In Vitro Cellular Handling and *in Vivo* Targeting of E-Selectin-Directed Immunoconjugates and Immunoliposomes Used for Drug Delivery to Inflamed Endothelium

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Purpose. Drug targeting to activated endothelial cells is now being explored as a new approach to interfere with chronic inflammation. This study compares a dexamethasone–anti-E-selectin immunoconjugate (dexa-Ab_{Esel}) with anti-E-selectin immunoliposomes (Ab_{Esel}-immunoliposomes) that contain dexamethasone, regarding *in vitro* binding and internalization as well as *in vivo* accumulation in activated endothelial cells.

Methods. *In vitro* binding and internalization of dexa-Ab_{Esel} and the Ab_{Esel}-immunoliposomes into TNFα-activated HUVECs was studied using confocal laser scanning microscopy and radiolabeled compounds. Tissue accumulation of both compounds was studied in a murine delayed-type hypersensitivity model using immunohistochemistry.

Results and Conclusions. Both preparations were selectively internalized by activated endothelial cells. Dexa-Ab_{Esel} was internalized by activated HUVECs to a larger extent than the Ab_{Esel}-immunoliposomes, although in theory the high drug-loading capacity of the liposomes may enable a larger amount of dexamethasone to be delivered intracellularly. Both dexa-Ab_{Esel} and Ab_{Esel}-immunoliposomes accumulated in activated endothelial cells in murine inflamed skin. Ab_{Esel}-immunoliposomes, but not dexa-Ab_{Esel}, were additionally detected in control skin, though to a lesser extent, and in macrophages of the liver and the spleen. Studies on therapeutic effects and side effects in models of chronic inflammation are now necessary to establish pharmacodynamics of dexa-Ab_{Esel} and/or Ab_{Esel}-immunoliposomes in the treatment of chronic inflammation.

KEY WORDS: Drug targeting; E-selectin; immunoconjugate; immunoliposome; activated endothelial cells.

INTRODUCTION

In chronic inflammation, the endothelium plays an important role in the vicious circle of ongoing leukocyte recruitment (1). An attractive therapeutic approach is to selectively target antiinflammatory drugs to activated endothelium, thereby increasing the effectiveness of the targeted drug and simultaneously diminishing systemic side effects (2). To selectively deliver drugs into activated endothelial cells, E-selectin is a suitable target molecule because its expression is restricted to activated endothelial cells (3). Furthermore, it is an internalizing molecule, which is a prerequisite for intracellular degradation of the drug-targeting preparation and release of the targeted drug, thereby leading to local pharmacologic activity (2,4,5).

In drug-targeting research, several types of drug carriers have been exploited, including particulate, soluble, and cellular carriers, with each carrier having its own advantages and limitations (6). Examples of particulate carriers are liposomes, which are regarded as useful drug-targeting vehicles because of their high drug-loading capacity, their structural versatility, and the innocuous nature of their components (7). In addition, long-circulating poly(ethylene glycol) (PEG)-coated liposomes have been developed, which circumvent rapid clearance by cells of the mononuclear phagocyte system, leading to increased systemic circulation times of these carriers (8). Specific targeting of liposomes can be achieved by attaching ligands such as antibodies to the liposomal surface, resulting in immunoliposomes. Immunoconjugates consist of drugs directly coupled to antibodies, which belong to the family of soluble carriers. They have a relatively low drug-loading capacity, especially compared to liposomes. Increasing drug loading may not only significantly hamper antigen recognition of the antibody (9) but also strongly affect the normal pharmacokinetic behavior of the carrier *in vivo* (10). Advantages of immunoconjugates are the long circulation times after systemic administration (11) and enhanced tissue penetration capacity compared to liposomes (12). This is especially important in strategies that aim at the delivery of drugs to cells located within the diseased tissues, i.e., behind the vascular wall. This is, however, of minor importance in endothelial cell-targeting strategies because these cells are readily accessible from the circulation. Consequently, no extravasation is required for the drug carrier to bind to its target cell.

Direct comparative studies on drug delivery capacity between different types of carriers are scarce, thereby impeding the choice of the optimal drug-targeting strategy for a given drug and/or disease in order to achieve the best therapeutic effects. In this study, E-selectin-directed immunoliposomes and immunoconjugates have been prepared, both preparations containing the glucocorticoid dexamethasone as a model antiinflammatory compound. These two types of drug-targeting carriers are compared regarding *in vitro* internalization kinetics into activated endothelial cells and *in vivo* homing characteristics to endothelium in inflamed tissue. This

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ABBREVIATIONS: Ab_{hEsel}, mouse antihuman E-selectin antibody (H18/7); $Ab_{mEsel(1)}$, rat antimouse E-selectin antibody (10E9.6); $Ab_{mEsel(2)}$, rat antimouse E-selectin antibody (MES-1); Dexa, dexamethasone; Dexa-P, dexamethasone phosphate; CLSM, confocal laser scanning microscopy; DTH, delayed-type hypersensitivity; PEG, polyethylene glycol.

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comparison may help to choose the most suitable carrier for endothelium-directed drug-targeting strategies for therapy of chronic inflammatory diseases.

MATERIALS AND METHODS

Antibodies

The H18/7 (mouse IgG2a antihuman E-selectin) monoclonal Ab-producing hybridoma (Ab_{hEsel}) was kindly provided by Dr. M. Gimbrone, Jr. (Boston, MA) and used for the *in vitro* studies described here. The monoclonal Ab-producing hybridoma 10E9.6 was used for production of rat IgG2a antimouse E-selectin ($Ab_{mEsel(1)}$); the rat IgG2a antimouse E-selectin antibody MES-1 ($Ab_{mEsel(2)}$) was kindly provided by Dr. D. Brown (Celltech Group, UK). Both antimouse E-selectin antibodies were used for the present *in vivo* studies. Ab_{hEsel} and $Ab_{mEsel(1)}$ were purified from the culture medium by protein A and protein G affinity chromatography, respectively (protein A and protein G sepharose fast flow, Pharmacia, Roosendaal, the Netherlands), followed by dialysis against PBS. An irrelevant control antibody (MOC31, mouse IgG1 antihuman EGP-2), hereafter referred to as Ab_{Ctrl}, was kindly provided by IQProducts (Groningen, the Netherlands).

