Site-specific Inhibition of Glomerulonephritis Progression by Targeted Delivery of Dexamethasone to Glomerular Endothelium

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AbEsel; anti E-selectin antibody, BUN; blood urea nitrogen, Ct; threshold cycle value, dexa; dexamethasone, GBM; glomerular basement membrane, LDM; laser dissection microscopy, PAS; periodic acid-Schiff base, PEG; polyethylene glycol
Abstract

Glomerulonephritis represents a group of renal diseases with glomerular inflammation as a common pathologic finding. Due to the underlying immunologic character of these disorders, they are frequently treated with glucocorticoids and cytotoxic immunosuppressive agents. Although effective, use of these compounds has limitations due to toxicity and systemic side effects. In the current study we tested the hypothesis that targeted delivery of dexamethasone (dexa) by immunoliposomes to activated glomerular endothelium decreases renal injury while preventing its systemic side effects. E-selectin was chosen as a target molecule based on its disease specific expression on activated glomerular endothelium in a mouse anti-glomerular basement membrane glomerulonephritis. Site selective delivery of AbEsel-liposomes encapsulated dexamethasone strongly reduced glomerular proinflammatory gene expression without affecting blood glucose levels, a severe side effect of administration of free dexamethasone. Dexa-AbEsel-liposomes reduced renal injury as shown by a reduction of blood ureum nitrogen levels, decreased glomerular crescent formation and downregulation of disease associated genes. Immunoliposomal drug delivery to glomerular endothelium presents a powerful new strategy for treatment of glomerulonephritis to sustain efficacy and prevent side effects of potent anti-inflammatory drugs.
Introduction

The glomerulonephritides comprise a spectrum of inflammatory diseases specifically affecting the glomeruli (Eremina et al., 2003). In general, glomerular inflammation is characterized by activation of various common pro-inflammatory pathways including complement activation, production of pro-inflammatory cytokines, endothelial activation and influx of circulating leukocytes (Falk et al., 2004). As a consequence, glomerular injury and proteinuria develop.

Based on the underlying immunologic nature of glomerulonephritides, these disorders are frequently treated with glucocorticosteroids in combination with cytotoxic agents (Javaid and Quigg, 2005). Glucocorticosteroids and cytotoxic agents, such as cyclophosphamide, are potent anti-inflammatory and immunosuppressive agents, but have their limitation with regard to toxicity and side effects (Tam, 2006; Chadban and Atkins, 2005). Identifying more effective and less toxic treatment for glomerulonephritis still remains a major aim. One way to increase drug efficacy and decrease side effects of treatment is to selectively deliver the therapeutic entity into the diseased tissue. Endothelial cells have obtained considerable attention as targets for this purpose (Ulbrich et al., 2003; Kuldo et al., 2005; Kamps et al., 1997). Not only are endothelial cells in direct contact with the blood stream and consequently accessible for systemically administered therapeutics, but, they also play an essential role in the pathological process associated with glomerulonephritis. The interaction of leukocytes with activated endothelial cells is well orchestrated via processes of tethering and rolling, mediated by endothelial expression of P- and E-selectin, followed by firm adhesion through interaction between integrins of the leukocytes,
and endothelial members of the immunoglobulin superfamily, including VCAM-1 and ICAM-1 (Vestweber, 2002).

Liposomes are high capacity drug carriers that, when modified with monoclonal antibodies, can be targeted to a selective target epitope of choice (Spragg et al., 1997; Kamps and Molema, 2006). Inflammation related cell adhesion molecules, including E-selectin and VCAM-1, are attractive candidates as target epitopes on activated endothelium because of their increased expression during inflammation and their predominant endothelial location.

The current study was designed to explore the feasibility of local immunoliposomal delivery of the glucocorticosteroid dexamethasone into activated glomerular endothelium in glomerulonephritis.
Material and Methods

Preparation of Immunoliposomes

Synthesis of immunoliposomes was as described earlier (Bartsch et al., 2005; Kamps et al., 1996). In short, lipids from stock solutions of 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]-maleimide (DSPE-PEG-Mal) in chloroform : methanol (9 : 1), were mixed in a molar ratio of 55 : 40 : 4 : 1, dried under reduced nitrogen pressure, dissolved in cyclohexane, and lyophilized. The lipids were then hydrated in HN buffer (10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (Hepes), 135 mM NaCl, pH 6.7) or when appropriate in an aqueous solution of 75-100 mg/ml dexamethasone disodium phosphate. The liposomes formed were sized by repeated extrusion (13 times) through polycarbonate filters (Costar, Cambridge MA, USA), pore size 50 nm, using a high pressure extruder (Lipex, Vancouver, Canada). The monoclonal rat anti-mouse E-selectin antibody, (MES-1, kindly provided by Dr. D. Brown, Celltech group, UK) and irrelevant rat IgG (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) were thiolated by means of N-succinimidyl-S-acetylthioacetate, and coupled to a maleimide group at the distal end of the polyethylene glycol chain (PEG) by a sulphydryl-maleimide coupling technique. The immunoliposomes were characterized by determining protein content, using mouse IgG as a standard (Peterson, 1977), and phospholipid phosphorus content (Bottcher et al., 1961). Total liposomal lipid concentrations were adjusted for the amount of cholesterol present in the liposome preparations. Per mol lipid 52.1 ± 25.6 g anti E-selectin antibody and 53.6 ± 23.1 g irrelevant rat IgG was coupled, respectively. Particle size was analyzed

