

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

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ABSTRACT

Glomerulonephritis represents a group of renal diseases with glomerular inflammation as a common pathologic finding. Because of 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 as a result of 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 but prevents 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} liposome-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 urea nitrogen levels, decreased glomerular crescent formation, and down-regulation 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.

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 proinflammatory 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 limitations with regard to toxicity and side effects (Chadban and Atkins, 2005; Tam, 2006). 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 (Kamps et al., 1997; Ulbrich et al., 2003; Kuldo et al., 2005). Not only are endothelial cells in direct contact with the blood stream and consequently accessible for systemically administered therapeutics but also they play an essential role in

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ABBREVIATIONS: VCAM, vascular cell adhesion molecule; ICAM, intercellular adhesion molecule; Ab_{Esel}, anti E-selectin antibody; TNF, tumor necrosis factor; PBS, phosphate-buffered saline; PAS, periodic acid-Schiff base; BUN, blood urea nitrogen; RT, reverse transcription; PCR, polymerase chain reaction; GBM, glomerular basement membrane; TGF, transforming growth factor; MCP1, monocyte chemoattractant protein-1; IL, interleukin; Dexa, dexamethasone.

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

Preparation of Immunoliposomes. Synthesis of immunoliposomes was as described previously (Kamps et al., 1996; Bartsch et al., 2005). In short, lipids from stock solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, cholesterol, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]-maleimide 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 10 mM HEPES and 135 mM NaCl, pH 6.7, or, when appropriate, in an aqueous solution of 75 to 100 mg/ml dexamethasone disodium phosphate. The liposomes formed were sized by repeated extrusion (13 times) through polycarbonate filters (Costar; Corning Life Sciences, Acton, MA), pore size 50 nm, using a high-pressure extruder (Lipex, Vancouver, BC, Canada). The monoclonal rat anti-mouse E-selectin antibody, (MES-1, kindly provided by Dr. D. Brown, UCB Celltech, Slough, 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 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. Anti-E-selectin antibody (52.1 ± 25.6 g/mol of lipid) and irrelevant rat IgG (53.6 ± 23.1 g/mol of lipid) was coupled. Particle size was analyzed by dynamic light scattering using a Nicomp model 370 submicron particle analyzer (Santa Barbara, CA) in the volume weighing model. The diameter of Ab_{Esel} liposomes was 114.0 ± 31.1 nm and that 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 (grams per mol of lipid) and diameter (nanometers) of the immunoliposomes (R) and the assumption 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 mass of the antibody molecule (M_{Ab}) of 150 kDa, the number of antibodies coupled to the liposomes was estimated to be 20 Ab_{Esel} molecules per liposome and 17 IgG molecules per liposome by geometric arguments according to the following formula: $\pi/6 \times C_{Ab} \times (3d_{bl} \times R^2 - 3R \times d_{bl}^2 + d_{bl}^3) \times M_{Ab}^{-1} \times V_{Ls}^{-1}$ (Adrian et al., 2007). The content of encapsulated dexamethasone

For morphological analysis of renal injury, formaldehyde-fixed, paraffin-embedded 4- μ m sections were stained with periodic acid-Schiff base (PAS). The amount of PAS-positive material within glomeruli was semiquantitatively scored as described previously (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-

<i>E-selectin</i>	
Forward	CAACGCTCTAGGTTCAAAAACAATCAG
Probe	CACAAATGCATCGTGGGA
Reverse	TTAAGCAGGCAAGAGGAACCA
<i>VCAM-1</i>	
Forward	TGAAGTTGGCTCACAAATTAAGAAGTT
Probe	AACACTTGATGTAAAAGGA
Reverse	TGCGCAGTAGAGTGCAAGGA
<i>ICAM-1</i>	
Forward	ATGGGAATGTCCACGGAATG
Probe	CAGTACTGTACCACCTCTC
Reverse	GCACCAAGATGATTATAGTCCAGTTATT
<i>P-selectin</i>	Mm00441295_m1 (Applied Biosystems)
<i>TNFα</i>	Mm00443258_m1 (Applied Biosystems)
<i>IL-1β</i>	Mm00434228_m1 (Applied Biosystems)
<i>IL-6</i>	Mm00446190_m1 (Applied Biosystems)
<i>MCPI</i>	Mm00441242_m1 (Applied Biosystems)
<i>TGFβ</i>	Mm00441724_m1 (Applied Biosystems)
<i>GAPDH</i>	Mm99999915_g1 (Applied Biosystems)

positive material and glomerular crescent was 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 μ g per mouse; $n = 7$), Ab_{Esel} liposomes (0.4 μ mol of lipid; $n = 4$), IgG liposomes (0.4 μ mol of lipid; $n = 3$), and Dexa-Ab_{Esel} liposomes (0.4 μ mol of lipid, 25 μ g of 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 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 & Johnson, South Brunswick, NJ). Glucose concentration was determined in plasma using a glucose oxidase analyzer (2300 STAT; YSI Inc., Yellow Springs, OH). For collection of urine, mice were placed for 24 h in individual metabolic cages. Urinary albumin content was measured in 96-well microplates (Nalge Nunc International, Rochester, NY) with the mouse albumin enzyme-linked immunosorbent assay quantitation kit (Bethyl Laboratories, Montgomery, TX) using purified mouse albumin as a reference. Plasma and