The antidexamethasone polyclonal antibody, selectively recognizing protein-conjugated dexamethasone, was prepared and purified in our laboratory (13,14).

Preparation of Immunoconjugate and Immunoliposomes

Materials

Dexamethasone was obtained from Genfarma (Maarssen, the Netherlands), dexamethasone phosphate from Bufa (Hilversum, the Netherlands), dipalmitoylphosphatidylcholine (DPPC) from Lipoid GmbH (Ludwigshafen, Germany), cholesterol and 2-mercapto(S-acetyl)acetic acid N-hydroxysuccinimide ester (SATA) from Sigma (St. Louis, MO), PEG_{2000}-DSPE from Avanti Polar Lipids (Alabaster, AL), maleimide-PEG_{2000}-DSPE from Shearwater Polymers (Huntsville, AL), and [^{3}H]cholesteryloleylether from Amersham (Buckinghamshire, UK). All other chemicals were of analytic grade or the best grade available. Polycarbonate filters for liposome extrusion were from Costar (Cambridge, MA).

Preparation of Dexa-Ab Conjugates

Dexa-Ab conjugates were prepared and characterized as described previously (14). In short, dexamethasone-21-hemisuccinate was prepared according to McLeod (15). The introduced carboxylic acid group was subsequently reacted to primary amino groups of the Ab. After dialysis and filtration through an 0.2-μm filter, dexa-Ab conjugates were analyzed for protein content (Lowry), dexamethasone content (HPLC), and molecular size (SDS PAGE and antidexamethasone Western blotting). The prepared conjugates (dexa-Ab_{hEsel} and dexa-$Ab_{mEsel(1)}$) both contained approximately 2 dexamethasone molecules per Ab_{Esel} molecule and were stored at −20°C.

Preparation of Ab_{Esel}-(dexa-)Immunoliposomes

Stealth immuno(dexa)liposomes, with antibodies coupled at the distal end of the PEG chain, were prepared as described previously (16). In short, liposomes were composed of DPPC:cholesterol:PEG_{2000}-DSPE:maleimide-PEG_{2000}-DSPE (1.85:1:0.075:0.075). Lipids were dissolved in ethanol, mixed and dried under nitrogen pressure, and were hydrated in dexamethasone-phosphate (dexa-P)-containing HN-buffer (10 mM Hepes, 135 mM NaCl), pH 6.7, and extruded through 50-nm filters. Primary amino groups of the Ab were modified using SATA (8:1 SATA:Ab mole:mole ratio) and subsequently reacted to maleimide groups on the liposomal PEG chains. Uncoupled Ab was separated from immunoliposomes by gel permeation chromatography using Sepharose CL-4B with HN-buffer, pH 7.4, as eluent. Liposomal lipid concentration was determined by a colorimetric phosphate determination after perchloric acid destruction according to Rouser (17). Particle size and size distribution were determined by dynamic laser light scattering using a Malvern Autosizer 4700 Spectrometer (Malvern Instruments, Worcs, UK). Liposomal dexa-P content was determined by reversed-phase high-performance liquid chromatography (RP-HPLC) after extraction of the liposomes, according to Bligh and Dyer (18). Samples of the water/methanol phase were analyzed by RP-HPLC (Waters LC Module Iplus) over an Alltima RP18 column with a mobile phase consisting of water (acidified to pH 2 with phosphoric acid/acetonitrile (75:25 v/v). The HPLC method allows analysis of both dexa-P and its hydrolyzed derivative dexamethasone. The latter product, however, was never observed in the prepared liposomes. Antibody density on the liposomes was determined by protein assay according to Peterson (19). Liposomes were stored at 4°C under nitrogen and used within 3 weeks after preparation.

In this way Ab_{hEsel}- and Ab_{Ctrl}-immunoliposomes containing dexamethasone and radioactive Ab_{hEsel}-, $Ab_{mEsel(1)}$-, and $Ab_{mEsel(2)}$-immunoliposomes were prepared containing 97, 121, 42, 94, and 68 μg Ab per μmol lipid, respectively. The average particle size of PEG liposomes containing dexamethasone was 112 nm (polydispersity (pd) 0.09), which increased by 38 (pd 0.20) and 17 (pd 0.13) nm upon coupling of Ab_{hEsel} and Ab_{Ctrl}, respectively. Particle size of the radioactively labeled PEG liposomes was 105 nm (pd 0.06) and, upon coupling of Ab_{hEsel}, became 141 nm (pd 0.08), whereas the size of the $Ab_{mEsel(1)}$- and $Ab_{mEsel(2)}$-liposomes was 168 (pd 0.29) and 158 (pd 0.33) nm, respectively. Dexamethasone content of liposomes was determined to be 49.5 μg dexa-P/μmol lipid.

Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as previously described (14) and obtained from the Endothelial Cell Facility RuG/AZG (Groningen, The Netherlands). Primary isolates were cultured on 1% gelatin-precoated tissue culture flasks (Corning, Costar, The Netherlands) at 37°C under 5% CO_2/95% air. The culture medium consisted of RPMI 1640 supplemented with 20% heat-inactivated FCS, 2 mM L-glutamine, 5 U/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml endothelial cell growth factor supplement extracted from bovine brain. For the experiments described, HUVECs were used up to passage 3. The H5V mouse endothelioma cell line was kindly provided by Dr. A. Vecchi (Milan, Italy). These cells were grown in tissue culture flasks at 37°C under 5% CO_2/95% air. The culture medium consisted of DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine and 300 μg/ml gentamicin.