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by dynamic light scattering using a Nicomp model 370 submicron particle analyzer (Santa Barbara, CA, USA) in the volume weighing model. The diameter size of AbEsel-liposomes was 114.0 ± 31.1 nm and those of IgG-liposomes 103.3 ± 16.4 nm. The number of antibody molecules coupled per liposome was calculated based on the measured concentration (C_{Ab}) of antibody (g/mol lipid) and diameter of the immunoliposomes (R) and the assumptions that the liposomes were approximately spherical. From this, and literature values for specific lipid volume (V_{ls}) of 1.25nm^3, liposome bilayer thickness (d_{bl}) of 3.7 nm (Enoch and Strittmatter, 1979) and the molecular weight of the antibody molecule (M_{Ab}) of 150 kDa, the number of antibodies coupled to the liposomes was estimated by geometric arguments according to the following formula: \( \pi/6 * C_{Ab} * (3d_{bl} * R^2 - 3R * d_{bl}^2 + d_{bl}^3) * M_{Ab}^{-1} * V_{ls}^{-1} \) (Adrian et al., 2007) to be 20 AbEsel molecules per liposome and 17 IgG molecules per liposome.

The content of encapsulated dexamethasone disodium-phosphate (Bufa, Hilversum, The Netherlands) was determined after Bligh and Dyer extraction in the resulting methanol/H_2O phase by HPLC (Melgert et al., 2000). Per µmol lipid 55.4 ± 6.5 µg dexamethasone was encapsulated.

**Animals**

C57bl/6 female mice were purchased from Harlan (Zeist, The Netherlands). Animals were maintained on mouse chow and tap water *ad libitum* in a temperature-controlled chamber at 24°C with a 12-hr light/dark cycle. All animal experiments were performed upon approval of the local Animal Care and Use Committee of Groningen University. After an adaptation period of one week, mice were allocated to the experiments as described below.
Induction of Accelerated Anti-GBM Glomerulonephritis

The IgG fraction was isolated from polyclonal sheep anti-mouse GBM serum by HiTrap Sephadex-Protein G column (GE Healthcare Europe GmbH, Diegem, Belgium). Induction of anti-GBM glomerulonephritis was modified from the earlier described protocol (Assmann et al., 1985; Heeringa et al., 2000). In short, immunization was initiated by 100 µl (2 mg/ml) commercially available sheep IgG (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) mixed with 100 µl complete Freund’s adjuvant (BD Pharmingen, Alphen aan den Rijn, The Netherlands) and injected intraperitoneally. After 6.5 days glomerulonephritis was induced by i.v. injection with 1 mg sheep anti-mouse GBM antibodies and 200 ng recombinant mouse tumor necrosis factor alpha (TNFα; BioSource Europe, Nivelles, Belgium). The total volume injected was 200 µl. Mice were sacrificed at 2 h (n=12), 24 h (n=6), 48 h (n=9), 7 days (n=5) and 14 days (n=11). Organs were perfused with ice-cold phosphate buffered saline (PBS) and harvested. One kidney was fixed in 4% formaldehyde/PBS and embedded in paraffin for morphological analysis, the other kidney, and liver, spleen and heart were snap-frozen in liquid nitrogen and stored at -80°C.

Glomerulonephritis Development and Disease Progression

Five-µm cryosections were fixed in acetone for 10 minutes and incubated with fluorescein isothiocyanate (FITC)-labeled rabbit anti-sheep IgG or -anti-mouse IgG antibodies (DAKO, Glostrup, Denmark). Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI; Boehringer, Mannheim, Germany). Sections were examined using a fluorescence microscope (DM RXA, Leica, Cambridge, UK) and Leica Q600 Qwin V01.06 software.
For morphological analysis of renal injury 4-µm formaldehyde fixed, paraffin embedded sections were stained with periodic acid-Schiff base (PAS). The amount of PAS-positive material within glomeruli was semiquantitatively scored as previously described (Heeringa et al., 2000). A glomerular crescent score was determined for each animal. Only glomeruli that had two or more layers of cells in Bowman’s space were considered crescent. The presence of PAS-positive material and glomerular crescent were expressed as mean scores obtained by two experimentally blinded observers counting between 50 and 100 glomeruli per kidney section.

**Homing Studies and Pharmacological Effects of Targeted Delivery of Dexamethasone Versus Free Dexamethasone**

For homing studies and acute pharmacological effects, dexamethasone (Genfarma, Maarsen, The Netherlands; 25 µg per mouse; n=7), AbEsel-liposomes (0.4 µmol lipid; n=4), IgG-liposomes (0.4 µmol lipid; n=3) and Dexa-AbEsel-liposomes (0.4 µmol lipid, 25 µg dexamethasone phosphate; n=6), respectively, were i.v. injected at the onset of glomerulonephritis induction. To analyze pharmacological effects at day 14, mice (n=6 per treatment) were i.v. injected twice with, respectively, AbEsel-liposomes, Dexa-AbEsel-liposomes, Dexa-IgG-liposomes and free dexamethasone, at the onset of glomerulonephritis induction, and at day 7 (dosages as above). In the group treated with empty AbEsel-liposomes, one mouse lost more than 15% body weight and was taken out of the experiment at day 4.