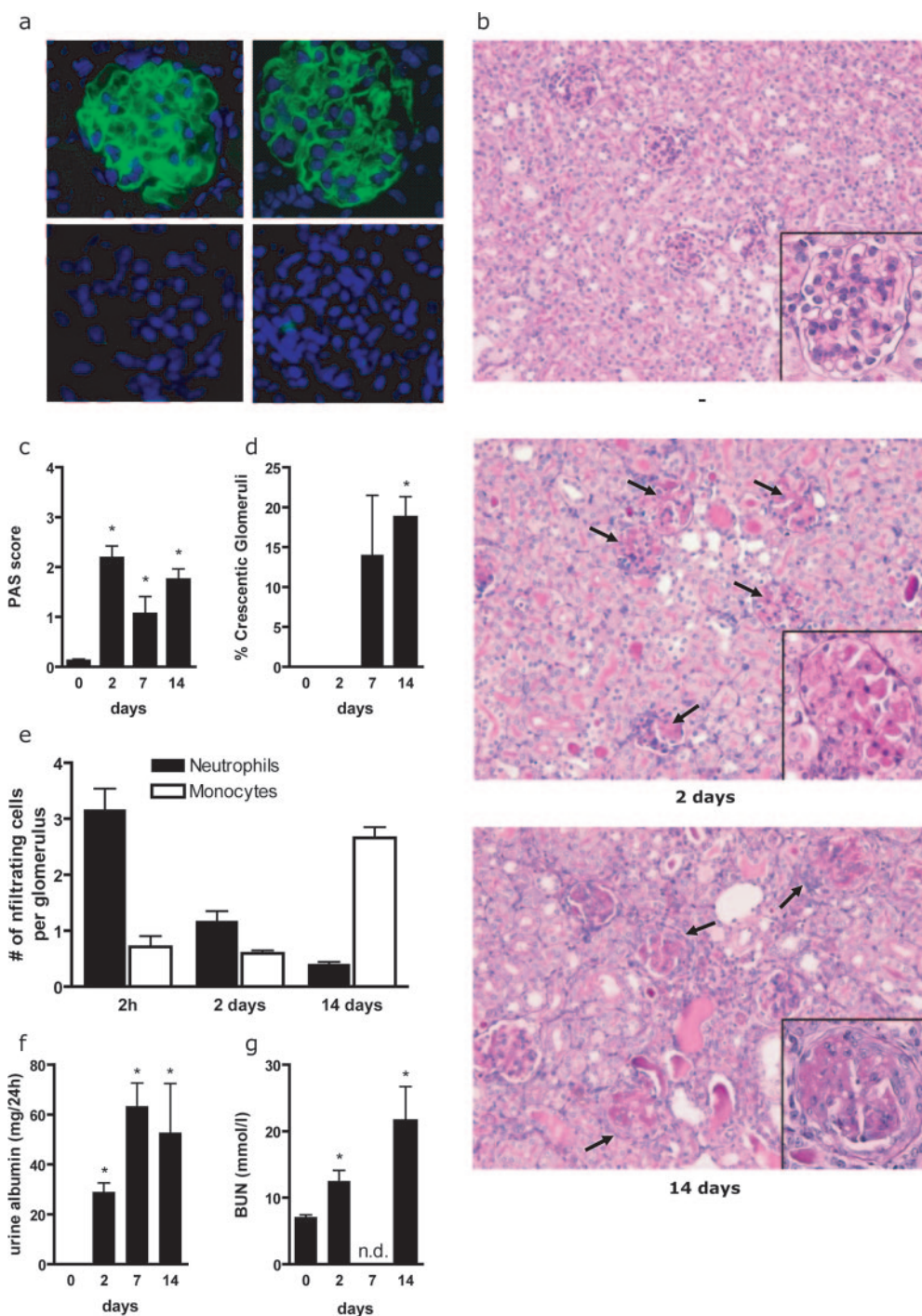


Fig. 1. Development of glomerulonephritis. a, glomerular deposition of sheep (left) and mouse (right) immunoglobulin at 1 day after glomerulonephritis induction (top). Immunofluorescent staining along the glomerular capillary wall was observed at all time points from 2 h until 14 days. No staining was observed in healthy control mice (bottom). Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole. b, light microscopy (PAS stain) of kidney from untreated mouse (top), and mice sacrificed 2 (middle) and 14 days (bottom) after induction of glomerulonephritis. Arrows indicate glomerular thrombus formation at day 2 and crescent formation at day 14. Insets indicate a normal glomerulus with open capillaries and Bowman capsule (top), PAS-positive glomerulus with intracapillary thrombus (middle), and glomerular crescent formation (bottom). 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, 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 with healthy control mice in c, d, f, and g.

urinary creatinine levels were measured by enzymatic colorimetric method using Creatinine plus (Roche, Woerden, The Netherlands), which allows precise and specific quantification of creatinine (Kessler et al., 2007). The creatinine clearance (C_{Cr}) was calculated according to the formula $C_{Cr} = [Cr_{urine}] \times V_{volume\ urine} \times [Cr_{plasma}]^{-1}$. Urine volume was measured in milliliters per minute.

