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GENE DELIVERY

Targeted adenovirus mediated inhibition of NF-κB-dependent inflammatory gene expression in endothelial cells *in vitro* and *in vivo*

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ABSTRACT

In chronic inflammatory diseases the endothelium expresses mediators responsible for harmful leukocyte infiltration. We investigated whether targeted delivery of a therapeutic transgene that inhibits nuclear factor κB signal transduction could silence the proinflammatory activation status of endothelial cells. For this, an adenovirus encoding dominant-negative IκB (dnIκB) as a therapeutic transgene was employed. Selectivity for the endothelial cells was achieved by introduction of antibodies specific for inflammatory endothelial adhesion molecules E-selectin or VCAM-1 chemically linked to the virus via polyethylene glycol. *In vitro*, the retargeted adenoviruses selectively infected cytokine-activated endothelial cells to express functional transgene. The comparison of transductional capacity of both retargeted viruses revealed that E-selectin based transgene delivery exerted superior pharmacological effects. Targeted delivery mediated dnIκB transgene expression in endothelial cells inhibited the induced expression of several inflammatory genes, including adhesion molecules, cytokines, and chemokines. *In vivo*, in mice suffering from glomerulonephritis, E-selectin-retargeted adenovirus selectively homed in the kidney to microvascular glomerular endothelium. Subsequent downregulation of endothelial adhesion molecule expression 2 days after induction of inflammation demonstrated the pharmacological potential of this gene therapy approach. The data justify further studies towards therapeutic virus design and optimization of treatment schedules to investigate their capacity to interfere with inflammatory disease progression.

1. Introduction

Vascular endothelial cells are crucially involved in physiological and pathological processes taking place in the body. Their good accessibility for therapeutics and their genetic stability make them an important target for therapeutic interventions [1]. Upon inflammatory activation microvascular endothelial cells facilitate infiltration of leukocytes. This process occurs by cell–cell interaction, and involves adhesion molecules, cytokines, chemokines, and blood coagulation factors expressed by both endothelial cells and leukocytes. Expression of these molecules is often triggered by proinflammatory cytokines such as tumor necrosis factor (TNF)α and interleukin (IL)-1 upon binding to their respective receptors, and occurs mainly as a result of nuclear factor (NF)-κB and p38 mitogen-activated protein kinase (MAPK) signaling, as evidenced in various inflammatory diseases including glomerulonephritis [2–4]. Inhibition of NF-κB or p38MAPK would block many downstream effector molecules, which from a pharmacological point of view is beneficial compared to mono-target therapy with e.g., blocking antibodies against a single inflammatory protein [5].

At present, treatment of inflammatory kidney diseases is based on the use of nonspecific immune suppressing agents like corticosteroids and cytotoxic drugs and is associated with significant side effects [6,7]. In recent years, several classes of new anti-inflammatory therapeutics have been developed that interfere with the signal transduction kinases, including small chemical inhibitors and adenovirally encoded therapeutic proteins [8,9]. By targeted delivery of the therapeutic entities into the cells involved in the patho(physio)logy, local pharmacological effects can be created without concurrent toxic side effects [10–12]. The utility of the recombinant adenoviral system *in vivo* is, however, limited by rapid clearance from the blood and vector accumulation in the liver and other clearance organs [13]. To abolish this natural, Coxsackie and adenovirus receptor (CAR)-mediated tropism and to improve pharmacokinetic behavior *in vivo*, the viral capsid can be

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modified chemically with polyethylene glycol (PEG). This leads to a 17-fold increase in Area Under the plasma concentration curve [14] and has the additional advantage that it can prevent the association of neutralizing antibodies against the adenovirus [15]. Subsequent chemical conjugation with cell-specific ligands can provide the virus with new cell selectivity.

In the current study, we aimed to develop a gene therapy modality that is able to selectively interfere with endothelial activation in glomerulonephritis using endothelium-retargeted adenovirus encoding a dominant-negative mutant of IκB (dnIκB) as a transgene. Upon expression, the transgene should compete with endogenous IκB for NF-κB binding. As a consequence, NF-κB translocation and target gene transcription is blocked. To direct the adenoviruses toward activated endothelium, we chemically modified the capsid with PEG and antibodies against E-selectin or vascular cell adhesion molecule-1 (VCAM-1), adhesion molecules selectively expressed by endothelial cells at inflammatory sites. *In vitro*, we compared the transductional capacity and pharmacological effects of both retargeted viruses. Homing and pharmacological effects of E-selectin retargeted adenovirus were studied *in vivo* in mouse glomerulonephritis. This study demonstrates the potential of tropism-modified adenoviruses to selectively deliver therapeutic genes into microvascular endothelial cells in inflammation to interfere with disease associated cellular activation.