**Blood and Urine Examinations**

Blood urea nitrogen (BUN) was measured in plasma collected at the time of sacrifice by an enzymatic degradation assay on a Vitros 250 automated analyzer (Johnson &
Johnson, South Brunswick, NJ). Glucose concentration was determined in plasma using YSI 2300 STAT glucose oxidase analyzer. For the collection of urine mice were placed for 24 h in individual metabolic cages. Urinary albumin content was measured in 96-wells microplates (NalgeNUNC International Rochester, NY) with the mouse albumin enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories, Montgomery, TX) using purified mouse albumin as a reference. Plasma and urinary creatinine levels were measured by enzymatic colorimetric method using Creatinine plus (Roche, Woerden, The Netherlands), which allows precise and specific quantification of creatinine (Keppler et al., 2007). The creatinine clearance (C_Cr) was calculated according to the formula C_Cr = C_r urine x V_ urine (ml per min) x [C_r plasma]^{-1}.

*Quantitative Gene Expression Analysis by Realtime RT-PCR*

Total RNA was isolated from ten 8-µm cryosections with RNaseqy mini kit (Qiagen Benelux B.V., Venlo, The Netherlands) including a DNase treatment on the column. RNA integrity was studied by gel electrophoresis and RNA yield (OD260) and purity (OD260/ OD280) were measured by ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). One microgram RNA was reverse transcribed using SuperscriptIII reverse transcriptase (Invitrogen, Breda, The Netherlands) and random hexamer primers (Promege, Leiden, The Netherlands). Quantitative PCR amplifications were performed according to manufacturers protocol on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Primers and probes for E-selectin, VCAM-1 and ICAM-1 were purchased as customized assays from Applied Biosystems. These primer/probes span multiple exons and were designed according to the same quality
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criteria as the Assays-on-Demand primer/probes used. Sequences and accession numbers are shown in Table 1. All PCR reactions were carried out in triplicate.

Relative quantitation of gene expression was calculated based on the comparative Ct method ($\Delta C_t = C_t \text{gene of interest} - C_t \text{housekeeping gene}$). Comparison of gene expressions in different samples was performed based on the differences in $\Delta C_t$ of individual samples ($\Delta \Delta C_t$).

Immunohistochemical Analysis of Cell Adhesion Molecules, Immune Cell Infiltrates and Localization of Immunoliposomes

Five-µm cryosections were fixed in acetone for 10 min and incubated for 45 min with primary rat anti-mouse monoclonal antibodies: anti-E-selectin (MES-1), anti-VCAM-1 (clone M/K-2.7; ATCC, Manassas VA, USA) anti-neutrophil (NIMP-R14) and anti-monocyte/macrophage (FA-11). Endogenous biotin was blocked by Biotin Blocking System (Dako) and peroxidase activity was blocked by incubation with 0.1% H$_2$O$_2$ in PBS for 10 min. Subsequently, sections were incubated for 1 h with biotin labeled rabbit anti-rat antibodies (DAKO; dilution 1:300 in PBS) in the presence of 5% normal mouse serum and 5% normal sheep serum, and incubated for 30 min with Streptavidine-ABComplex-HRP (DAKO). Peroxidase activity was detected with 3-amino-9-ethylcarbazole (AEC; Sigma-Aldrich Chemie) and sections were counterstained with Mayer’s hematoxylin (Klinipath, Duiven, The Netherlands). Isotype matched controls were carried out for all primary antibodies and consistently found to be devoid of staining. Immunostaining of E-selectin in glomeruli was quantified by counting the number of glomerular cross sections expressing E-selectin. Glomerular infiltrates were evaluated by counting the number of stained cells in...
glomeruli. Results were expressed as mean scores obtained by two experimentally blinded observers counting between 50 and 100 glomeruli per mouse.

To analyze accumulation of immunoliposomes in kidney, heart, liver and spleen immunohistochemical staining for i.v. injected liposomal anti-E-selectin antibodies was performed as described above, except for omitting incubation with primary antibody. For quantitative analysis of staining of immunoliposomes in glomeruli morphometric analysis of 50 glomeruli per mouse was carried out using Leica QWin image analysis software. Numbers represent percentage of stained area within glomeruli.

*Laser Dissection Microscopy (LDM) to Isolate Glomeruli*

Five-µm cryosections were mounted on 1.35 µm thin polyethylene-naphtalene membranes attached to normal 1 mm slides (P.A.L.M. Microlaser Technology AG, Bernried, Germany), fixed in acetone (3 minutes) and stained with Mayer’s hematoxylin (2 min, 25 µl per section), washed with DEPC-treated water (10 sec, 25 µl per section) and air-dried. Glomeruli were dissected using the Laser Robot Microbeam System (P.A.L.M. Microlaser Technology). Eight hundred dissected glomeruli were collected directly in RNA lysis buffer (Stratagene Europe, Amsterdam, The Netherlands) in the cap of a microcentrifuge tube via laser pressure catapulting. Extraction of total RNA and DNase treatment were carried out according to the protocol of StrataPrep total RNA microprep kit (Stratagene). Quantitative RT-PCR was carried out as described above.