Confocal Laser Scanning Microscopy

Uptake of dexa-Ab_{hEsel} or dexamethasone containing Ab_{hEsel}-immunoliposomes by activated endothelial cells was assessed using Confocal Laser Scanning Microscopy (CLSM). For this, HUVECs were grown to confluence on fibronectin-coated chamber slides (Nunc, Napierville, IL) and stimulated with TNFα (rhTNFα; Boehringer, Germany), 100 ng/ml. After 4 h, dexa-Ab_{hEsel} (10 μg/ml) or dexa-P containing Ab_{hEsel}-immunoliposomes (200 nmol lipid/ml) were added to the medium. After various incubation periods, cells were washed with cold PBS and fixed with 2% paraformaldehyde/PBS overnight at 4°C. Cells were stained for Ab as described previously (14). In short, cells were preincubated with 10% normal goat serum followed by permeabilization using 0.1% saponin/PBS. Ab was detected by TRITC-labeled goat antimouse IgG (Southern Biotechnology Associates Inc., ITK Diagnostics, The Netherlands). Slides were embedded in freshly prepared antifading medium consisting of 0.25% Dabco (1,4-diazabicyclo[2.2.2]octane, Merck, Darmstadt, Germany) in 90% glycerol/PBS. Slides were examined using a confocal laser scanning microscope equiped with 488-nm argon, 568-nm krypton, and 633-nm HeNe lasers (Leica TCS-SP, Leica Microsystems, Rijswijk, The Netherlands). Images were analyzed using Leica TCS-SP Power Scan software.

Cellular Handling of Radiolabeled Dexa-Ab_{hEsel} and Ab_{hEsel}-Immunoliposomes

Dexa-Ab_{hEsel} was labeled with ^{125}I to a specific activity of 10 μCi/μg using the chloramine T method, leading to introduction of ^{125}I into the protein part of the conjugate (20). Ab_{hEsel}-immunoliposomes were prepared as described above, including a trace amount (1 μCi/μmol lipid) of [^{3}H]cholesteryloleylether as a nonexchangeable liposome marker. HUVECs were grown to confluence in gelatin-coated 12-well plates and stimulated with 100 ng/ml TNFα. Radioactivity was added simultaneously with TNFα or after 4 h of stimulation, depending on the issue being addressed in the particular experiment. At indicated time points, cells were washed three times with 1% BSA/PBS and incubated with 0.1 M HCl for 10 min at 4°C to dissociate membrane-bound radioactivity (21). After two additional washes with HCl, cells were lysed by incubation with 1 M NaOH for 30 min at 37°C to release the remaining, intracellularly trapped radioactivity.

When iodinated dexa-Ab_{hEsel} was incubated, samples of the incubation medium were precipitated by adding trichloroacetic acid to a final concentration of 10% (v/v), to determine the amount of nonprecipitable, i.e., degraded, conjugate. Spontaneous release of ^{125}I from radiolabeled products was determined by incubation of the conjugate in medium without cells, at 37°C.

^{125}I samples were counted in a γ counter (RiastarTM Gamma Counting System, Packard Instrument Company, Meriden, CT); ^{3}H samples were mixed with 3.5 ml scintillation fluid (Ultimal Gold XR, Packard Biosciences, Groningen, The Netherlands), vigorously shaken for 2 h, and counted in a calibrated scintillation counter (Minaxi Tri-Carb 4000 series, Packard).

Analysis of Binding of Ab_{mEsel}-Conjugate and -Liposomes to Endothelial Cells

Binding of mouse E-selectin-directed drug-targeting preparations to H5V endothelioma cells was investigated using immunohistochemistry. H5V cells were grown to confluence on six-well plates and stimulated with 250 ng/ml TNFα for 4 h. After three washes with cold PBS, cells were trypsinized and, for immunohistochemical analysis, spun down on slides using the Shandon Cytospin 3 Cell Preparation System (Life Sciences International (Benelux) BV, the Netherlands). Cytospots were acetone-fixed, preincubated with 10% normal rabbit serum, and subsequently incubated with 10 μg/ml $Ab_{mEsel(1)}$, 10 μg/ml dexa-$Ab_{mEsel(1)}$, 1 μmol lipid/ml $Ab_{mEsel(1)}$-liposomes, 10 μg/ml $Ab_{mEsel(2)}$, or 1 μmol lipid/ml $Ab_{mEsel(2)}$-liposomes for 1 h at room temperature in a humid chamber. After extensive washing with PBS, Ab_{mEsel} was detected by incubation with horseradish peroxidase (HRP)-conjugated goat antirat IgG (Southern Biotechnology Associates Inc.), followed by HRP-conjugated rabbit antigoat IgG (DAKO, ITK Diagnostics, The Netherlands). After color development, counterstaining was performed with Mayers' hematoxylin, and slides were mounted with glycerin.

Homing of Dexa-Ab_{mEsel} and Ab_{mEsel}-Immunoliposomes in Delayed-Type Hypersensitivity Mice

Animals

Male BALB/c mice (20–25 g) were purchased from Harlan (Zeist, The Netherlands) and housed under standard laboratory conditions with free access to standard chow and acidified water. All experiments were approved by the Local Committee on Animal Experimentation and adhered to the "Principles of Laboratory Animal Care."

Delayed-Type Hypersensitivity (DTH) Model

On days 1 and 2, mice were sensitized by skin painting on the shaved abdomen with 20 μl 0.5% (v/v) 2,4-dinitro-1-fluorobenzene (DNFB, Sigma) in acetone:olive oil (4:1). On day 15, animals were challenged by application of 20 μl 0.2% (v/v) DNFB in acetone:olive oil (4:1) to shaved flank skin. Acetone:olive oil (4:1) without DNFB was applied to the other shaved flank, which served as a negative, noninflamed control.