2. Materials and methods

2.1. Adenoviruses

The adenoviral transformed Human Embryonic Kidney (HEK)293 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) F12 (Gibco BRL, Division of Invitrogen, Breda, The Netherlands) containing 10% FBS, 2 mM L-glutamine (Gibco BRL) and penicillin (100 IU/ml)/streptomycin (100 μg/ml) (Gibco BRL) at 37 °C in humidified 5% CO_2/95% air. Recombinant, replication-deficient adenovirus Ad5IκBAA encodes hemagglutinin (HA)-tagged dnIκB under the control of cytomegalovirus (CMV) promoter [16]. It was propagated in HEK293 cells and purified by double centrifugation in CsCl gradient and dialysis against HEPES/sucrose buffer, pH 8.0. The number of viral particles (vp) was calculated from the optical density at 260 nm. Viral titer was determined by plaque formation assay using HEK293 cells and expressed as plaque-forming units. E1- and E3-deleted AdTL reporter adenovirus containing a CMV promoter in front of green fluorescent protein and luciferase genes [17] were grown and purified as described above.

2.2. Antibody modification of adenovirus

For the *in vitro* studies, adenoviruses were modified with H18/7 (mouse IgG2a anti-human E-selectin, Ab_{Esel}) and E1/6aa2 (mouse IgG1 anti-human VCAM-1, Ab_{VCAM1}) monoclonal antibodies, kindly provided by Dr. M.A. Gimbrone from Harvard Medical School, Boston, MA. For mouse cell and *in vivo* studies, the rat IgG2a anti-mouse E-selectin antibody MES-1 (Ab_{mEsel}, kindly provided by Dr. D. Brown, UCB-Celltech, Slough, UK) was conjugated to the virus. Virus modified with rat IgG (Ab_{IgG}, Sigma-Aldrich Chemie, Zwijdrecht, The Netherlands) was used as a control in the *in vivo* experiments.

Conjugation of PEG and antibody coupling to the adenoviruses were essentially performed as described previously [14]. Briefly, an aliquot of PEG linker (3.4 kDa) modified with N-hydroxysuccinimide ester and vinyl sulfone group at its distal ends (NEKTAR Therapeutics, Huntsville, AL) was dissolved in dimethylformamide (DMF; 1 mg PEG per 10 μl DMF), and added slowly to the ice-cold virus (10^{12} vp) in a molar ratio of 10^5 PEG/vp. The reaction mixture was protected from light and gently mixed for 90 min at 4 °C and purified using a PD-10 column (GE Healthcare, Amersham Biosciences Europe Gmbh, Uppsala, Sweden) and HEPES/sucrose buffer as an eluent. Anti-E-selectin, anti-VCAM-1, or control antibodies were reacted with N-succinimidyl-S-acetylthioacetate (SATA; Sigma-Aldrich Chemie) for 1 h. Free SATA was separated from the antibodies by gel permeation chromatography and antibodies were deacetylated as described before for albumin [18]. The thioacetyl-antibodies were allowed to react with PEGylated adenovirus (molar ratio of 10^3 antibodies/1 PEGylated virus) for 4 h at 4 °C under gentle mixing. Unreacted reagents were removed by dialysis (DispoDialyzers 300kD MWCO, Spectrum Laboratories, Rancho Dominguez, CA) against HEPES/sucrose buffer at 4 °C. The final virus preparation was collected and stored at −80 °C in small aliquots until use.

2.3. Cell culture

HUVEC, obtained from the Endothelial Cell Facility UMCG (Groningen, The Netherlands), were isolated from at least two umbilical cords to circumvent donor bias, mixed and cultured as previously described [19]. Cells were cultured on 1% gelatin-precoated tissue culture flasks (Corning, Costar, The Netherlands) at 37 °C under 5% CO_2/95% air. In the experiments performed, HUVEC were used up to passage 4.