*Statistic Analysis*
Statistical significance of differences was studied by means of the two-sided Student’s t-test, assuming equal variances, except for semiquantitative scoring of PAS positive material. In the latter, statistical significance of differences was calculated by Mann-Witney nonparametric test. Differences were considered to be significant when p < 0.05.
Results

Induction and Development of Glomerulonephritis

Injection of heterologous antibodies directed against GBM resulted in onset of glomerulonephritis in mice preimmunized with non-specific sheep antibodies. We adapted the classical accelerated anti-GBM glomerulonephritis model (Assmann et al., 1985) by including TNFα in the anti-GBM antibody injection. This resulted in fast, reproducible and progressive inflammation in the glomeruli, whereas TNFα or anti-GBM antibodies alone did not result in significant leukocyte influx or glomerular injury (data not shown). Figure 1 demonstrates the development of progressive glomerulonephritis in our model at different time points from 2h to 2 weeks.

Glomerular depositions of sheep and mouse immunoglobulin were observed at all time points (Fig. 1a) with formation of glomerular intracapillary PAS-positive thrombus starting at 2 days (Fig. 1b,c). Additionally, mild extracapillary proliferation and intratubular protein casts were noted, all indicative of glomerulonephritis induction (Fig. 1b). Glomerular accumulations of PAS-positive material was highest at 2 days and remained present for 14 days (Fig. 1c). Crescent formation was detected at 7 and 14 days after disease induction (Fig. 1b,d). Two hrs after glomerulonephritis induction, a peak of intraglomerular neutrophils was observed (Fig. 1e). The glomerular accumulation of neutrophils subsequently decreased in time, which was paralleled by an increase of intraglomerular monocytes (Fig. 1e), a time dependent pattern that is characteristic for the experimental anti-GBM glomerulonephritis model (Assmann et al., 1985; Masaki et al., 2003). Albuminuria was markedly increased after induction of glomerulonephritis to > 50 mg per 24h at day 7 and 14, while untreated mice secreted less than 0.1 mg albumin per 24 h (Fig. 1f). At the same time glomerular filtration function deteriorated (Fig. 1g).
To further examine the disease process, expression of proinflammatory genes was analyzed by quantitative RT-PCR. First, threshold values (C\textsubscript{t}) in kidneys of untreated mice were determined. Expression of cytokines and P- and E-selectin was low whereas VCAM-1, ICAM-1 and TGF\textbeta were moderately expressed in healthy kidneys (Table 2). A rapid upregulation of cell adhesion molecules and cytokines was observed 2h after onset of glomerulonephritis (Fig. 2). The expression of cell adhesion molecules and cytokines was significantly higher compared to untreated animals throughout the experiment. Expression of the fibrosis related TGF\textbeta enhanced slightly in time, indicative of ongoing glomerular injury and crescent formation (Border and Noble, 1994).

To characterize the proinflammatory gene expression profile specifically in glomeruli at the early stages of disease, we analyzed laser microdissected glomeruli. Interestingly, a spatial and temporal heterogenic regulation of proinflammatory genes in response to disease induction was observed. E-selectin was preferentially upregulated in glomeruli at all time points (Fig. 3a). Initially P-selectin was more abundantly expressed in the glomeruli than in total kidney isolates, whereas upregulation of VCAM-1 was more pronounced in the non-glomerular vascular compartments at all time points. Similarly to VCAM-1, glomerulonephritis induced expression of MCP1 was predominantly located outside the glomeruli (Fig. 3a).

Gene expression data were corroborated by immunohistochemical analysis for E-selectin and VCAM-1 (Fig. 3b). E-selectin protein was solely produced in glomeruli whereas VCAM-1 was present in glomerular capillaries and in microvascular beds outside the glomeruli. The immunostaining of VCAM-1 was weak in glomeruli and stronger in arterioles. While E-selectin protein was undetectable in untreated mice, 2 h after disease induction 75 ± 5 % of all glomeruli were E-selectin positive. After 2
days the presence of glomerular E-selectin expression was decreased to 39 ± 13 % of all glomeruli.

I.V. Administered AbEsel- liposomes Selectively Homed to Hlomeruli

The disease specific glomerular expression of E-selectin provided it a promising target molecule on glomerular endothelial cells. Immunohistochemical staining demonstrated selective homing of i.v. administered immunoliposomes containing monoclonal anti-E-selectin antibodies (AbEsel) to the diseased glomeruli (Fig 4a). No staining was observed elsewhere in the kidney. This localization remained during disease development, although in time the staining became less pronounced.

