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

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Ab$_{Esel}$; anti E-selectin antibody, BUN; blood urea nitrogen, C$_t$; 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 Ab$_{Esel}$-liposomes encapsulated dexamethasone strongly reduced glomerular proinflammatory gene expression without affecting blood glucose levels, a severe side effect of administration of free dexamethasone. Dexa-Ab$_{Esel}$-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 Iimmunoliposomes

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 sulfhydryl-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

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

Ab_{Esel}-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 $1.25 nm^3$,

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 Ab_{Esel} 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), Ab_{Esel}-liposomes (0.4 µmol lipid;

n=4), IgG-liposomes (0.4 µmol lipid; n=3) and Dexa-Ab_{Esel}-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, Ab_{Esel}-liposomes,

Dexa-Ab_{Esel}-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 Ab_{Esel}-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 &

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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 $C_{Cr} = [Cr_{urine}] \times V_{olume\ urine}$ (ml per min) $\times [Cr_{plasma}]^{-1}$.

Quantitative Gene Expression Analysis by Realtime RT-PCR

Total RNA was isolated from ten 8-μm cryosections with RNeasy 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 (OD_{260}) and purity (OD_{260}/OD_{280}) 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 (Promega, Leiden, The Netherlands). Quantitative PCR amplifications were performed according to manufacturers protocol on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Applera Nederland, 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 ($\Delta Ct = Ct_{\text{gene of interest}} - Ct_{\text{housekeeping gene}}$). Comparison of gene expressions in

different samples was performed based on the differences in ΔCt of individual

samples ($\Delta\Delta Ct$).

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_2O_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).

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To further examine the disease process, expression of proinflammatory genes was analyzed by quantitative RT-PCR. First, treshold values (C_t) in kidneys of untreated mice were determined. Expression of cytokines and P- and E-selectin was low whereas VCAM-1, ICAM-1 and TGFβ 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 TGFβ 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 Ab$_{Esel}$- 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 (Ab$_{Esel}$) 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 Ab$_{Esel}$-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 Ab$_{Esel}$-liposomes localized in the heart, whereas some association of Ab$_{Esel}$-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 [^{3}H]cholesteryloleyl ether labeled Ab$_{Esel}$-liposomes or IgG-liposomes, and analyzed the kidney uptake of both liposome-preparations. The experiment demonstrated a targeting index of the lipid part of Ab$_{Esel}$-liposomes : IgG-liposomes of 3.6 : 1 (Ásgeirsdóttir et al., unpublished results), which corresponded

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nicely to the glomerular surface staining by immunohistochemistry as shown in Fig 4b.

Based on this selective homing to inflamed glomerular endothelium Ab$_{Esel}$-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 Eeffects 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 PEG-ylated Ab$_{Esel}$-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 Ab$_{Esel}$-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

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this discrepancy was due to local pharmacological effects of Dexa-Ab$_{Esel}$-liposomes in the glomeruli, combined with a relative low expression of VCAM-1 therein. We therefore studied the effect of Dexa-Ab$_{Esel}$-liposome on the expression levels of VCAM-1 in microdissected glomeruli and demonstrated that glomerular expression of VCAM-1 was indeed downregulated by Dexa-Ab$_{Esel}$-liposomes (Fig. 5b). Similarly, glomerular expression of MCP1 was downregulated by Dexa-Ab$_{Esel}$-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-Ab$_{Esel}$-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

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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 $30 \pm 4\%$ to $17 \pm 4\%$ (mean $\pm$ 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 $\pm$ 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 ($29 \pm 3\%$; mean $\pm$ 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

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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

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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 *in vitro* (Voinea et

al., 2005), and *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 Dexa-Ab$_{Esel}$-

liposomes, whereas the expression levels of these genes in whole kidney isolates were

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not altered. Clearly, analysis in whole kidney isolates would lead to wrong

conclusions about the pharmacological efficacy of Dexa-Ab$_{Esel}$-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-Ab$_{Esel}$-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.

