. Therefore, we incubated the calcein liposomes with the HUVEC simultaneously at 4°C and 37°C for 90 min, after thoroughly replacing surface-bound liposomes by 'acid wash' [33] microscopic images were taken.

At 4°C (Fig. 7, upper) a certain amount of calcein is released homogenously within the cytosol, attributed to fusion of the liposomes with the cells. This finding might be unexpected since normally liposomes should not fuse with cells without trigger mechanisms. Because we could confirm that this result is not an artefact from cell handling or liposome incubation (no release of calcein outside the cell with subsequent uptake of the free dye), fusion might be interpreted in terms of the missing sterical protection of these liposomes by PEG. Furthermore, no studies about liposome fusion with endothelial cells are available for comparison. The lower image in Fig. 7 illustrates the behaviour at 37°C, where additionally to fusion endocytosis may occur. This picture is dominated by a spot-like distribution of calcein within the cells, demonstrating the localization of calcein within intracellular vacuoles, which are most likely endosomes. Plain liposomes and those coupled with unspecific antibodies behave similar to the specific IL.

These studies give an qualitative insight into the liposomal fusion process, but do not allow a quantification. Considering the passive liposome internalization (Fig. 3C), it is not reasonable that these data are solely caused by fusion.

An additional evidence for liposome fusion is the

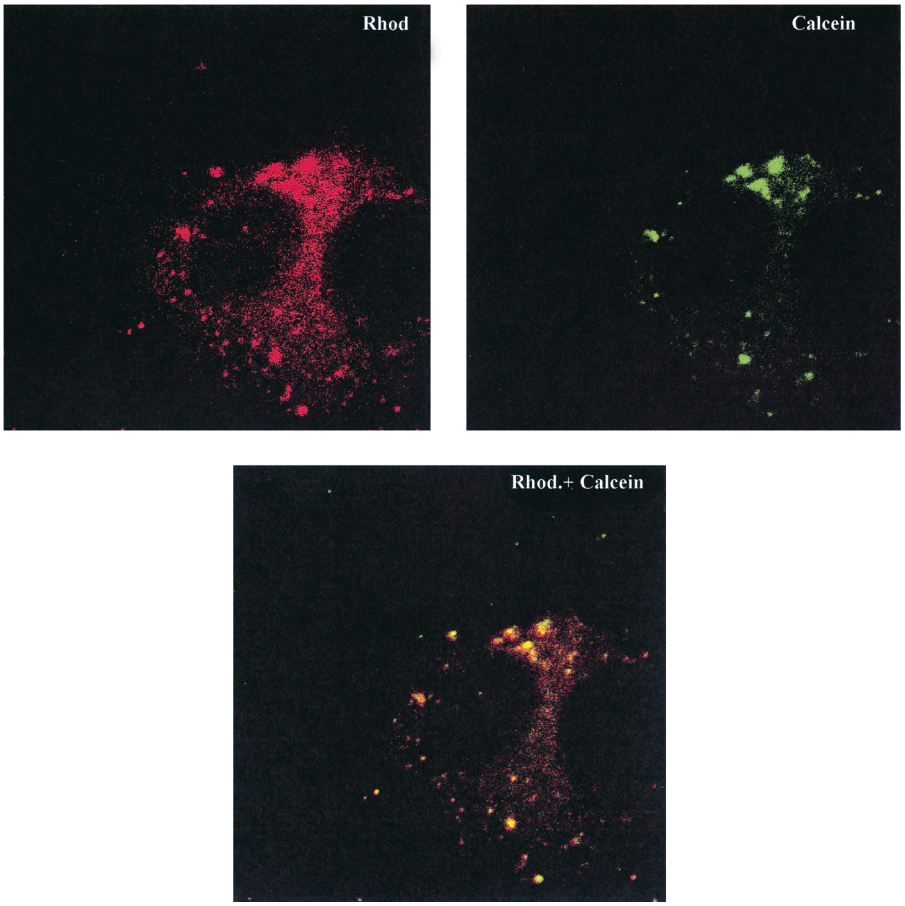

Fig. 8. Interaction of Rhodamine-PE- labelled and calcein-loaded conventional IL (without PEG-PE 2000) with stimulated HUVEC for 90 min at 37°C. Both labels were simultaneously detected (upper images); the superimposed picture of both filter settings resulting in orange is illustrated in the lower image to demonstrate the co-localization of the markers.