Immunoconjugate and Immunoliposome Homing Studies

Antimouse E-selectin immunoliposomes were prepared, containing either $Ab_{mEsel(1)}$ or $Ab_{mEsel(2)}$. Distribution of the native Ab_{mEsel} antibodies, the dexa-$Ab_{mEsel(1)}$ conjugate, and both types of immunoliposomes was studied immunohistochemically in DTH mice 24 h after DNFB challenge. Mice were anesthetized (Isoflurane/N_2O/O_2 inhalation), and 10 μg $Ab_{mEsel(1)}$, $Ab_{mEsel(2)}$ or dexa-$Ab_{mEsel(1)}$, or 1 μmol total lipid $Ab_{mEsel(1)}$-liposomes or $Ab_{mEsel(2)}$-immunoliposomes was administered via the penile vein. One hour after administration, mice were killed, and organs were excised and frozen in isopentane (–80°C). Acetone-fixed cryostat sections (4 μm) were blocked with 10% goat serum and subsequently double-stained for the endothelial cell marker CD31 (rat antimouse CD31-FITC, BD Pharmingen, Alphen a/d Rijn, The Netherlands) and the presence of Ab_{mEsel} (TRITC-conjugated goat antirat IgG, SBA). In the case of dexa-Ab_{mEsel}, double staining was also performed for CD31 and conjugated dexamethasone [antidexamethasone polyclonal

antiserum, followed by TRITC-conjugated swine antirabbit IgG (DAKO) and TRITC-labeled goat antiswine IgG (Jackson Immunoresearch)]. Nuclear counterstaining was performed using 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, Boehringer Mannheim, Mannheim, Germany). Sections were embedded in glycerol/PBS (9/1 v/v) and examined using a fluorescence microscope (DM RXA, Leica) equiped with a Kappa CF8/1 FMC camera (Kappa Optoelectronics, Gleichen, Germany) and Leica Q600 Qwin software (Qwin V01.06, Leica, Cambridge, UK).

RESULTS

Confocal Laser Scanning Microscopy (CLSM)

CLSM, which allows three-dimensional visualization of the endothelial cells, was used to determine the internalization pathway in endothelial cells of the drug-targeting preparations under investigation. Ab_{hEsel}-dexa-immunoliposomes (Fig. 1A) were present intracellularly in vesicle-like structures in preactivated HUVECs after 1 h of incubation, as determined by detection of the Ab_{hEsel} molecule. A similar vesicle-like punctuate staining pattern was observed for dexa-Ab_{hEsel} (Fig. 1B) and unmodified Ab_{hEsel} (Fig. 1C). Control drug-targeting preparations containing an irrelevant antibody (Ab_{Ctrl}-liposomes and dexa-Ab_{Ctrl} conjugate) did not bind to and were not taken up by activated HUVECs. Furthermore, both dexa-Ab_{hEsel} and Ab_{hEsel}-immunoliposomes were not taken up by resting endothelial cells (data not shown), which is in accordance with the absence of E-selectin expression by resting cells, and the results obtained with the cell-binding experiments using radiolabeled compounds.

Cellular Handling of Radiolabeled Dexa-Ab_{hEsel} and Ab_{hEsel}-Immunoliposomes

Radioactive binding experiments were performed to investigate specificity of binding to E-selectin and to further quantify binding and internalization characteristics of the protein and liposome drug-targeting preparations.

E-Selectin Specificity

Radiolabeled Ab_{hEsel}-immunoliposomes (10 nmol lipid/ml) bound specifically to activated HUVECs via E-selectin because binding was inhibited by excess unlabeled Ab_{hEsel} (10 μg/ml) but not by excess irrelevant Ab_{Ctrl} (10 μg/ml) (Fig. 2). PEG-liposomes without conjugated Ab_{hEsel} did not bind to either resting or activated endothelial cells, as expected. Similarly, binding of [^{125}I]dexa-Ab_{hEsel} to activated HUVECs was inhibited by excess unlabeled Ab_{hEsel}, not by excess irrelevant Ab_{Ctrl} (data not shown, (33)).

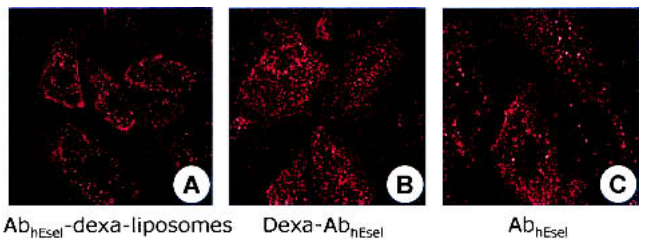

Fig. 1. Binding and internalization of Ab_{hEsel}-dexa-immunoliposomes (A), dexa-Ab_{hEsel} (B), and unconjugated Ab_{hEsel} (C) (in)to vesicle-like structures by activated HUVECs, as determined by confocal laser scanning microscopy. After 1 h of incubation with TNFα-stimulated HUVECs, Ab_{hEsel} was detected using TRITC-conjugated goat antimouse IgG (original magnification ×630). Ab_{Ctrl}-immunoliposomes did not bind to activated endothelial cells, nor did Ab_{Esel}-immunoliposomes bind to resting endothelial cells (data not shown).

Internalization Kinetics

Next, internalization kinetics were compared by incubating the immunoconjugate and the immunoliposomes at non-saturating conditions (tracer doses of 10 ng/ml and 10 nmol lipid/ml, respectively) with cells already expressing E-selectin. As seen from the steeper slope of the line that represents internalized conjugate (Fig. 3B, black triangles) compared to the line that represents internalized liposomes (Fig. 3A, black triangles), dexa-Ab_{hEsel} was internalized faster than Ab_{hEsel}-immunoliposomes. Dexa-Ab_{hEsel} was also internalized to a larger extent than Ab_{hEsel}-immunoliposomes within the studied time frame, with 46.8% versus 19.2% of total (intracellular plus membrane bound) cell-associated radioactivity being intracellularly present after 2 h of incubation (Fig. 3).