The immortalized glomerulus-associated human mesangial cells were cultured as described previously [20].

The mouse endothelial cell line H5V (kindly provided by Dr. A. Vecchi, Mario Negri Institute for Pharmacological Research, Milan, Italy) were grown in tissue culture flasks at 37 °C under 5% CO_2/95% air. The culture medium consisted of DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, and 300 μg/ml gentamycin.

Upon confluence, primary cells and cell lines were detached from the surface using trypsin/EDTA (0.5/0.2 mg/ml in PBS) and split in appropriate dilutions.

2.4. Flow cytometric analysis of target epitope expression and binding of retargeted virus to cells

The expression levels of E-selectin and VCAM-1 on the surface of human endothelial and mesangial cells were determined by flow cytometry. Confluent cells were activated with 10 ng/ml TNFα (F. Hoffmann-La Roche Ltd, Basel, Switzerland) or IL-1β (R&D Systems, Minneapolis, MN), or both activators simultaneously added for 4 h, and subsequently detached from the wells by short treatment with trypsin. After washing with PBS/5% FCS, cells were stained for 45 min on ice with mouse anti-human E-selectin (H18/7), VCAM-1 (E1/6-aa2), and CD31 (DakoCytomation, Glostrup, Denmark). After washing, detection was performed with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (DakoCytomation) for 45 min on ice. Cells were washed and fixed with 0.5% para-formaldehyde/PBS, after which flow cytometric data acquisition took place on an Epics-Elite Flow cytometer (Coulter Electronics, Mijdrecht, The Netherlands). A total of 5000 events was analyzed per sample. Non-specific staining was assessed by incubation of cells with mouse isotype control monoclonal antibodies as primary antibody. The mean fluorescence intensity (MFI) values of these controls were subtracted from MFI of particular samples.

To determine Ab_{mEsel}-PEG-AddnIκB binding to mouse endothelium, confluent layers of H5V cells were activated with mouse recombinant TNFα (BioSource Europe, Nivelles, Belgium) and mouse recombinant IL-1β (BioSource Europe), both at 100 ng/ml for 4 h, and subsequently detached from the wells by short treatment with trypsin. After washing with PBS/5% FCS, cells were incubated for 45 min on ice either with rat anti-mouse E-selectin antibody (MES-1) or with Ab_{mEsel}-PEG-AddnIκB at 10^3 vp/cell. After washing with PBS/5% FCS antibody/antibody-modified virus, detection was performed with FITC-conjugated rabbit anti-rat antibody (DakoCytomation) for 45 min on ice. Cells were washed with PBS/5%FCS and fixed with 0.5% para-formaldehyde/PBS, after which flow cytometric data acquisition took place as described above.

2.5. AdTL adenovirus transduction protocols

For virus transduction experiments using reporter gene viruses, AdTL, PEG-AdTL, Ab_{Esel}-PEG-AdTL, HUVEC and mesangial cells were plated in 96-well tissue culture plates (Costar) at 10 000 cells/well. After overnight culture cells were stimulated for 4 h at 37 °C with 10 ng/ml TNFα, 10 ng/ml IL-1β, or a combination of both cytokines. For Ab_{VCAM1}-PEG-AdTL, HUVEC activation by cytokines was over a 24 h time period. Next, medium was replaced by viral vectors diluted in DMEM/5% FCS at 10^3 vp/cell and cells were incubated for 60 min at 37 °C. The incubation medium was replaced by normal culture medium and cells were further incubated for 48 h. Cells were lysed in Cell Culture Lysis Buffer (Promega, Leiden, The Netherlands) and luciferase activity was measured in LumiCount (Packard Bioscience, Meriden, CT) according to standard protocols. In competition experiments, HUVEC were incubated at room temperature with 20 μg/ml unconjugated antibodies diluted in medium containing 5% FCS for 10 min prior to addition of viruses.