Glomerular staining of AbEsel-liposomes was detected at 2h in 93±4%, at 1 day in 90±1%, and at 2 days in 81±6% of all glomeruli with approximately 10% of glomerular surface being positive for the specific immunoliposome (Fig. 4b). Control IgG-liposomes localized in glomeruli, likely due to local inflammation associated vascular permeability increase, but to a much lower extent (Fig 4a,b). No AbEsel-liposomes localized in the heart, whereas some association of AbEsel-liposomes was detected with cells in the red pulp in the spleen and with Kupffer cells in the liver, which reflects the regular elimination route of immunoliposomes in the body (data not shown).

To rule out the possibility that detecting the immunoliposomes using secondary antibody does not represent the liposomes themselves, we applied in a parallel experiment radioactive [³H]cholesterylloleyl ether labeled AbEsel-liposomes or IgG-liposomes, and analyzed the kidney uptake of both liposome-preparations. The experiment demonstrated a targeting index of the lipid part of AbEsel-liposomes : IgG-liposomes of 3.6 : 1 (Ásgeirsdóttir et al., unpublished results), which corresponded
nicely to the glomerular surface staining by immunohistochemistry as shown in Fig 4b.

Based on this selective homing to inflamed glomerular endothelium AbEsel-liposomes were next employed as carriers for a targeted delivery of dexamethasone to interfere with glomerular endothelial cell activation and hence to counteract glomerulonephritis development and progression.

Targeted Dexamethasone Exerted Strong, Local Pharmacological Effects Without Alteration of Blood Glucose Level

Dexamethasone has pleiotropic anti-inflammatory effects, including an interaction with the transcription factors NF-κB and AP-1, thereby preventing expression of proinflammatory genes (Barnes, 2006). We therefore compared the effects of PEGylated AbEsel-liposomes encapsulated dexamethasone and free dexamethasone using mRNA levels of proinflammatory genes as read out. As expected, none of the treatments prevented depositions of sheep and mouse immunoglobulin in glomeruli (data not shown). Kidneys were harvested after 2h, a time point when proinflammatory genes were robustly upregulated. Free dexamethasone significantly inhibited gene expression induction of cell adhesion molecules and cytokines (Fig. 5a). Targeted liposomal delivery of dexamethasone similarly resulted in significant downregulation of P- and E-selectin, whereas cytokine expression was slightly but not statistically significantly, affected. No effects on proinflammatory gene expression were observed in mice treated with empty AbEsel-liposomes (Fig. 5a).

Interestingly, expression of VCAM-1 was inhibited by free dexamethasone but not by cell selective delivery into glomerular endothelium. Based on our data on predominant non-glomerular expression of VCAM-1 (Fig. 3a) we hypothesized that
this discrepancy was due to local pharmacological effects of Dexa-AbEsel-liposomes in the glomeruli, combined with a relative low expression of VCAM-1 therein. We therefore studied the effect of Dexa-AbEsel-liposome on the expression levels of VCAM-1 in microdissected glomeruli and demonstrated that glomerular expression of VCAM-1 was indeed downregulated by Dexa-AbEsel-liposomes (Fig. 5b). Similarly, glomerular expression of MCP1 was downregulated by Dexa-AbEsel-liposomes, whereas no effect on MCP1 expression was observed in whole kidney isolates (Fig. 5). Downregulation of P- and E-selectin in microdissected glomeruli was comparable to that observed in whole kidney isolates (data not shown), corroborating the predominant expression of these cell adhesion molecules in glomeruli (Fig 3a).

Dexamethasone is known to cause many side effects, such as osteoporosis, growth retardation in children, skin fragility and metabolic effects (Barnes, 2006).

Hyperglycemia, caused by induction of hepatic gluconeogenesis and inhibition of cellular glucose uptake, is one of the most acute clinically relevant side effects of dexamethasone (Feldman-Billard et al., 2006; Hans et al., 2006; Weinstein et al., 1995). Indeed, in mice treated with free dexamethasone the plasma glucose concentration 2h after i.v. injection of the drug was significantly increased (Fig. 6). This was not observed in mice i.v. injected with dexamethasone encapsulated in immunoliposomes (Fig. 6).

Treatment with Dexa-AbEsel-liposomes Ameliorates Glomerulonephritis Progression

To study whether targeted inhibition of acute inflammatory gene expression in glomeruli would be able to counteract the occurrence of renal injury during glomerulonephritis development, mice were treated at day 0 and day 7 with free
dexamethasone, Dexa-Ab_{Esel}-liposomes, or empty Ab_{Esel}-liposomes and sacrificed at day 14.

Plasma BUN levels in mice treated with Dexa-Ab_{Esel}-liposomes were decreased as compared to levels in mice treated with Ab_{Esel}-liposomes (Fig. 7a). A similar reduction in BUN levels was observed in mice treated with free dexamethasone. A trend towards improved glomerular function exemplified by increased creatinine clearance was observed in both Dexa-Ab_{Esel}-liposomes and dexamethasone treated mice (Fig. 7b). Moreover, Dexa-Ab_{Esel}-liposomes were able to strongly inhibit the formation of crescents in glomeruli (Fig. 7c,d). The percentages of crescentic glomeruli decreased significantly from 30 ± 4% to 17 ± 4% (mean ± s.e.m.). Free dexamethasone showed a similar trend, however with a greater variation between mice (Fig. 7c). Overall, renal injury was notably counteracted by treatment with Dexa-Ab_{Esel}-liposomes, demonstrated by less glomerular lesions, intratubular protein casts, and tubulointerstitial damage (Fig. 7d). Control Dexa-IgG-liposomes that did end up in the kidney to a 4-fold lower extent than targeted Dexa-Ab_{Esel}-liposomes (see Fig. 4b), did reduce BUN levels to 13.4 ± 2.4 (mean ± s.e.m), a pharmacological effect which is most likely associated with inflammation related enhanced permeability and retention based passive accumulation and subsequent local slow drug release. In contrast, these non-targeted liposomes did not result in reduction of the formation of crescentic glomeruli (29 ± 3%; mean ± s.e.m), nor in improvement of creatinine clearance.