Acknowledgments

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MOL #34140

Footnotes

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 $\pm$ 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

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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 Ab_{Esel}-liposomes selectively homed to glomeruli at the onset of

glomerulonephritis. (a) Immunohistochemical detection of Ab_{Esel}-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 Ab_{Esel}-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 Ab_{Esel}-liposomes at different time

points.

Figure 5

Dexamethasone and Dexa-Ab$_{Esel}$-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-Ab$_{Esel}$-liposomes and empty Ab$_{Esel}$-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-Ab$_{Esel}$-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-Ab$_{Esel}$-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-Ab$_{Esel}$-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 Ab$_{Esel}$-liposomes (O), Dexa-Ab$_{Esel}$-liposomes (●) and free

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dexamethasone ($\blacktriangledown$). (a) Plasma BUN levels at day 14 after glomerulonephritis induction were reduced in mice treated with Dexa-Ab$_{Esel}$-liposomes and dexamethasone. (b) A trend towards increased creatinine clearance at day 14 after glomerulonephritis induction was observed in mice treated with Dexa-Ab$_{Esel}$-liposomes and dexamethasone. (c) Glomerular crescent formation at day 14 after glomerulonephritis induction was significantly reduced in mice treated with Dexa-Ab$_{Esel}$-liposomes. (d) Representative PAS staining of kidney induction from mice treated with empty Ab$_{Esel}$-liposomes (left panel) and Dexa-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 Dexa-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 ($\bigcirc$), Dexa- Ab$_{Esel}$-liposomes ($\bullet$) and free dexamethasone ($\blacktriangledown$). Gene expression was analyzed in whole kidney isolates at day 14. Expression levels of mRNA in healthy control mice ($\blacksquare$) were arbitrarily set at one.

Table 1: Primers used for quantitative RT-PCR

E-selectin	
forward	CAACGTCTAGGTTCAAAACAATCAG
probe	CACAAATGCATCGTGGGA
reverse	TTAAGCAGGCAAGAGGAACCA
VCAM-1	
forward	TGAAGTTGGCTCACAATTAAGAAGTT
probe	AACACTTGATGTAAAAGGA
reverse	TGCGCAGTAGAGTGCAAGGA
ICAM-1	
forward	ATGGGAATGTCACCAGGAATG
probe	CAGTACTGTACCACTCTC
reverse	GCACCAGAATGATTATAGTCCAGTTATT
P-selectin	Mm00441295_m1 (Applied Biosystems)
TNFα	Mm00443258_m1 (Applied Biosystems)
IL-1β	Mm00434228_m1 (Applied Biosystems)
IL-6	Mm00446190_m1 (Applied Biosystems)
MCP1	Mm00441242_m1 (Applied Biosystems)
TGFβ	Mm00441724_m1 (Applied Biosystems)
GAPDH	Mm99999915_g1 (Applied Biosystems)

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* (C_t) in quantitative RT-PCR. Values represent mean $C_t \pm$ SD, n = 3.

GAPDH (house keeping gene)	19.4 ± 0.5
P-selectin	33.0 ± 0.6
E-selectin	31.3 ± 0.6
VCAM-1	27.5 ± 0.5
ICAM-1	27.3 ± 0.5
TNFα	31.9 ± 0.5
IL-1β	32.5 ± 0.7
MCP1	32.0 ± 0.4
TGFβ	26.3 ± 0.6