For transduction with dnIκB adenovirus, HUVEC were plated at 12500 cells/cm^2. After overnight culture, cells were incubated for 1 h at 37 °C with dnIκB viral vectors diluted in DMEM/5% FCS at different vp/cell ratios, to determine pharmacological efficacy, i.e., the vp/cell concentration, or dose, at which 50% of effects are obtained (EC_{50}). Incubation medium was replaced by endothelial cell culture medium and cells were further incubated for 48 h to allow transgene expression. For dnIκB effector studies, after this period cells were activated for 4 h with 10 ng/ml IL-1β before collection of samples for protein or gene expression by Western blot and real-time RT-PCR, respectively. Transduction experiments with PEGylated and retargeted Ab_{Esel}- and Ab_{VCAM1}-PEG-AddIκB adenoviruses were performed as described above with HUVEC activated for 4 h respectively 24 h with 10 ng/ml TNFα prior to the infection, to allow the inflammatory target epitopes under study to become expressed.

2.6. Detection of dnIκB transgene protein by Western blot

Cells were detached from the wells by trypsinization and after washing with ice-cold PBS they were lysed in 100 μl of freshly prepared SDS sample buffer as described previously [19]. 30 μg of protein was loaded and run on SDS-PAGE 12.5% acrylamide gel. Gels were electro-blotted onto a nitrocellulose membrane, and blots were incubated for 1 h in 10 ml of blocking buffer (PBS/0.1% Tween20/5% skim milk). Blots were washed three times in wash buffer (PBS/0.1% Tween20) and incubated overnight at 4°C with different antibodies: anti-HA-tag antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:200 in blocking buffer), anti-IκB antibody (Santa Cruz Biotechnology; dilution 1:20 in blocking buffer), or goat anti-VCAM-1 polyclonal antibody (C-19, Santa Cruz Biotechnology; dilution 1:500 in blocking buffer). Blots were washed three times, incubated with horse-radish peroxidase-conjugated swine anti-rabbit-IgG (DAKO Cytomation, dilution 1:1000 in blocking buffer) for 1 h at room temperature while shaking, and washed 4 times in wash buffer. Detection was done by chemiluminescence (Amersham Corp., Arlington Heights, IL, USA) and captured on X-ray film.

2.7. Gene expression analysis by quantitative RT-PCR

RNA was isolated by RNeasy minikit (Qiagen, Benelux B.V., Venlo, The Netherlands). cDNA synthesis was performed with 1 μg RNA using 250 ng random hexamers (Promega), 40 units RNase OUT inhibitor (Invitrogen, Breda, The Netherlands) and SuperScript III RNase H^- Reverse Transcriptase (Invitrogen) according to standard laboratory protocols. Real-time PCR reactions were performed and data were analyzed as described previously [19]. Primers and probes were obtained as Assay-on-Demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands): housekeeping gene GAPDH (assay ID Hs99999905_m1), E-selectin (Hs00174057_m1), VCAM-1 (Hs00365486_m1), ICAM-1 (Hs00164932_m1), IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), MCP-1 (Hs00234140_m1).

2.8. Glomerulonephritis mouse model and in vivo adenoviral experimental procedures

All animal experiments were approved by the Local Committee on Animal Experimentation. Female C57bl/6 mice (9–10 weeks) were purchased from Harlan (Zeist, The Netherlands) and housed under standard laboratory conditions with free access to standard chow and acidified water. To induce glomerulonephritis, mice were immunized by intraperitoneal injection of 100 μl (2 mg/ml) sheep IgG (Sigma-Aldrich Chemie) mixed with 100 μl complete Freund's adjuvant (BD Pharmingen, Alphen aan den Rijn, The Netherlands). Six and a half days later (termed day 0) 1 mg of sheep anti-mouse glomerular basement membrane (anti-GBM) antibody together with 200 ng/ of recombinant mouse TNFα (BioSource Europe, Nivelles, Belgium) were *i.v.* injected to induce the disease [21]. Morphological analysis of renal injury was performed on formaldehyde-fixed, paraffin-embedded sections, which were stained with Periodic acid/Schiff (PAS), with PAS-positive material within glomeruli appearing within 48 h after induction of the inflammatory process.

At 2 h after induction of inflammation, 1×10^{11} respectively 2×10^{10} vp of Ab_{mEsel}-PEG-AddnIκB or Ab_{IgG}-PEG-AddnIκB (calculated based on densitometric analysis of the DNA of the adenovirus batches) were administered *i.v.* Animals were sacrificed at 3 h after injection of the adenoviruses for homing and viral DNA copy number determination, and at 46 h for viral DNA copy number determination and analysis of effects on gene expression. Organs were perfused with PBS/saline, harvested, snap frozen on liquid nitrogen, and stored in −80 °C.