Quantitative Gene Expression Analysis by Real-Time RT-PCR. Total RNA was isolated from 10 8- μ m 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). One microgram of RNA was reverse-transcribed using SuperScript III 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, 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 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 cycle threshold (C_t) method ($\Delta C_t = C_{t_{gene\ of\ interest}} - C_{t_{housekeeping\ gene}}$). Comparison of gene expressions in different samples was performed based on the differences in ΔC_t of individual samples ($\Delta\Delta C_t$).

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 $C_t \pm$ S.D., $n = 3$. Higher C_t value represents lower expression level. One C_t value difference = 2-fold expression level difference.

<i>GAPDH</i> (housekeeping gene)	19.4 \pm 0.5
<i>P-selectin</i>	33.0 \pm 0.6
<i>E-selectin</i>	31.3 \pm 0.6
<i>VCAM-1</i>	27.5 \pm 0.5
<i>ICAM-1</i>	27.3 \pm 0.5
<i>TNFα</i>	31.9 \pm 0.5
<i>IL-1β</i>	32.5 \pm 0.7
<i>MCP1</i>	32.0 \pm 0.4
<i>TGFβ</i>	26.3 \pm 0.6

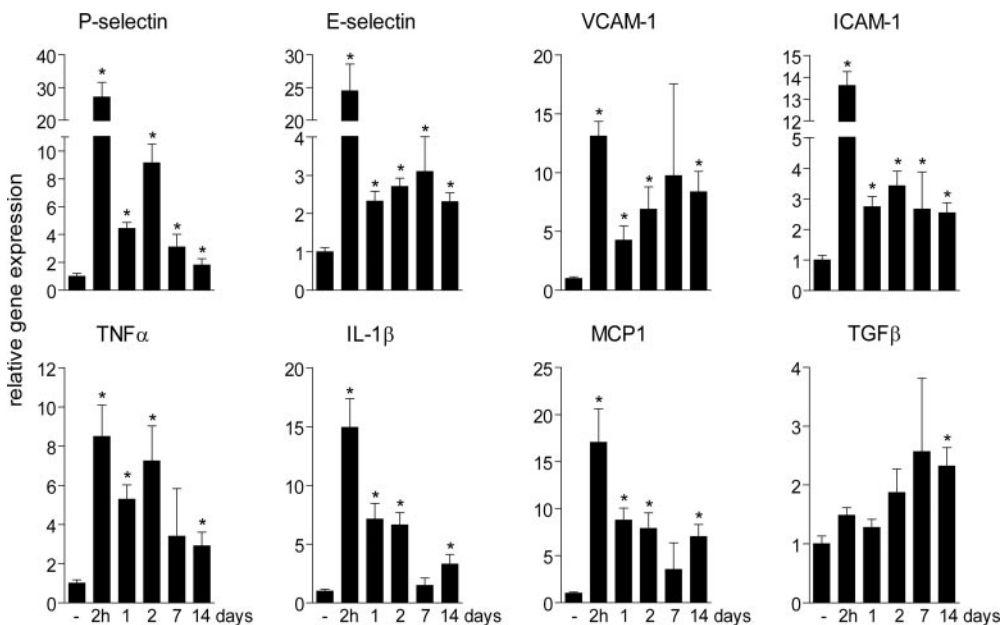


Fig. 2. Quantitative gene expression analysis of the cell adhesion molecules P- and E-selectin, VCAM-1, and ICAM-1, cytokines TNF α , IL-1 β , IL-6, MCP1, and TGF β in kidney of untreated mice (–), and at 2 h and 1, 2, 7, and 14 days after induction of glomerulonephritis. Expression levels of mRNA in untreated mice were arbitrarily set at 1. Data are presented as mean values \pm S.E.M. of 5–12 animals per treatment. *, $p < 0.05$ compared with nontreated animals.

Immunohistochemical Analysis of Cell Adhesion Molecules, Immune Cell Infiltrates, and Localization of Immunoliposomes. Five-micrometer 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; American Type Culture Collection, Manassas VA) anti-neutrophil (NIMP-R14), and anti-monocyte/macrophage (FA-11). Endogenous biotin was blocked by Biotin Blocking System (DakoCytomation Denmark A/S), and peroxidase activity was blocked by incubation with 0.1% H_2O_2 in PBS for 10 min. Thereafter, sections were incubated for 1 h with biotin labeled rabbit anti-rat antibodies (dilution 1:300 in PBS; DakoCytomation Denmark A/S) in the presence of 5% normal mouse serum and 5% normal sheep serum and incubated for 30 min with Streptavidin-ABCComplex-horseradish peroxidase (DakoCytomation Denmark A/S). Peroxidase activity was detected with 3-amino-9-ethylcarbazole (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 to Isolate Glomeruli. Five-micrometer cryosections were mounted on 1.35- μ m polyethylene-naphthalene membranes attached to normal 1-mm slides (P.A.L.M. Microlaser Technologies GmbH, Bernried, Germany), fixed in acetone (3 min), and stained with Mayer's hematoxylin (2 min, 25 μ l per section), washed with diethyl pyrocarbonate-treated water (10 s, 25 μ l 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.