* Higher C_t value represents lower expression level. One C_t value difference = 2 fold expression level difference.
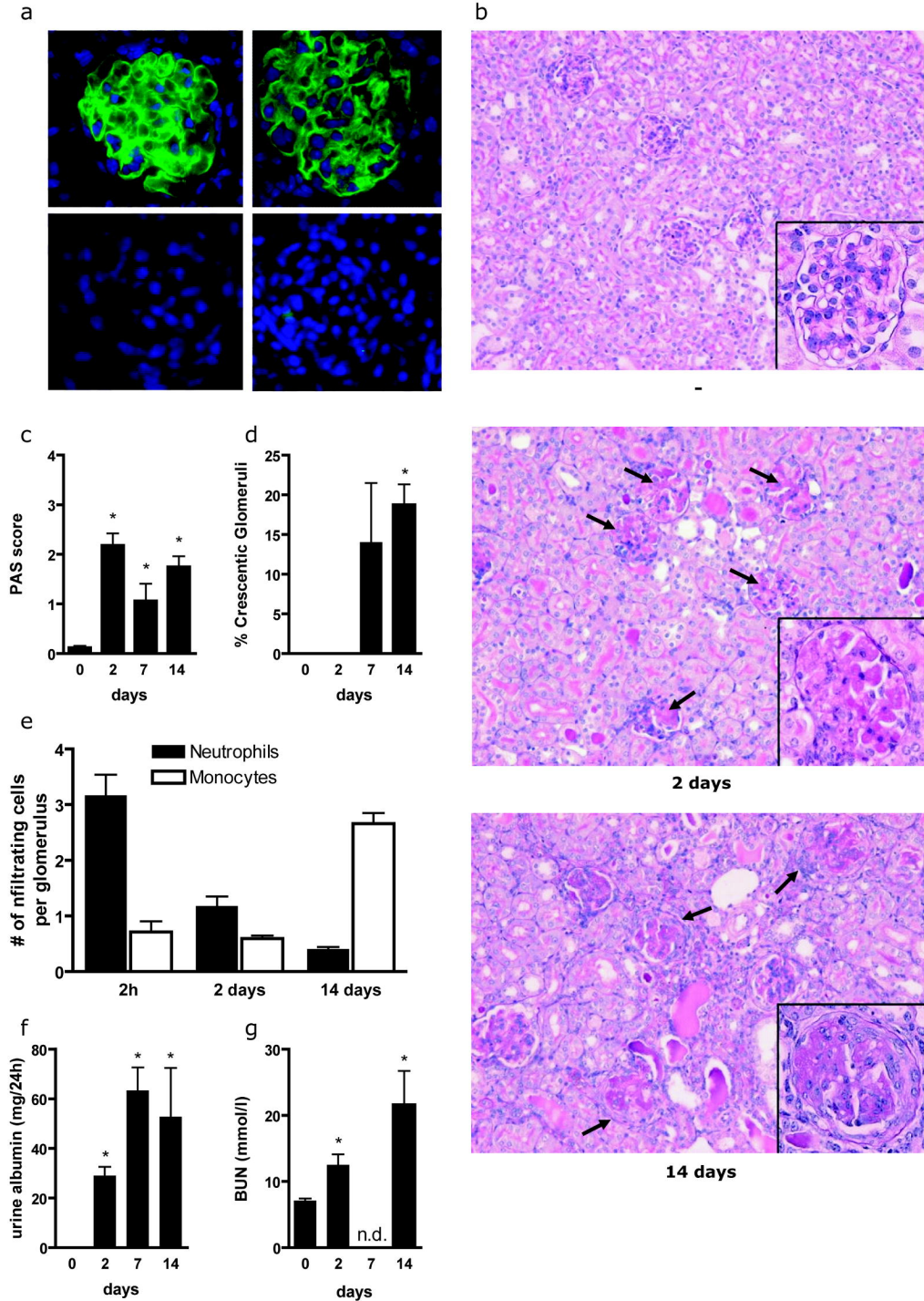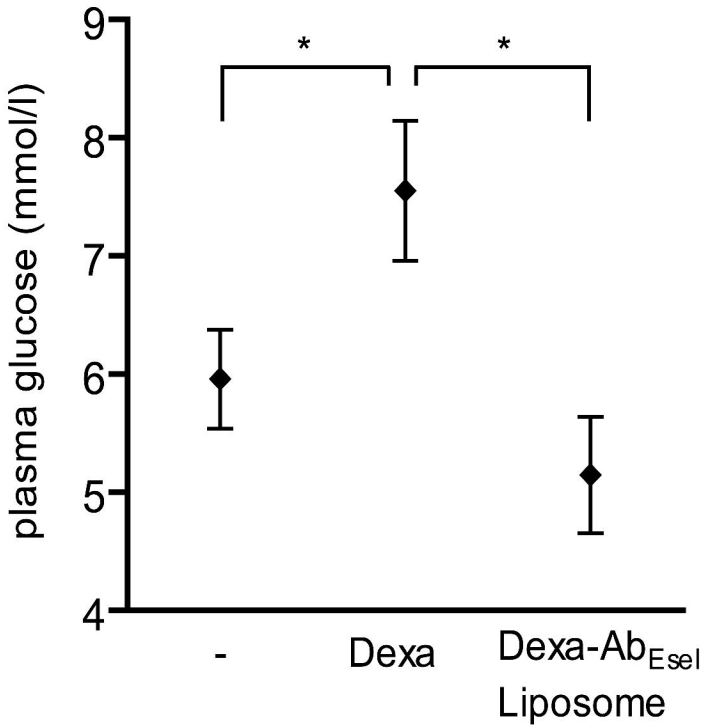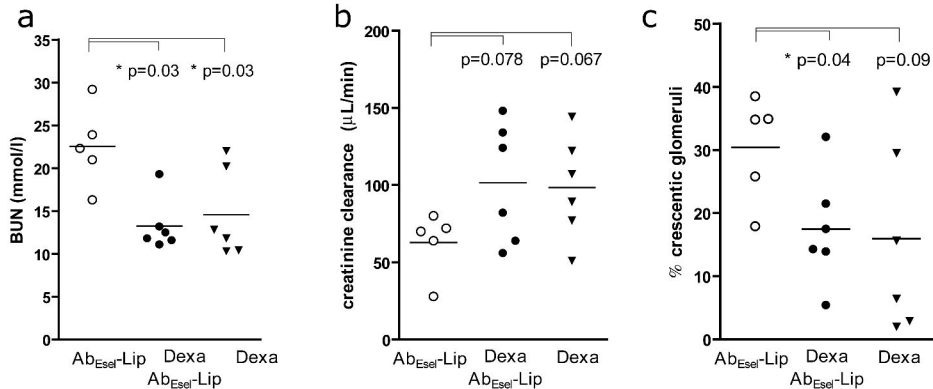
Figure 1