2.9. Immunohistochemical detection of Ab_{mEsel}-PEG-AddnIκB adenovirus localization in vivo

Acetone-fixed 5 μm cryostat sections of kidneys of mice sacrificed 3 h after virus injection were blocked with 10% normal sheep serum (NSS) and 5% NMS for 1 h, washed with PBS, and incubated for 45 min with Alexa fluor 594 donkey anti-rat-IgG (H + L, Molecular Probes Invitrogen Detection Technologies, Eugene, Oregon; diluted 1:200 in PBS/5% FCS/5% NSS/1% NMS) to detect Ab_{mEsel}-conjugated adenovirus. After washing with PBS, nuclear counter staining was performed using DAPI (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Sections were embedded in Citifluor (Citifluor Ltd., London, UK) and examined using a fluorescence microscope (DM RXA, Leica) equipped with a Kappa CF8/1 FMC camera (Kappa Opto-Electronics, Gleichen, Germany).

2.10. Quantification of the viral genome in mouse tissues

For the quantification of adenovirus, 15–20 mg of kidney tissue cryosections were lysed in buffer from the DNeasy blood and tissue kit (Qiagen), and DNA was isolated according to the manufacturer's protocols. Quantitative PCR specific for the adenovirus serotype 5 hexon-gene was performed on the DNA isolate using the LightCycler FastStart DNA master^{PLUS} SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany) with wild type adenovirus DNA as an external standard to generate a calibration curve [22]. The PCR program consisted of 10 min hot start at 95 °C (Taq DNA polymerase activation), 40 cycles of 10 sec denaturation at 95 °C, 10 sec annealing at 60 °C, 10 sec extension at 72 °C. Melting curve analysis was performed at the end of the PCR program by denaturation at 95 °C, 10 sec annealing at 60 °C and gradual heating to 95 °C at 0.1 °C/sec. The hexon copy numbers were expressed as copies per ng DNA.

2.11. Laser microdissection of glomeruli and subsequent gene expression analysis

Five μm kidney cryosections were mounted on polyethylene-naphtalene membranes attached to glass slides (P.A.L.M. Microlaser Technology AG, Bernried, Germany), fixed in acetone for 5 min and stained with Mayer's hematoxylin, washed with diethyl pyrocarbonate treated water (0.1% DEPC), and air-dried. Glomeruli were marked along the Bowman's capsule and total areas of approximately 3×10^6 μm^2 were dissected using the Laser Robot Microbeam System (P.A.L.M. Microlaser Technology). Dissected areas were collected directly in the cap of a P.A.L.M. AdhesiveCaps tube (P.A.L.M. Microlaser Technology) via laser pressure catapulting. RNA extraction was performed according to the protocol of Absolutely RNA Microprep kit (Stratagene, Amsterdam, The Netherlands). Reverse transcription was performed as described above, and 10 μl cDNA was mixed with 40 μl water and 50 μl 2× TaqMan PCR Master Mix (Applied Biosystems) and loaded into Taqman MicroFluidicCard Low Density Array, custom made with regard to the gene analysis (Applied Biosystems). Quantitative PCR reaction was performed in duplicates for each sample in a TaqMan ABI7900HT. Gene expression was normalized to the housekeeping gene GAPDH.

2.12. Statistics

The two-sided Student's *t* test (assuming equal variances) was performed to evaluate statistical significance. Differences were considered significant when $p<0.05$.

3. Results

3.1. Effectiveness of the IκB mutant to silence inflammatory gene expression in HUVEC

To study the capacity of the therapeutic virus to block inflammatory gene expression in endothelial cells, we first determined human umbilical vein endothelial cells (HUVEC) responses to TNFα after infection with non-modified AddnIκB. E-selectin and VCAM-1 were chosen as effector genes, since their cytokine-inducible expression is NF-κB-driven [19]. Plain virus was able to infect resting cells, with concurrent expression of the dnIκB transgene protein as shown by Western blot analysis (Fig. 1a). Moreover, dnIκB effectively blocked the cytokine-induced expression of both E-selectin and VCAM-1 in a dose-dependent fashion, with EC_{50} values of 8.4×10^2 and 5.0×10^3 vp/cell, respectively (Fig. 1b). A dose of 10^4 vp/cell nearly abolished the expression of both genes, thereby confirming the potential of dnIκB inhibitory protein as an anti-inflammatory therapeutic protein.