Concomitantly with improved renal function, treatment with Dexa-Ab_{Esel}-liposomes decreased the expression of disease related inflammatory genes at day 14. Expression levels of VCAM-1 and fibrosis related TGFβ were markedly lower in mice receiving Dexa-Ab_{Esel}-liposomes compared to empty Ab_{Esel}-liposomes (Fig. 8). Similarly, a
significant reduction in MCP1 and IL-6 mRNA levels was observed, whereas a trend
towards downregulation of IL-1β and TNFα was detected. Administration of free
dexamethasone resulted in significant downregulation of VCAM-1 and MCP1, while
TGFβ, IL-6, IL-1β and TNFα were not statistically significantly affected.
Discussion

In this study we demonstrate that targeted delivery of dexamethasone into glomerular endothelium by liposomes, modified with E-selectin specific monoclonal antibodies as homing device, selectively inhibited proinflammatory gene expression and improved renal function in progressive glomerulonephritis. Furthermore, the selective glomerular delivery of dexamethasone prevented unwanted systemic side effects which were observed after administration of free dexamethasone.

Anti-GBM crescentic glomerulonephritis can be treated with immunotherapy (Tam, 2006). Nonetheless, the need for more specific therapies is evident. The functional redundancy of molecules involved in leukocyte-endothelial cell interactions, as well as the complexity of inflammatory gene expression control and protein function, justifies cell specific therapeutic intervention with a broad pharmacological spectrum (Rosenkranz and Mayadas, 1999; Kuldo et al., 2005). Therefore we postulated that selective delivery of a potent anti-inflammatory drug with pleiotropic pharmacological effects into glomerular endothelium could exert a broad, yet local, inhibitory effect while preventing systemic side effects.

The current study is the first report describing a successful targeted delivery of drug loaded immunoliposomes to activated glomerular endothelial cells in glomerulonephritis in which local pharmacological activity did not interfere with vascular integrity. The local and disease specific expression of E-selectin on glomerular endothelium rendered it an excellent target molecule for drug delivery based therapy in glomerulonephritis. Previously, we reported a rapid receptor mediated internalization of anti E-selectin drug conjugates via E-selectin on activated endothelial cells (Kok et al., 2002; Asgeirsdottir et al., 2003; Everts et al., 2003). This intracellular delivery allows endothelial cell specific pharmacological interference of
regulatory mechanisms to control and inhibit the expression of proinflammatory mediators such as cytokines and adhesion molecules, as demonstrated by our current data.

The fact that elevated E-selectin expression has been detected in the kidney of patients with IgA nephropathy, lupus nephritis and diabetic nephropathy supports the clinical relevance of the development of E-selectin based drug targeting strategies (Honkanen et al., 1998; Hirata et al., 1998). The use of endothelial target epitopes other than E-selectin can, however, be taken into account. Immunoliposomes directed toward VCAM-1 were internalized by activated endothelial cells \textit{in vitro} (Voinea et al., 2005), and \textit{in vivo} demonstrated beneficial effects in an atherosclerosis mouse model (Homem de Bittencourt PI Jr et al., 2006). The use of VCAM-1 selective immunoliposomes in glomerulonephritis to interfere with microvascular endothelial cells distinct from the glomerular capillaries is an attractive strategy, especially since VCAM-1 is highly expressed throughout disease progression (Allen et al., 1999).

Laser capture microdissection is a valuable tool in (patho)physiology research as it, in combination with qRT-PCR, allows the detection of local disease activity that otherwise would be masked in analysis of a whole organ. For drug delivery research, microdissection is also instrumental in revealing local molecular targets of the therapeutic strategy studied. Using this approach we demonstrated that in our glomerulonephritis model upregulation of proinflammatory genes differed between the microvascular beds in the kidney. Strong upregulation of E-selectin was observed on glomerular endothelium, whereas VCAM-1 and MCP1 expression was mainly localized outside the glomeruli. Furthermore, RNA isolates of microdissected glomeruli revealed local downregulation of VCAM-1 and MCP1 by Dexta-AbEsel− liposomes, whereas the expression levels of these genes in whole kidney isolates were
not altered. Clearly, analysis in whole kidney isolates would lead to wrong conclusions about the pharmacological efficacy of Dexa-AbEsel-liposomes.