Figure 2

Figure 3

a

b

glomerular surface stained (%)

IgG-Lip   Ab$_{Esel}$-Lip

15

10

5

0

2h   2h   1   2 days

after glomerulonephritis induction

* #
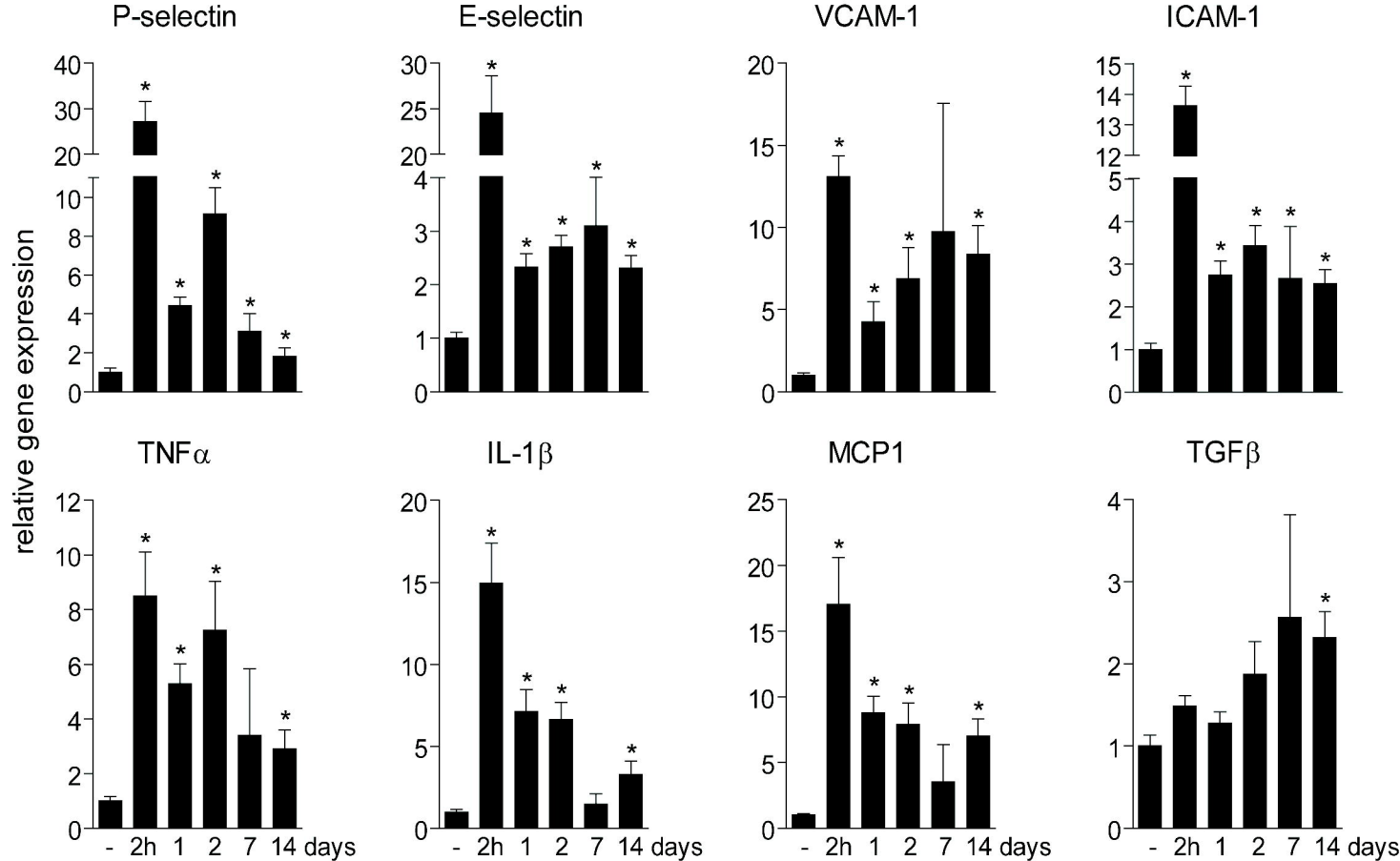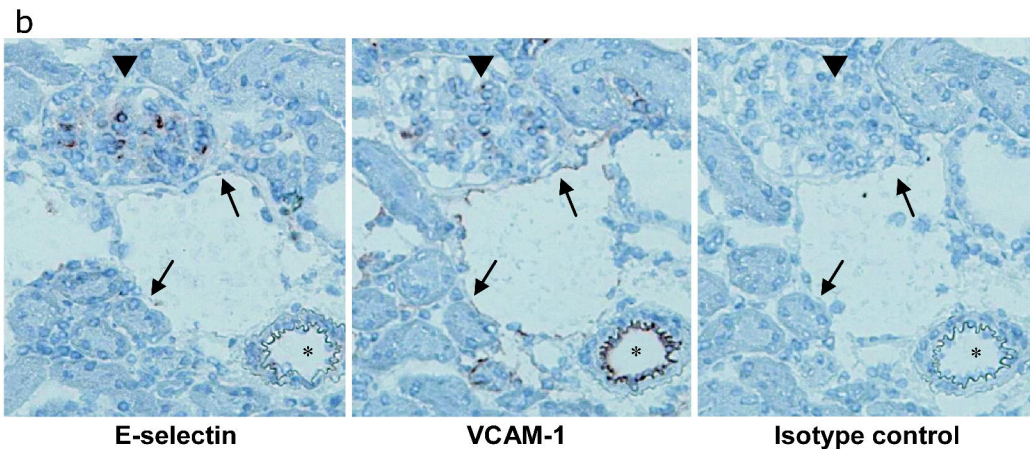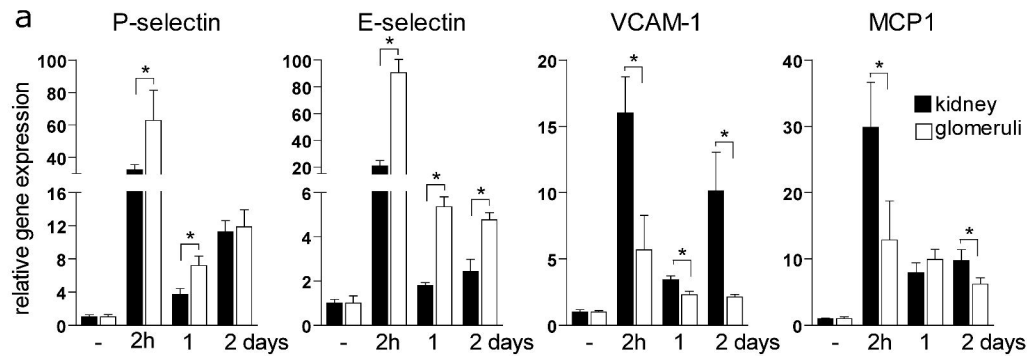