Concentration-Dependent Dexamethasone Uptake

To further compare the amount of dexamethasone that can be delivered intracellularly by both types of drug carrier, activated endothelial cells were incubated with a concentration range of both drug-targeting preparations. Subsequently, membrane-bound and intracellularly present protein or lipo-

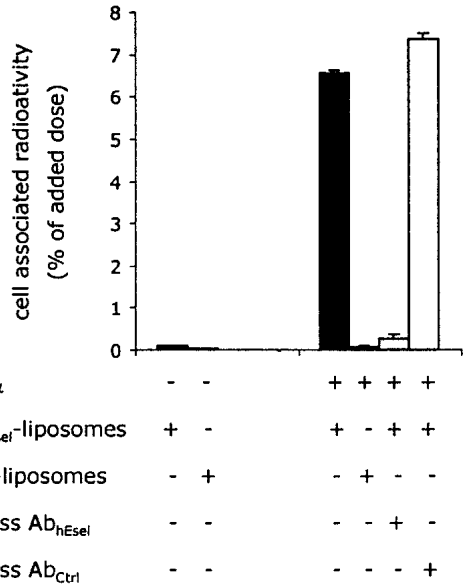

Fig. 2. Radiolabeled Ab_{hEsel}-immunoliposomes bind specifically to E-selectin expressed by activated endothelial cells. Ab_{hEsel}-immunoliposomes or unmodified PEG-liposomes (both 10 nmol lipid/ml) were incubated with HUVECs for 6 h in the absence or presence of TNFα (100 ng/ml). Binding and/or uptake of Ab_{hEsel}-immunoliposomes (in)to activated endothelial cells could be blocked with excess Ab_{hEsel} and not with excess Ab_{Ctrl} (both 10 μg/ml). Values represent mean ± S.D. (n = 3); + indicates incubation with the particular compound. Similarly, dexa-Ab_{hEsel} bound specifically to activated endothelial cells (33).

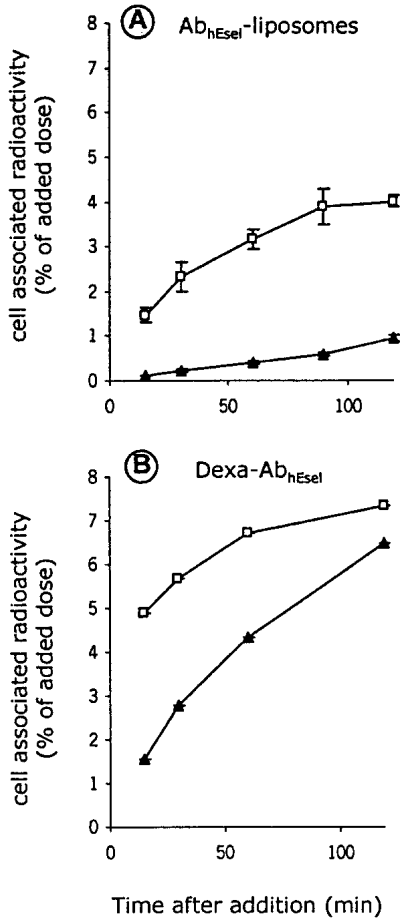

Fig. 3. Time-dependent binding and uptake of radiolabeled Ab$_{hEsel}$-immunoliposomes (A) and dexa-Ab$_{hEsel}$ (B) (in)to activated endothelial cells. Ab$_{hEsel}$-immunoliposomes (10 nmol/ml) and dexa-Ab$_{hEsel}$ (10 ng/ml) were incubated for different time periods with TNFα-activated HUVECs (100 ng/ml, 4 h). Surface-bound *(white squares)* and internalized *(black triangles)* radiolabel was subsequently determined as described in the text. Values represent mean ± S.D. (n = 3).

somal preparations were determined. The Ab$_{hEsel}$-immunoliposomes used did not contain dexa-P in this particular experiment, but on the basis of earlier studies such as the confocal laser scanning microscopy, it is assumed that drug loading of liposomes does not affect binding and internalization characteristics. The theoretical amount of delivered drug was calculated assuming a drug loading based on achieved encapsulation efficiencies of 50 μg dexa-P/μmol lipid. To compare the delivery capacity, we calculated the absolute number of added carrier molecules by converting conjugate and liposome concentrations from micrograms of protein per milliliter and micromoles lipid per milliliter, respectively, to moles of carrier molecules per milliliter. For liposomes, this calculation was performed assuming unilamellar liposomes with a diameter of 105 nm and an average area of a phospholipid molecule of 75 Å^2, resulting in approximately 80,000 lipid molecules (22) and 8,000 dexamethasone molecules per liposome. For the conjugates, the calculation was performed assuming a molecular weight of 150,000 for the antibody.

As can be observed in Fig. 4, both carriers showed saturation of dexamethasone delivery at the highest concentration

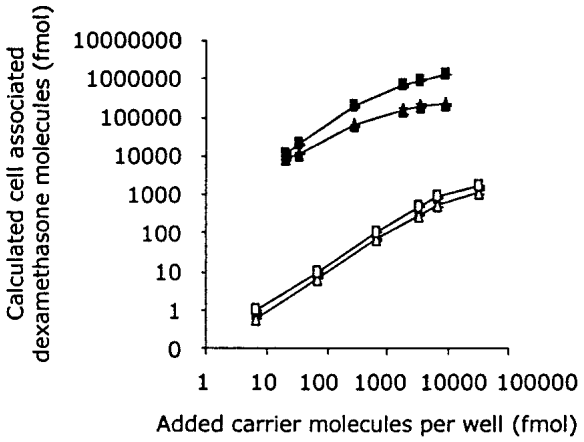

Fig. 4. Concentration-dependent binding and internalization of radiolabeled Ab$_{hEsel}$-immunoliposomes and dexa-Ab$_{hEsel}$ (in)to activated endothelial cells. Liposome and conjugate concentrations were converted from micromoles of lipid and micrograms of protein, respectively, to moles of carrier molecules. If a drug load of 50 μg dexamethasone phosphate is achieved per micromole of lipid, there will be approximately 8,000 dexamethasone molecules per liposome and 2 moles of dexamethasone per mole of Ab$_{hEsel}$ protein. The binding *(squares)* and internalization *(triangles)* of Ab$_{hEsel}$-immunoliposomes *(black)* resulted in more extensive delivery of dexamethasone than delivery by dexa-Ab$_{hEsel}$ *(white)*. Values represent mean ± S.D. (n = 3).

tested. However, maximal binding and uptake were reached at a lower number of added liposomes compared to the conjugate. Furthermore, a strikingly large difference was observed in dexamethasone delivery capacity between the two carrier types, favoring the Ab$_{hEsel}$-immunoliposomes approximately 1,000-fold over the dexa-Ab$_{hEsel}$ immunoconjugate. This difference mainly results from the difference in drug:carrier loading ratio, which is approximately 8,000, and 2 moles of dexamethasone per mole Ab$_{hEsel}$-immunoliposome and dexa-Ab$_{hEsel}$ conjugate, respectively.