Statistical 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-Whitney 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 nonspecific sheep antibodies. We adapted the classic accelerated anti-GBM glomerulonephritis model (Assmann et al., 1985) by including $\text{TNF}\alpha$ in the anti-GBM antibody injection. This resulted in fast, reproducible, and progressive inflammation in the glomeruli, whereas $\text{TNF}\alpha$ 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 2 h 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. 1, b and c). In addition, mild extracapillary proliferation and intratubular protein casts were noted, all indicative of glomerulonephritis induction (Fig. 1b). Glomerular accumulations of PAS-positive material were 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. 1, b and d). Two hours after glomer-

ulonephritis 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 24 h at days 7 and 14, whereas untreated mice secreted less than 0.1 mg of 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 C_t values in kidneys of untreated mice were determined. Expression of cytokines and P- and E-selectin was low, whereas VCAM-1, ICAM-1, and $\text{TGF}\beta$ were moderately expressed in healthy kidneys (Table 2). A rapid up-regulation of cell adhesion molecules and cytokines was observed 2 h after onset of glomerulonephritis (Fig. 2). The expression of cell adhesion molecules and cytokines was significantly higher compared with untreated animals throughout the experiment. Expression of the fibrosis related $\text{TGF}\beta$ 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. It is noteworthy that a spatial and temporal heterogenic regulation of proinflammatory genes in response to disease induction was observed. E-selectin was preferentially up-regulated in glomeruli at all time points (Fig. 3a). Initially P-selectin was more abundantly expressed in the glomeruli than in total kidney isolates, whereas up-regulation of VCAM-1 was more pronounced in the nonglomerular vascular compartments at all

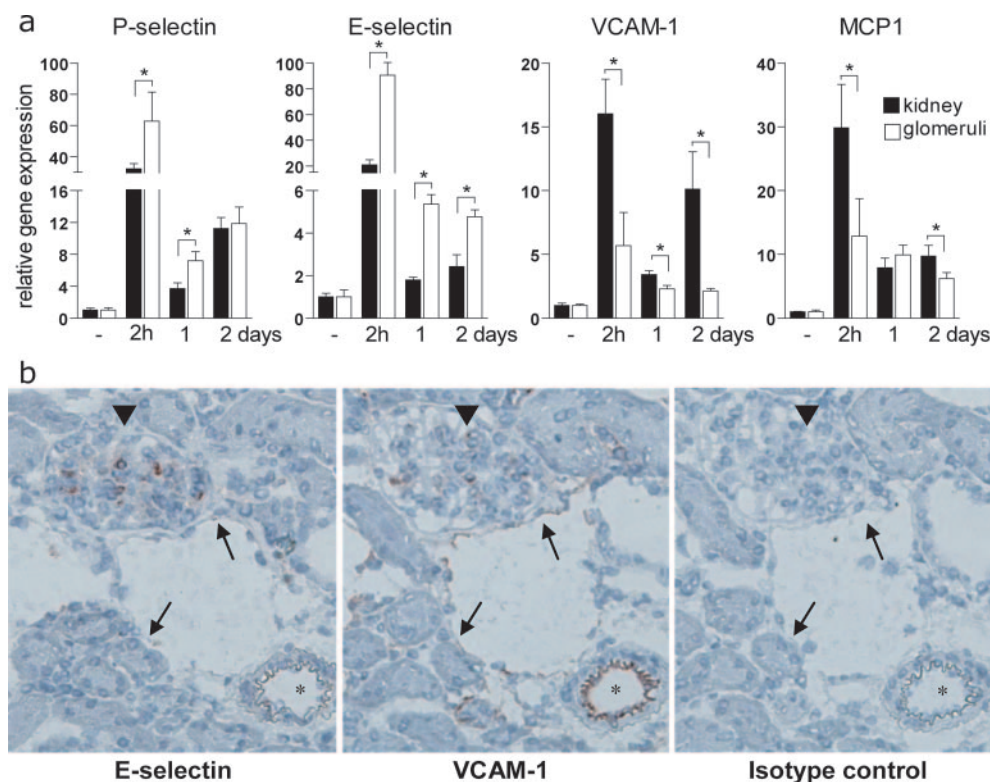


Fig. 3. Glomerular up-regulation of cell adhesion molecules after induction of glomerulonephritis. a, up-regulation of P- and E-selectin, VCAM-1, and MCP1 in microdissected glomeruli and total kidney isolates at 2 h, 1 day, and 2 days after glomerulonephritis induction. Expression levels of mRNA in untreated mice (–) were arbitrarily set at one for total kidney isolates and microdissected glomeruli, respectively. Data are presented as mean values \pm S.E.M. of six animals per treatment. *, $p < 0.05$ up-regulation in glomeruli compared with whole-kidney isolates. b, immunohistochemical staining with anti-E-selectin antibodies, anti-VCAM-1 antibodies, and isotype-matched control antibodies at 2 h after glomerulonephritis induction. Asterisk, arteriole; arrowhead, glomerulus; arrow, venule.

time points. Similar to up-regulation of 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. Although E-selectin protein was undetectable in untreated mice, 2 h after disease induction $75 \pm 5\%$ of all glomeruli were positive for E-selectin. After 2 days, the presence of glomerular E-selectin expression was decreased to $39 \pm 13\%$ of all glomeruli.