Targeted delivery to glomerular endothelium resulted in strong local pharmacological effects. In our study we used dexamethasone, a corticosteroid that is generally well tolerated after systemic administration, as a model drug to demonstrate proof of concept. However, today many potent anti inflammatory drugs do not make it through clinical trials because of severe side-effects (Peifer et al., 2006). For these classes of drugs the targeted delivery approach described in this study represents an important added value for future clinical applications. The challenge for the future drug delivery will be to formulate new chemical entities that are rapidly being discovered and are currently making their ways into basic research and clinical trials. These new drugs include tyrosine kinase inhibitors, MAPKinase inhibitors, and other agents interfering with proinflammatory signal transduction pathways (Bergers et al., 2003; Karin, 2006; Fabian et al., 2005). The recent observation that their molecular targets are fundamental in whole body homeostasis, makes their incorporation into cell-type specific drug delivery systems of essential value for clinical utility (Imming et al., 2006).

We showed that Dexa-AbEsel-liposomes were highly effective in improving renal function in glomerulonephritis, as reflected by reduced BUN levels, glomerular crescent formation and proinflammatory gene expression. Although free dexamethasone was also effective, encapsulation of dexamethasone into immunoliposomes prevented the occurrence of increased plasma glucose levels which was observed after administration of free dexamethasone. Hence, immunoliposomal delivery of potent drugs into inflamed microvascular endothelium maintains the pharmacological quality of the drug while eliminating systemic side effects and as
such represents a new therapeutic strategy for the treatment of glomerulonephritis.
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Reference List


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Footnotes

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Legends

Figure 1

Development of glomerulonephritis. (a) Glomerular deposition of sheep (left) and mouse (right) immunoglobulin at 1 day after glomerulonephritis induction (top panels). Immunofluorescent staining along the glomerular capillary wall was observed at all time points from 2h until 14 days. No staining was observed in healthy control mice (bottom panels). Nuclear counterstaining was performed with DAPI. (b) Light microscopy (PAS stain) of kidney from untreated mouse (top), and mice sacrificed 2 (middle) and 14 days (below) after induction of glomerulonephritis. Arrows indicate glomerular thrombus formation at day 2 and crescent formation at day 14. Inserts indicate a normal glomerulus with open capillaries and Bowman capsule (top), PAS-positive glomerulus with intracapillary thrombus (middle) and glomerular crescent formation (below). (c) Semiquantitative scoring of glomerular deposits of PAS-positive material as a measure of glomerular injury. (d) Presence of glomerular crescents was observed at 7 and 14 days after glomerulonephritis induction. (e) Glomerular infiltration of neutrophils and monocytes. (f) Development of albuminuria after induction of glomerulonephritis. (g) Blood urea nitrogen (BUN) levels after induction of glomerulonephritis. BUN levels were not determined (n.d.) at day 7. Data in (c) – (g) are presented as mean values ± s.e.m. of 5 – 12 animals per treatment. *, p < 0.05 compared to healthy control mice in (c), (d), (f), and (g).

Figure 2

Quantitative gene expression analysis of the cell adhesion molecules P- and E-selectin, VCAM-1, and ICAM-1, cytokines TNFα, IL-1β, IL-6, MCP1, and TGFβ in kidney of untreated mice (-), and at 2 h, and 1, 2, 7, and 14 days after induction of
glomerulonephritis. Expression levels of mRNA in untreated mice were arbitrarily set at one. Data are presented as mean values ± s.e.m. of 5 - 12 animals per treatment. *, p < 0.05 compared to non-treated animals.

Figure 3
Glomerular upregulation of cell adhesion molecules after induction of glomerulonephritis. (a) Upregulation of P- and E-selectin, VCAM-1 and MCP1 in microdissected glomeruli and total kidney isolates at 2h, 1 and 2 days after glomerulonephritis induction. Expression levels of mRNA in untreated mice (-) were arbitrarily set at one for total kidney isolates and microdissected glomeruli, respectively. Data are presented as mean values ± s.e.m. of 6 animals per treatment. *, p < 0.05 upregulation in glomeruli compared to whole kidney isolates. (b) Immunohistochemical staining with anti-E-selectin antibodies, anti-VCAM-1 antibodies, and isotype matched control antibodies at 2h after glomerulonephritis induction. Asterisk indicates arteriole, arrowhead glomerulus, arrow venule.

Figure 4
Intravenously injected AbEsel-liposomes selectively homed to glomeruli at the onset of glomerulonephritis. (a) Immunohistochemical detection of AbEsel-liposomes (top panel) and control IgG-liposomes (lower panel) 2h after glomerulonephritis induction. (b) Quantitative morphometric analysis of intraglomerular immunohistochemical staining of control IgG-liposomes and AbEsel-liposomes. Values indicate mean ± s.e.m. of 3 animals per treatment group. *, p < 0.05 compared to control Ig-liposomes. #, no significant difference was observed between AbEsel-liposomes at different time points.
Figure 5
Dexamethasone and Dexa-AbEsel-liposomes strongly downregulated proinflammatory gene expression in glomerulonephritis. (a) Gene expression levels in whole kidney isolates of untreated mice and 2 h after induction of glomerulonephritis in the absence or presence of dexamethasone, Dexa-AbEsel-liposomes and empty AbEsel-liposomes, respectively. Expression levels of mRNA in untreated mice were arbitrarily set at one. Data are presented as mean values ± s.e.m. of 4 - 12 mice, *, p < 0.05. (b)
Downregulation of glomerular expression of VCAM-1 and MCP1 by Dexa-AbEsel-liposomes. RNA was isolated from microdissected glomeruli. Expression levels of mRNA in untreated mice (hatched bar) were arbitrarily set at one. Disease associated glomerular expression (black bar) of VCAM-1 and MCP1 was prevented by treatment with Dexa-AbEsel-liposomes (white bar). Data are presented as mean values ± s.e.m. of 6 mice, *, p < 0.05