Homing of Dexa-Ab$_{mEsel}$ and Ab$_{mEsel}$-Immunoliposomes in Delayed-Type Hypersensitivity Mice

The rationale behind drug-targeting strategies is to selectively deliver drugs into the cell type of interest while simultaneously decreasing distribution of the drug to other sites in the body, thereby diminishing drug-associated side effects. In this respect, the *in vivo* tissue accumulation of drug-targeting preparations is an important feature to be studied in an early phase of their development. Here, the targeting of dexa-Ab$_{mEsel}$ and Ab$_{mEsel}$-immunoliposomes to inflamed tissue was studied in a murine delayed-type hypersensitivity model in which E-selectin is expressed on endothelial cells in the affected skin at the particular time point studied (23).

One hour after intravenous injection of Ab$_{mEsel(1)}$ or dexa-Ab$_{mEsel(1)}$, both preparations could clearly be detected in inflamed skin on staining for the carrier protein Ab$_{mEsel(1)}$ (Fig. 5A,B). In the case of dexa-Ab$_{mEsel(1)}$, conjugated dexamethasone was detected in inflamed skin (Fig. 5F). Staining colocalized with CD31 expression and was absent in unaffected skin (Fig. 5G,H,L), liver (Fig. 5M,N), and spleen (Fig. 5R,S), indicating endothelial cell-specific binding/uptake of both Ab$_{mEsel(1)}$ and dexa-Ab$_{mEsel(1)}$.

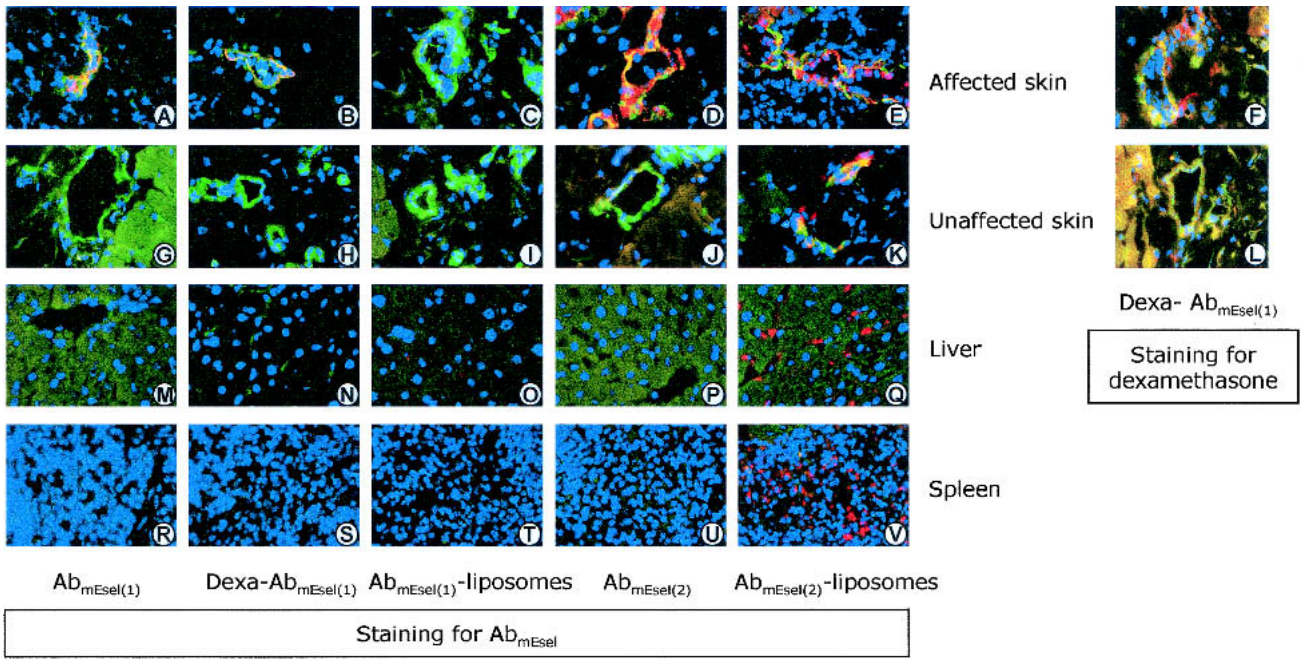

Fig. 5. Distribution of the various E-selectin-directed drug-targeting preparations in mice with a local skin inflammation (DTH model). Twenty-four hours after challenge, mice were intravenously injected with native $Ab_{mEsel(1)}$, dexa-$Ab_{mEsel(1)}$, $Ab_{mEsel(1)}$-immunoliposomes, native $Ab_{mEsel(2)}$, and $Ab_{mEsel(2)}$-immunoliposomes. As described in the text, staining in all tissues was performed for Ab_{mEsel} *(red)*. In the case of dexa-$Ab_{mEsel(1)}$, staining for protein-conjugated dexamethasone was also performed *(red)*, which colocalized with the endothelial cell marker CD31 *(green color)*. Nuclear counterstaining was performed using DAPI (original magnification ×400).

In the case of $Ab_{mEsel(1)}$-immunoliposomes, staining of Ab_{mEsel} was not detected in any of the tested organs (Fig. 5C,I,O,T). Subsequent binding experiments using TNFα-activated H5V murine endothelioma cells showed loss of E-selectin recognition of the prepared immunoliposomes and/or loss of detection of the coupled $Ab_{mEsel(1)}$ (Fig. 6E,F). This may likely account for lack of detection of the liposomes *in vivo*. Because $Ab_{mEsel(1)}$ appeared to be sensitive to radiolabeling (unpublished observations) as well as chemical reaction during the described immunoliposome preparation, another rat antimouse E-selectin antibody ($Ab_{mEsel(2)}$) was subsequently used for Ab_{mEsel}-immunoliposome preparation.