I.V. Administered Ab_{Esel}-Liposomes Selectively Homed to Glomeruli. 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 2 h in $93 \pm 4\%$, at 1 day in $90 \pm 1\%$, and at 2 days in $81 \pm 6\%$ of all glomeruli; approximately 10% of the glomerular surface was positive for the specific immunoliposome (Fig. 4b). Control IgG liposomes localized in glomeruli, probably as a result of local inflammation-associated vascular permeability increase, but to a much lower extent (Fig. 4, a and 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 [³H]cholesteryl-oleyl 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 (S. A. Asgeirsdóttir, J. A. A. M. Kamps, 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, 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 Effects without Alteration of Blood Glucose Level. Dexamethasone has pleiotropic anti-inflammatory effects, including an interaction with the transcription factors NF- κ B and AP-1, thereby preventing expression of proinflammatory genes (Barnes, 2006). We therefore compared the effects of Ab_{Esel} liposomes encapsulated dexamethasone and free dexamethasone by analyzing mRNA levels of proinflammatory genes. As expected, none of the treatments prevented depositions of sheep and mouse immunoglobulin in glomeruli (data not shown). Kidneys were harvested after

2 h, a time point when proinflammatory genes were robustly up-regulated. Free dexamethasone significantly inhibited gene expression induction of cell adhesion molecules and cytokines (Fig. 5a). Targeted liposomal delivery of dexamethasone similarly resulted in significant down-regulation of P- and E-selectin, whereas cytokine expression was affected slightly but not statistically significantly. No effects on proinflammatory gene expression were observed in mice treated with empty Ab_{Esel} liposomes (Fig. 5a).

It is noteworthy that expression of VCAM-1 was inhibited by free dexamethasone but not by cell-selective delivery into glomerular endothelium. Based on our data on predominant nonglomerular expression of VCAM-1 (Fig. 3a), we hypothesized that this discrepancy was due to local pharmacological effects of Dexa-Ab_{Esel} liposomes in the glomeruli, combined

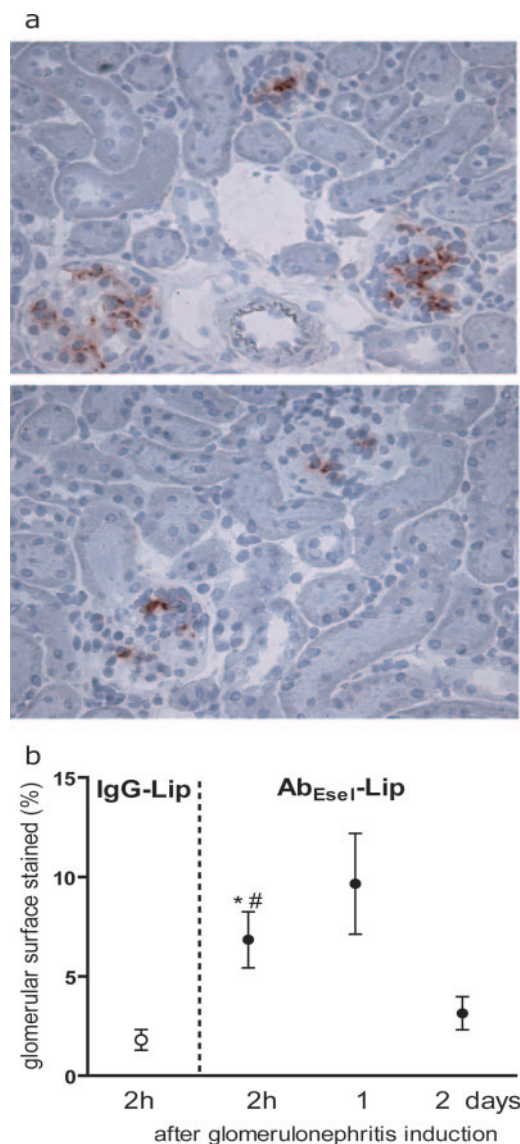


Fig. 4. Intravenously injected Ab_{Esel} liposomes selectively homed to glomeruli at the onset of glomerulonephritis. a, immunohistochemical detection of Ab_{Esel} liposomes (top) and control IgG liposomes (bottom) 2 h after glomerulonephritis induction. b, quantitative morphometric analysis of intraglomerular immunohistochemical staining of control IgG liposomes and Ab_{Esel} liposomes. Values indicate mean \pm S.E.M. of three animals per treatment group. *, $p < 0.05$ compared with control Ig liposomes. #, no significant difference was observed between Ab_{Esel} liposomes at different time points.