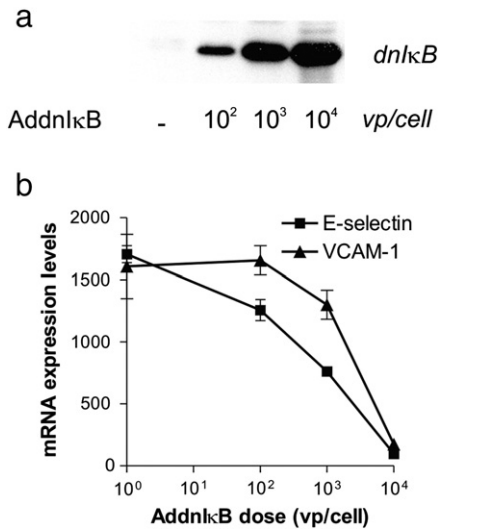

Fig. 1. Efficacy of dnIκB adenovirus to inhibit cytokine driven endothelial gene expression in vitro. Infection of HUVEC with different doses of AddnIκB (vp/cell) resulted in the expression of dnIκB transgene protein (a; Western blot), which was functional in inhibiting E-selectin and VCAM-1 expression in a dose dependent fashion (b; quantitative RT-PCR). HUVEC were infected for 1 h prior to activation. Cells were subsequently incubated for another 24 h. (a) Detection of the HA-tagged transgene dnIκB. (b) NF-κB-dependent genes E-selectin and VCAM-1 expression levels upon activation for 6 h with 10 ng/ml TNFα. E-selectin and VCAM-1 gene expression was adjusted to the expression of the housekeeping gene GAPDH, and calculated as fold increase in mRNA over the basal gene expression in quiescent endothelium arbitrarily set at 1. Mean values of triplicate mRNA levels ± SD.

3.2. In vitro target epitopes expression kinetics

To determine suitability of E-selectin and VCAM-1 as target epitopes for selective delivery of the adenovirus into endothelium under inflammatory conditions, we studied their surface expression kinetics by HUVEC at different time points. By means of flow cytometric analysis we observed that E-selectin and VCAM-1 were both induced in activated HUVEC (Fig. 2), while their expression was absent on resting cells. We further confirmed previously reported differential expression kinetics of both proteins in time. E-selectin was induced to a maximum level already at 4 h of activation, while VCAM-1 upregulation was visible at 4 h but increased significantly up to 24 h. From these data we

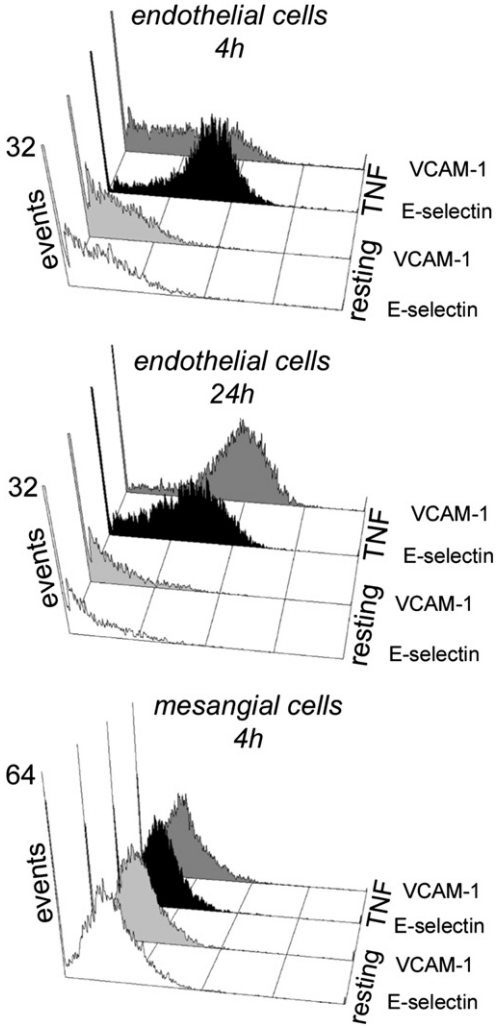

Fig. 2. Kinetics of in vitro endothelial E-selectin and VCAM-1 expression upon TNFα-activation. Flow cytometric analysis of cell adhesion molecule expression by endothelial cells activated for 4 h and 24 h and mesangial cells activated for 4 h with TNFα (10 ng/ml). Mean Fluorescence Intensity (MFI) of E-selectin and VCAM-1 expression was representative for 3 different experiments.

concluded that E-selectin is a more suitable epitope for targeted viral gene delivery at an early time point of activation, while VCAM-1 was being used as a target epitope at later time points.