In vitro analysis demonstrated that $Ab_{mEsel(2)}$-immunoliposomes associated with activated H5V endothelial cells and not with resting endothelial cells, indicating maintenance of E-selectin binding capacity of $Ab_{mEsel(2)}$ on liposome preparation (Fig. 6I,J). As a control for the $Ab_{mEsel(2)}$-liposomes, the native $Ab_{mEsel(2)}$ showed a similar distribution pattern as unmodified $Ab_{mEsel(1)}$, implying selective binding to activated endothelial cells in inflamed skin (Fig. 5D,J,P,U). In contrast, $Ab_{mEsel(2)}$-immunoliposomes were detected in both inflamed (Fig. 5E) and unaffected skin (Fig. 5K), although to a lesser extent in the latter tissue. The immunoliposomes were also detected in macrophages of the liver (Fig. 5Q) and the spleen (Fig. 5V). Because the antidexamethasone antibody recognizes only protein-conjugated dexamethasone and not soluble dexa-P incorporated in the immunoliposomes, the presence and colocalization of drug and liposome carrier could not be determined.

DISCUSSION

The study described here compared the immunoconjugate dexa-Ab_{Esel} and Ab_{Esel}-(dexa)-immunoliposomes regarding *in vitro* internalization and *in vivo* targeting to activated endothelial cells. *In vitro*, dexa-Ab_{Esel} was taken up by TNFα-activated endothelial cells to a larger extent compared to the Ab_{Esel}-immunoliposomes in the studied time frame. The percentage of added immunoliposomes that was internalized is comparable with values described in literature (24). Comparison of the concentration-dependent uptake of both drug-targeting preparations demonstrated saturation of E-selectin binding of the Ab_{hEsel}-immunoliposomes at a slightly lower concentration of added drug carrier molecules compared to dexa-Ab_{hEsel}. This difference is likely explained by the presence of multiple Ab_{hEsel} molecules on the surface of one liposome, thereby occupying multiple E-selectin molecules per liposome. However, because of their high drug-loading capacity, Ab_{hEsel}-immunoliposomes will be able to deliver a larger amount of dexamethasone into the endothelial cells than will dexa-Ab_{hEsel} despite their low internalization rate and saturation of binding at a lower concentration.

The relatively low extent of internalization of Ab_{Esel}-immunoliposomes, compared to the dexa-Ab_{hEsel} conjugate, may result from the presence of multiple E-selectin binding sites on the liposome. As is the case for leukocyte binding to E-selectin on activated endothelial cells, Ab_{Esel}-liposome binding to E-selectin may lead to cross-linking of E-selectin. Upon cross-linking, dephosphorylation of serine residues in the cytoplasmic tail of the E-selectin molecule occurs (25), which may lead to sustained E-selectin expression, as phosphorylation of cytoplasmic serines controls E-selectin internalization (26). Another possible disadvantageous consequence of E-selectin cross-linking by immunoliposomes may be prolonged stimulation of activated endothelial cells because transmembrane signaling upon cross-linking of E-selectin has recently been described (27). By formation of the

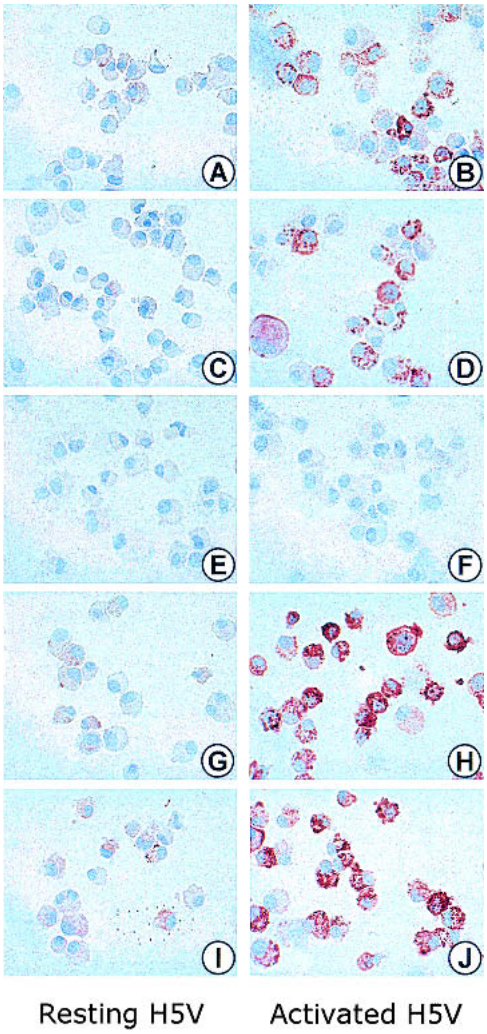

Fig. 6. Binding of $Ab_{mEsel(1)}$ (A,B), dexa-$Ab_{mEsel(1)}$ (C,D), $Ab_{mEsel(1)}$-liposomes (E,F), $Ab_{mEsel(2)}$ (G,H), and $Ab_{mEsel(2)}$-liposomes (I,J) to H5V cells, as determined by immunohistochemistry. Resting (left) or activated (right) H5V cells were incubated with 10 μg/ml (dexa-)Ab_{mEsel} or 1 μmol lipid/ml Ab_{mEsel}-liposomes, as described in the text. Binding was analyzed with anti-Ab_{mEsel} staining (original magnification ×400).

Ras/Raf-1/phospho-MEK macrocomplex, activation of extracellular regulated protein kinase (ERK1/2) and up-regulation of c-fos takes place. Future experiments studying pharmacologic effects will have to unravel the therapeutic consequences of the advantages and disadvantages of both drug-targeting preparations.