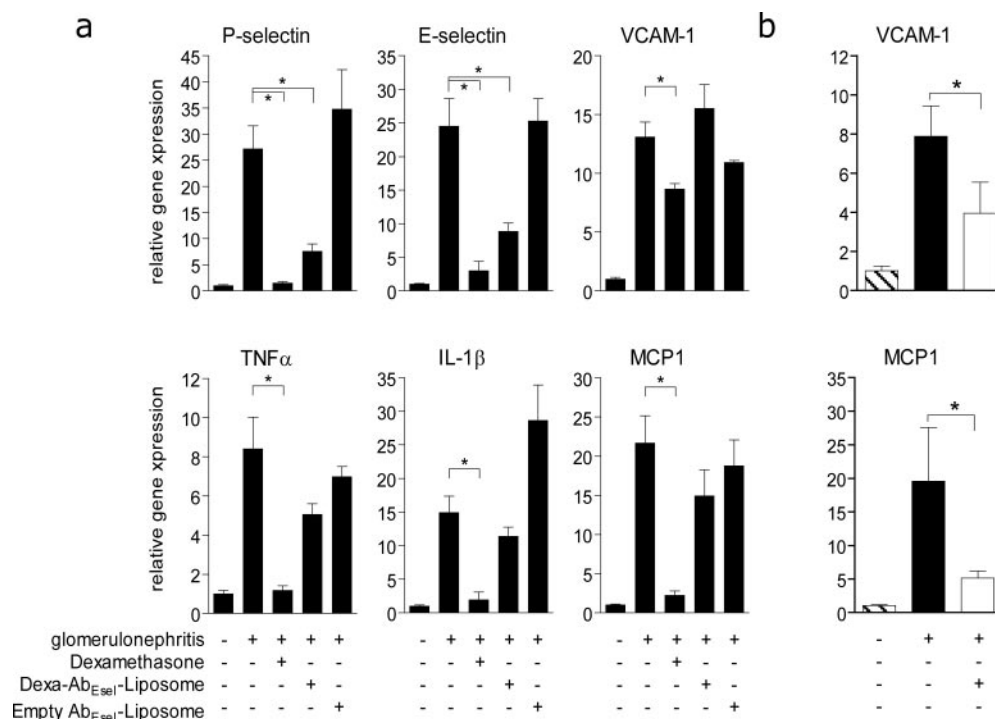


Fig. 5. Dexamethasone and DEXA-Ab_{Esel} liposomes strongly down-regulated proinflammatory gene expression in glomerulonephritis. a, gene expression levels in whole-kidney isolates of untreated mice and 2 h after induction of glomerulonephritis in the absence or presence of dexamethasone, DEXA-Ab_{Esel} liposomes and empty Ab_{Esel} liposomes, respectively. Expression levels of mRNA in untreated mice were arbitrarily set at 1. Data are presented as mean values \pm S.E.M. of 4 to 12 mice, *, $p < 0.05$. b, down-regulation 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 \pm S.E.M. of six mice. *, $p < 0.05$

with a relatively 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 down-regulated by DEXA-Ab_{Esel} liposomes (Fig. 5b). Likewise, glomerular expression of MCP1 was down-regulated by DEXA-Ab_{Esel} liposomes, whereas no effect on MCP1 expression was observed in whole-kidney isolates (Fig. 5). Down-regulation of P- and E-selectin in microdissected glomeruli was comparable with 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 earliest clinically relevant side effects of dexamethasone (Weinstein et al., 1995; Feldman-Billard et al., 2006; Hans et al., 2006).

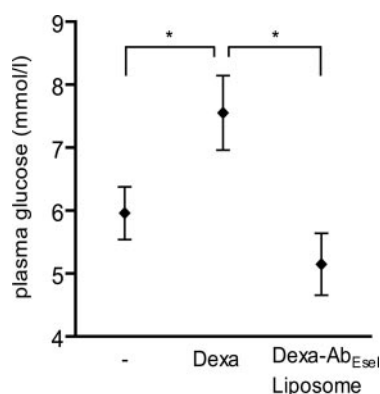


Fig. 6. Increased plasma glucose levels in mice treated with free dexamethasone; DEXA-Ab_{Esel} liposomes were devoid of this systemic side effect. Control mice (-): 2-h glomerulonephritis induction without drug treatment. Values represent mean \pm S.E.M. of 6 to 12 mice. *, $p < 0.05$.

Indeed, in mice treated with free dexamethasone, the plasma glucose concentration 2 h 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 inflammatory gene expression in glomeruli would be able to counteract the occurrence of renal injury during glomerulonephritis development, mice were treated at days 0 and 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 compared with 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 toward 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. 7, c and 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 but 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, which 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 that is most probably associated with inflammation-related enhanced permeability and retention-based passive accumulation and subsequent local

slow drug release. In contrast, these nontargeted liposomes did not result in reduction of the formation of crescentic glomeruli ($29 \pm 3\%$; mean \pm S.E.M.) or 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 TGF β were markedly lower in mice receiving Dexa-Ab_{Esel} liposomes compared with empty Ab_{Esel} liposomes (Fig. 8). Likewise, a significant reduction in MCP1 and IL-6 mRNA levels was observed, whereas a trend toward down-regulation of IL-1 β and TNF α was detected. Administration of free dexamethasone resulted in significant down-regulation of VCAM-1 and MCP1, whereas TGF β , IL-6, IL-1 β , and TNF α 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 a 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 that 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