Since our primary goal was the targeted delivery of AddnIκB to inhibit inflammatory endothelial activation in glomerulonephritis, we also studied possible regulation of the target epitopes in glomerulus-associated mesangial cells. Activation by TNFα did not induce E-selectin or VCAM-1 expression in mesangial cells.

3.3. E-selectin and VCAM-1 facilitated intracellular gene delivery by retargeted adenoviruses

We next analyzed whether antibody-PEG modified viruses with selectivity for E-selectin or VCAM-1 were able to deliver their transgene into target cells. Through PEGylation, adenoviruses lost their intrinsic infection potential (Fig. 3a), corroborating previous data from our laboratory [14]. By coupling anti-E-selectin or anti-VCAM-1 antibody to the distal end of the PEG molecule, both modified adenoviruses regained infectivity (Fig. 3a), which was now guided by the endothelial expression of their respective target molecules. The level of transfection with the antibody-coupled adenoviruses was dependent on the cytokine activator(s) used, and was higher for Ab_{Esel}-PEG-AdTL compared to Ab_{VCAM}-PEG-AdTL.

Target specificity of the retargeted adenoviruses, was investigated by co-incubation of parental anti-E-selectin and anti-VCAM-1 antibodies to compete with the binding of modified viruses to activated HUVEC. As shown in Fig. 3b, the transduction of Ab_{Esel}-PEG-AdTL and Ab_{VCAM}-PEG-AdTL was significantly inhibited by excess of parental antibody but not by irrelevant antibodies. These data demonstrate that the antibody-modified adenovirus was capable of infecting HUVEC by means of antibody-guided specificity and not by antibody conjugation *per se*.