Figure 6
Increased plasma glucose levels in mice treated with free dexamethasone, while Dexa-AbEsel-liposomes were devoid of this systemic side effect. Control mice (-): 2h glomerulonephritis induction without drug treatment. Values represent mean ± s.e.m. of 6 -12 mice, *, p < 0.05.

Figure 7
Selective delivery of dexamethasone into glomerular endothelial cells reduced renal injury during progression of glomerulonephritis. Mice were i.v. injected at day 0 and 7 with empty AbEsel-liposomes (○), Dexa-AbEsel-liposomes (●) and free
dexamethasone (▼). (a) Plasma BUN levels at day 14 after glomerulonephritis induction were reduced in mice treated with Dena-Ab_{Esel}-liposomes and dexamethasone. (b) A trend towards increased creatinine clearance at day 14 after glomerulonephritis induction was observed in mice treated with Dena-Ab_{Esel}-liposomes and dexamethasone. (c) Glomerular crescent formation at day 14 after glomerulonephritis induction was significantly reduced in mice treated with Dena-Ab_{Esel}-liposomes. (d) Representative PAS staining of kidney induction from mice treated with empty Ab_{Esel}-liposomes (left panel) and Dena-Ab_{Esel}-liposomes (right panel) at day 14 after glomerulonephritis. The frequency of glomerular crescent formation (arrows), intratubular protein casts and tubulointerstitial injury were reduced in the Dena-Ab_{Esel}-liposome treated mice.

Figure 8
Targeting dexamethasone to glomerular endothelium reduced expression of proinflammatory genes during progression of glomerulonephritis. Mice were i.v. injected at days 0 and 7 of glomerulonephritis development with empty Ab_{Esel}-liposomes (○), Dena-Ab_{Esel}-liposomes (●) and free dexamethasone (▼). Gene expression was analyzed in whole kidney isolates at day 14. Expression levels of mRNA in healthy control mice (■) were arbitrarily set at one.
Table 1: Primers used for quantitative RT-PCR

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<tr>
<th>E-selectin</th>
<th>CAACGTCTAGGGTTCAAAAACATCAG</th>
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<tr>
<td>probe</td>
<td>CACAAATGCATCGTGGA</td>
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<tr>
<td>reverse</td>
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<td>VCAM-1</td>
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<td>probe</td>
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<td>reverse</td>
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<tr>
<td>ICAM-1</td>
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<tr>
<td>probe</td>
<td>CAGTACTGTACCACTCTC</td>
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<tr>
<td>reverse</td>
<td>GCCCAGAATGATTAGTCCAGTTATT</td>
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<tr>
<td>P-selectin</td>
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<tr>
<td>TNFα</td>
<td>Mm00443258_m1 (Applied Biosystems)</td>
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<tr>
<td>IL-1β</td>
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<td>IL-6</td>
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<td>MCP1</td>
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<td>TGFβ</td>
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<tr>
<td>GAPDH</td>
<td>Mm99999915_g1 (Applied Biosystems)</td>
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Table 2: mRNA levels of proinflammatory genes in kidney of healthy control mice based on the number of cycles necessary for a fixed threshold value* (Ct) in quantitative RT-PCR. Values represent mean Ct ± SD, n = 3.

<table>
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<tr>
<th>Gene</th>
<th>Ct</th>
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<tr>
<td>GAPDH</td>
<td>19.4 ± 0.5</td>
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<tr>
<td>P-selectin</td>
<td>33.0 ± 0.6</td>
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<tr>
<td>E-selectin</td>
<td>31.3 ± 0.6</td>
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<tr>
<td>VCAM-1</td>
<td>27.5 ± 0.5</td>
</tr>
<tr>
<td>ICAM-1</td>
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<tr>
<td>TNFα</td>
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<tr>
<td>IL-1β</td>
<td>32.5 ± 0.7</td>
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<tr>
<td>MCP1</td>
<td>32.0 ± 0.4</td>
</tr>
<tr>
<td>TGFβ</td>
<td>26.3 ± 0.6</td>
</tr>
</tbody>
</table>

* Higher Ct value represents lower expression level. One Ct value difference = 2 fold expression level difference.
Figure 2
**Figure 3**

(a) Graphs showing relative gene expression of P-selectin, E-selectin, VCAM-1, and MCP1 over time (2h, 1 day, 2 days) in kidney and glomeruli. * indicates statistical significance between conditions.

(b) Images of tissue sections stained for E-selectin, VCAM-1, and isotype control, with black arrows pointing to areas of interest. Arrowheads indicate specific regions of interest for each stain.
Figure 4
Figure 5
Figure 6