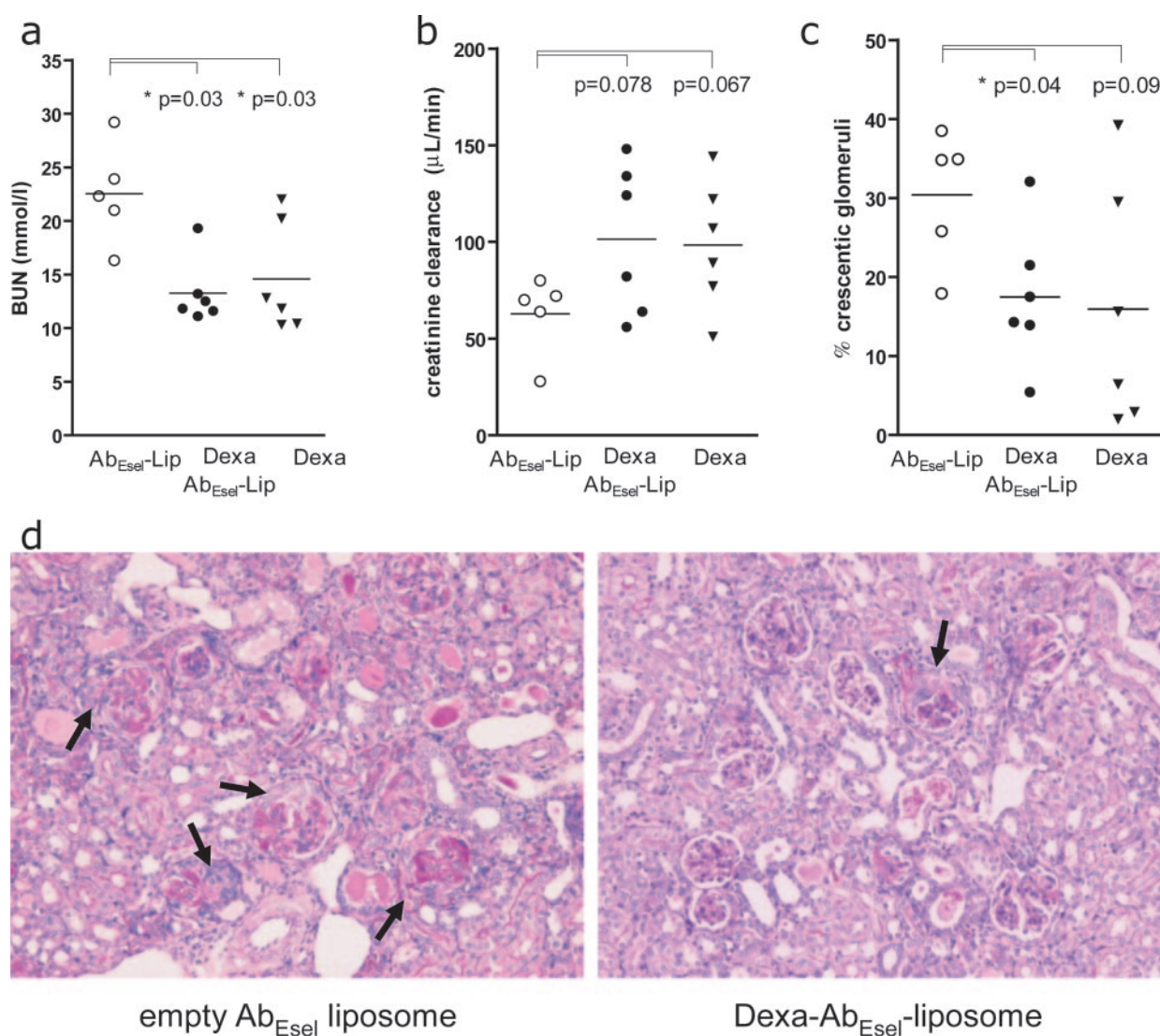


Fig. 7. Selective delivery of dexamethasone into glomerular endothelial cells reduced renal injury during progression of glomerulonephritis. Mice were i.v. injected at days 0 and 7 with empty Ab_{Esel} liposomes (○), Dexa-Ab_{Esel} liposomes (●) and free dexamethasone (▼). **a**, plasma BUN levels at day 14 after glomerulonephritis induction were reduced in mice treated with Dexa-Ab_{Esel} liposomes and dexamethasone. **b**, a trend toward 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) and Dexa-Ab_{Esel} liposomes (right) 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.