transfer of lipophilic markers from the liposomes to the cell membrane. Moreover, when combining a lipophilic label with an entrapped marker, which differ in their spectral characteristics, fusion can exactly be detected by separated localization of both markers, whereas a colocalization indicates the appearance of intact liposomes within the cell. Therefore, we used liposomes containing both Rhodamine-PE, as lipophilic label, and calcein. In order to detect simultaneously fusion and endocytosis, liposomes were incubated with HUVEC at 37°C for 90 min and analysed after an acid wash. The rhodamine fluorescence in Fig. 8 appears homogeneously along the cell surface, which might be indicative for fusion. The rhodamine spots within the cell are similar to Fig. 7 (37°C) were interpreted as the accumulation in vacuoles. The calcein spots with an analogue location are dominant which further confirms the appearance of vesicles inside of cellular compartments. This also becomes evident by superimposing both pictures, which results in orange spots. However, after desorbing external liposomes, no co-localization was detectable along the cell body. This separated localization of rhodamine in the cell membrane and calcein in the cytosol can be interpreted as a result of fusion.

In summary, the use of double-labelled liposomes confirms the calcein release experiments and demonstrates that IL tend to fuse with the cell membrane, whereas most of the liposomes were accumulated within the cell as a result of an active uptake.

4. Conclusions

E-Selectin-directed IL are regarded as very attractive vehicles to accumulate drugs at sites of inflammation. In the present study, we demonstrated that the IL are able to bind at interleukin-1-activated human endothelial cells (HUVEC) under simulated shear force conditions of the capillary blood flow. Binding is selectin-specific and does not occur on unstimulated cells. Since bound liposomes resist the flow for a therapy-relevant time, the fate of liposomes at the target site has to be evaluated to procure a therapeutical benefit from this behaviour. In this study, we analysed the liposomal uptake by the HUVEC with four different spectroscopical and microscopical methods.

To date, only a few studies have investigated the liposome uptake by endothelial cells. It is very difficult to compare our studies to these investigations because of the different origins of the endothelial cells. Nothing is known about the internalizing role of E-Selectin after targeting, whereas after activation E-Selectin on its own is replaced from the cell surface by endocytosis or by shedding [34].

To evaluate the influence of liposomal parameters on internalization, we used conventional IL with and without sterical stabilization, as well as IL with PEG-terminal coupled antibodies.

Despite a strong differentiated surface binding among the liposomes, with superiority being determined in terminal coupled ones, a constant fraction of about 25% of bound liposomes was taken up by the HUVEC for all types of liposomes. The dominant mechanism of liposome uptake is endocytic. Only the selectin-directed IL were be taken up by receptor-mediated endocytosis, whereas other liposomes were most likely ingested by phagocytosis. The appearance of the specific IL in the endosomal compartments after internalization could be demonstrated with the HPTS assay. The internalizing activity was directly attributed to E-Selectin and is considered relatively low. However, despite the similar internalization percentage of all of the bound liposomes, the much higher binding rate of the selectin-targeted IL leads to a considerable drug accumulation within the cell. Therefore, selectin targeting of IL toward the endothelium seems to be a suitable mode for intracellular trafficking of agents or as a basis for gene therapy which fundamentally enhances antiinflammatory therapies. This aspect can be emphasized further by the fact that the non-sterically stabilized liposomes fuse with the cell membrane and subsequently release liposomal content into the cytoplasm.

However, it must be kept in mind that despite successful liposomal internalization after targeting toward selectins, these liposomes have to be optimized for therapeutical means. An important aspect to modify the lipid composition of these liposomes is the avoidance of lysosomal degradation by creating pH-sensitive IL. These studies are currently under investigation.

In conclusion, when considering the internalization capacity of the targeted endothelial cells, a controlled

release of drugs out of the liposomes in the course of targeting and a subsequent uptake of the free drug might also be discussed as alternative.

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