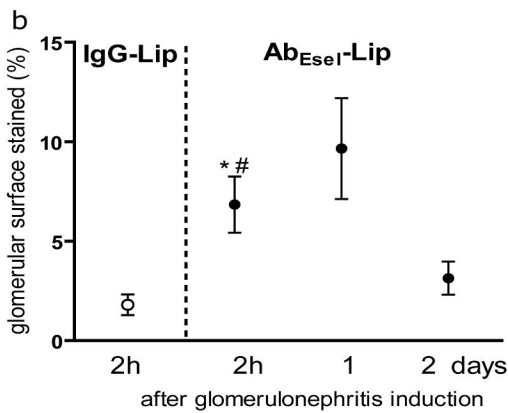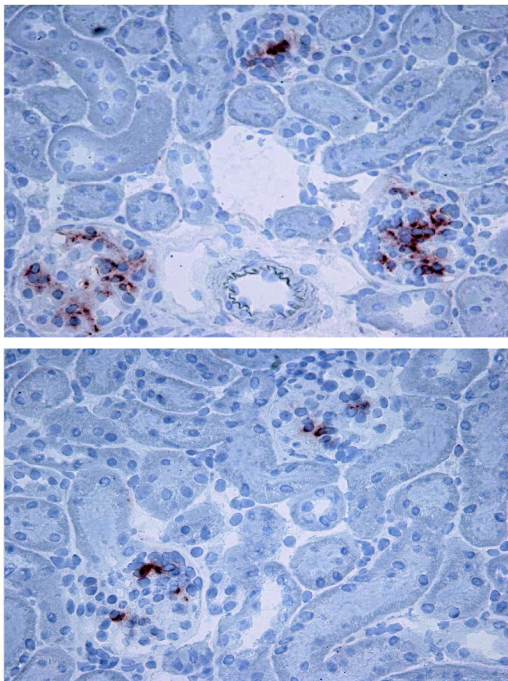
Figure 4
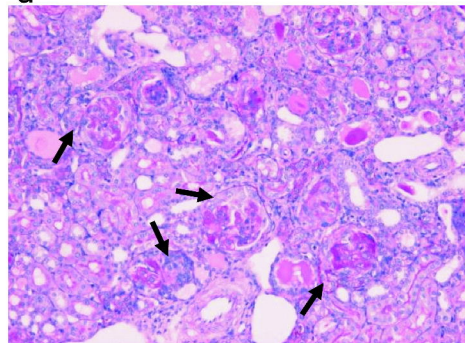
Figure 5

Figure 6

empty Ab$_{Esel}$ liposome
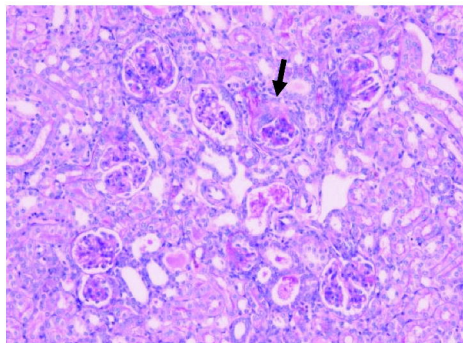
Dexa-Ab$_{Esel}$-liposome

Figure 7

Figure 8