The homing experiments using dexa-$Ab_{mEsel(1)}$-immunoconjugate and $Ab_{mEsel(2)}$-immunoliposomes showed accumulation of both drug-targeting preparations in endothelial cells in inflamed skin. In the case of dexa-$Ab_{mEsel(1)}$, both the antibody part as well as the dexamethasone part of the conjugate was clearly detected in the activated endothelial cells. This confirmed the selective homing of the intact conjugate. The conjugate was not detected in unaffected skin, spleen, and liver, whereas the $Ab_{mEsel(2)}$-immunoliposomes were detected in both affected and unaffected skin, although in the latter to a lesser extent. They were also detected in the liver and the spleen, most likely in macrophages present in these tissues. This suggests a more specific distribution of dexa-$Ab_{mEsel(1)}$ to activated endothelial cells compared to $Ab_{mEsel(2)}$-immunoliposomes. However, the possibility should be considered that, besides a difference in body distribution, a difference in immunohistochemical detection of the Ab_{mEsel} molecule in the drug-targeting preparations can be responsible for this observation. Likely, detection of multiple Ab_{mEsel} molecules present on the liposomal membrane is more sensitive than detection of a single Ab_{mEsel} molecule present in the conjugate. Direct quantification in tissue samples using radiolabeled drug-targeting constructs will allow determination of the exact amounts of both preparations in nontarget organs such as liver and spleen. However, the accumulation of drug-targeting preparations in activated vascular endothelium is difficult to quantify with the use of radiolabeled constructs because endothelial cells constitute only a small percentage of the total cell population in a particular tissue. Therefore, the use of a dual-labeling technique is required, where accumulation of a ^{125}I-labeled binding construct is compared to binding of a simultaneously administered ^{131}I-labeled nonbinding control construct (28). This technique will also allow correction for enhanced permeability at the site of inflammation and will clarify to what extent both drug-targeting preparations are taken up in both the affected and the unaffected skin. The qualitative data presented here show, nevertheless, that both the immunoconjugates and the immunoliposomes not only bind to E-selectin *in vitro* but also are able to reach the target site to certain levels *in vivo*. The unexpected accumulation of $Ab_{mEsel(2)}$-immunoliposomes in unaffected skin cannot be readily explained. *In vitro* experiments showed selective binding and/or uptake of these immunoliposomes by activated murine endothelioma cells, indicating maintenance of antibody–antigen recognition integrity. Ab_{mEsel}-immunoliposomes are coated with PEG and thus represent so-called "stealth" liposomes. Their prolonged circulation time endows them with an improved capacity to extravasate at sites of enhanced permeability such as tumor tissue or inflammatory sites. However, despite the application of the possibly irritating vehiculum (acetone:olive oil, 4:1) to the unaffected skin, no significant edema was present 24 h after challenge, thereby diminishing the possibility of vascular leakage as an explanation for this observation. In addition, previous investigations of the DTH model showed absence of E-selectin expression in the unaffected skin at this time, studied by both RT-PCR and immunohistochemistry (M. Everts *et al.*, submitted (23)), thus eliminating a possible interaction of the liposomes with an eventual low expression level of E-selectin. It has been reported, nevertheless, that long-circulating liposomes accumulate in the skin, resulting in the case of liposomal doxorubicin hydrochloride (Doxil) in the so-called hand–foot syndrome (29).

The observed increased accumulation of $Ab_{mEsel(2)}$-immunoliposomes in the liver and spleen compared to the dexa-$Ab_{mEsel(1)}$ conjugate and the native Ab_{mEsel} antibodies can be explained by the coupling of intact antibody molecules in a random orientation to the liposomes. This facilitates recognition by the Fc-receptors on macrophages because of clustering of Fc portions of the antibody on the liposomal membrane (30). The observed accumulation in macrophages may lead to side effects of this drug-targeting preparation. The use of $F(ab)_2$ fragments instead of whole antibody molecules can be recommended to avoid this problem to a certain extent (16). Binding of liposomes to tissue macrophages will also

decrease the circulation time of the liposomes (16). This effect is, however, modest with a limited antibody density, which is used in the liposome preparations described here. Moreover, a long circulation time is of great importance when the enhanced permeability and retention effect is used for liposome localization in the inflamed site, i.e., behind the endothelium, but likely of less importance for efficient targeting to a well-accessible target cell population such as the inflamed endothelium (31). In principle, association of the Fc portion of the antibody with Fc receptors expressed on circulating leukocytes could lead to transportation of the targeting constructs to the inflamed site. However, this does not occur to a significant extent because immunohistochemical analysis, as presented in Fig. 5, indicates no significant binding of targeting compounds to adherent leukocytes in the inflammatory lesion. Moreover, pharmacokinetic studies with immunoliposomes revealed that immunoliposomes, when in circulation, are present in the serum fraction and thus are not cell-associated (16).

Published studies directly comparing different types of drug-targeting constructs are scarce. In one report, Huwyler *et al.* compared immunoliposomes and conjugates directed at the rat transferrin receptor for brain-targeting purposes. It was shown that the immunoliposomes achieved lower brain delivery than did the conjugate. However, analogous to our results, because of their high drug-loading capacity, the liposomes were calculated to be able to deliver more drug molecules to the brain (32). This study, as well as our comparison between E-selectin-directed drug-targeting preparations reported here, helps to determine the advantages and disadvantages of both drug carrier systems. This aids the selection process for the optimal drug-targeting strategy regarding drug-delivery capacity as well as *in vivo* homing potential.

To summarize, the antibody-based dexa-Ab_{hEsel} conjugate was internalized at a higher rate by activated endothelial cells *in vitro* than the Ab_{hEsel}-immunoliposomes. However, the high number of dexamethasone phosphate molecules that can be entrapped into the liposomes will compensate for this low internalization efficiency. Importantly, the dexa-Ab_{mEsel} conjugate appeared to home more selectively to inflamed vasculature *in vivo* than the Ab_{mEsel}-immunoliposomes. Yet, the final balance between effects and side effects of Ab_{mEsel}-immunoliposomes as well as dexa-Ab_{Esel} needs to be determined and may still be favorable compared to that of unconjugated dexamethasone. Therefore, further studies investigating antiinflammatory effects in appropriate models of chronic inflammation should reveal the therapeutic benefits as well as the side-effects of both drug targeting preparations. This will be the basis to determine their potential as a novel therapeutic approach for the treatment of chronic inflammatory disorders.

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