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

relative gene expression

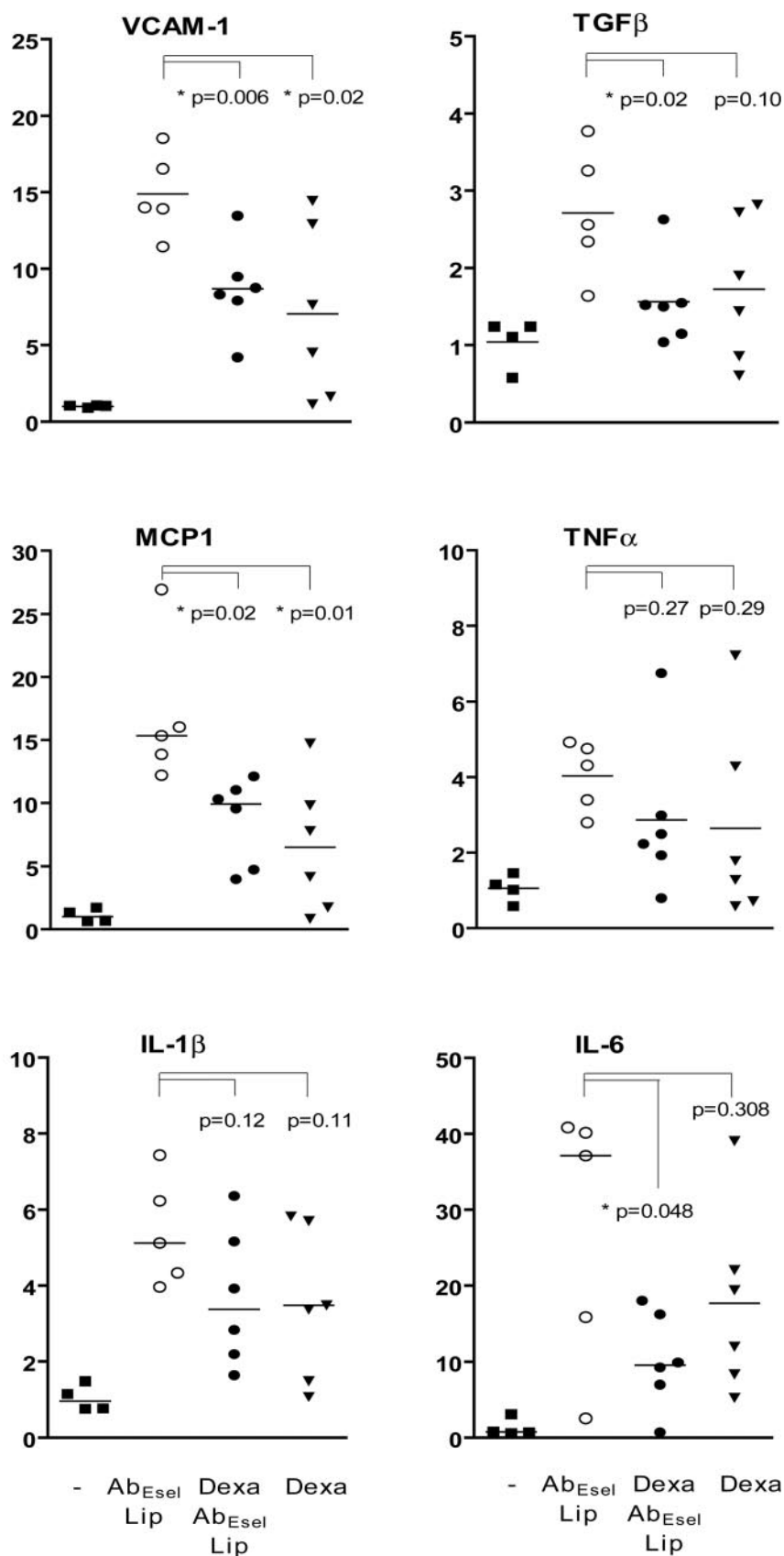


Fig. 8. Targeting dexamethasone to glomerular endothelium reduced expression of proinflammatory genes during progression of glomerulonephritis. Mice were i.v. injected at days 0 and 7 of glomerulonephritis development with empty Ab_{Esel} liposomes (○), DEXA-Ab_{Esel} liposomes (●) and free dexamethasone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

exert a broad, yet local, inhibitory effect but prevent 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. We have 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 (Hirata et al., 1998; Honkanen et al., 1998). The use of endothelial target epitopes other than E-selectin, however, can 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 et al., 2007). 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 because VCAM-1 is highly expressed throughout disease progression (Allen et al., 1999).

Laser capture microdissection is a valuable tool in (patho-)physiology research because it allows, in combination with qRT-PCR, the detection of local disease activity that would otherwise 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, up-regulation of proinflammatory genes differed between the microvascular beds in the kidney. Strong up-regulation 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 down-regulation of VCAM-1 and MCP1 by Dexa-Ab_{Esel} liposomes, whereas the expression levels of these genes in whole kidney isolates were not altered. It is clear that 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, many potent anti-inflammatory drugs do not make it through clinical trials today 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 deliv-

ery 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, mitogen-activated protein kinase inhibitors, and other agents interfering with proinflammatory signal transduction pathways (Bergers et al., 2003; Fabian et al., 2005; Karin, 2006). 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 the increased plasma glucose levels observed after administration of free dexamethasone. Hence, immunoliposomal delivery of potent drugs into inflamed microvascular endothelium maintains the pharmacological quality of the drug but eliminates systemic side effects and, as such, represents a new therapeutic strategy for the treatment of glomerulonephritis.

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