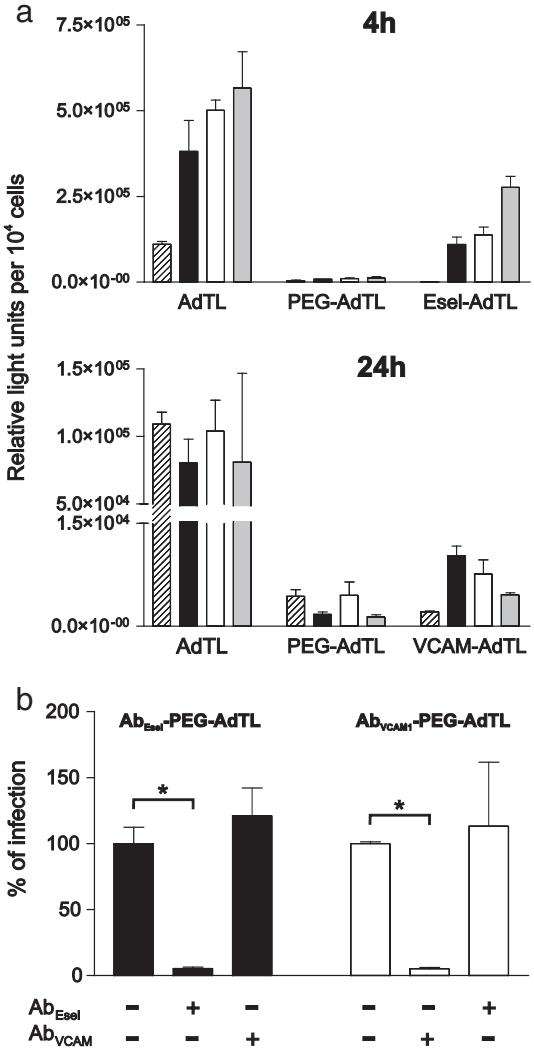

Fig. 3. E-selectin and VCAM-1 retargeted adenoviruses selectively infected cytokine-activated endothelial cells in vitro. Luciferase reporter gene assays were performed on lysates of endothelial cells at 48 h after infection by AdTL, PEG-AdTL, Ab_{Esel}-PEG-AdTL (Esel-AdTL) or Ab_{VCAM1}-PEG-AdTL (VCAM-AdTL) of resting (hatched bars) or activated HUVEC cells: 10 ng/ml TNFα (black bars), 10 ng/ml IL-1β (white bars), or 10 ng/ml of both cytokines (gray bars). Activation was performed for 4 h respectively 24 h. (a) Reporter enzyme activity measured in mean relative light units per 10^4 cells. (b) Competition between Ab_{Esel}-PEG-AdTL respectively Ab_{VCAM1}-PEG-AdTL and blocking antibodies Ab_{Esel} or Ab_{VCAM} (20 μg/ml) for binding to TNFα-activated HUVEC. Mean relative light units obtained for the competition data were calculated as percentage of the values obtained in the absence of the competing antibodies set at 100%. Mean values of triplicate wells ± SD. *$p<0.05$.

3.4. Targeted expression of dnIκB mutant inhibited inflammatory gene and protein expression in HUVEC

The therapeutic potential of the retargeted dnIκB encoding adenovirus was studied in cytokine-induced activated HUVEC. Since Ab_{Esel}-PEG-AdTL demonstrated better transfection efficiency than Ab_{VCAM}-PEG-AdTL, further studies were performed with E-selectin targeted virus. Based on flow cytometric analysis of E-selectin protein expression by HUVEC upon consecutive activation with different combinations of TNFα and IL-1β (data not shown), we established an experimental set-up as presented in Fig. 4a. By application of this scheme, transfection with PEGylated adenovirus resulted in limited transgene expression (Fig. 4b) and concomitant minor effects on NF-κB driven gene expression (Fig. 4c). By retargeting AddnIκB via Ab_{Esel}, the virus regained infectivity to facilitate dnIκB expression in activated cells (Fig. 4b), resulting in significant inhibition of inflammatory gene expression upon cytokine challenge (Fig. 4c). Antibody-PEG modification of AddnIκB not only inhibited intrinsic viral knob-CAR interaction to a major extent, but also provided the virus with target cell specificity of delivery of a therapeutically active transgene to inhibit the expression of multiple genes.

3.5. Ab_{Esel}-PEG-AddnIκB homed to inflammatory endothelium in vivo and affects early stage glomerulonephritis development

For *in vivo* studies, mouse Ab_{Esel} (Ab_{mEsel}) modified adenovirus was constructed and flow cytometric analysis showed that Ab_{mEsel} and Ab_{mEsel}-PEG-AddnIκB specifically bound to mouse endothelial cells activated with cytokines, resulting in a fluorescence signal corresponding to that obtained with 2 μg of Ab_{mEsel} binding (Fig. 5).

Next, we investigated homing of Ab_{mEsel}-modified adenovirus in mice with glomerulonephritis (Fig. 6a). This inflammatory kidney disease is characterized by leukocyte infiltration in glomeruli and impaired glomerular filtration. The activated endothelium expresses cell adhesion molecules to facilitate this leukocyte recruitment. We previously showed that E-selectin was expressed in glomeruli in the early phase of inflammation, while in healthy control mice E-selectin was absent [21]. Three hours after intravenous *i.v.* administration, Ab_{mEsel}-PEG-AddnIκB was found to be associated with cells in inflamed glomeruli (Fig. 6b-A), while in the kidney of healthy control animals no virus could be detected (data not shown). Ab_{mEsel}-PEG-AddnIκB colocalized with endothelial cell marker protein CD31 (Fig. 6b-C), although not all endothelial cells in the glomeruli were targeted by the virus. Rat IgG control modified virus (Ab_{IgG}-PEG-AddnIκB) was barely detectable in any of the vascular beds in the kidney using the same staining protocol (Fig. 6b-B). Furthermore, no Ab_{mEsel}-PEG-AddnIκB could be detected in other vascular beds in kidney, corroborating immunohistochemical data on glomerulus restricted E-selectin expression [21,10]. From these results, we concluded that introduction of E-selectin specificity in the adenovirus endowed the virus with homing specificity to endothelial cells present in inflamed tissue.

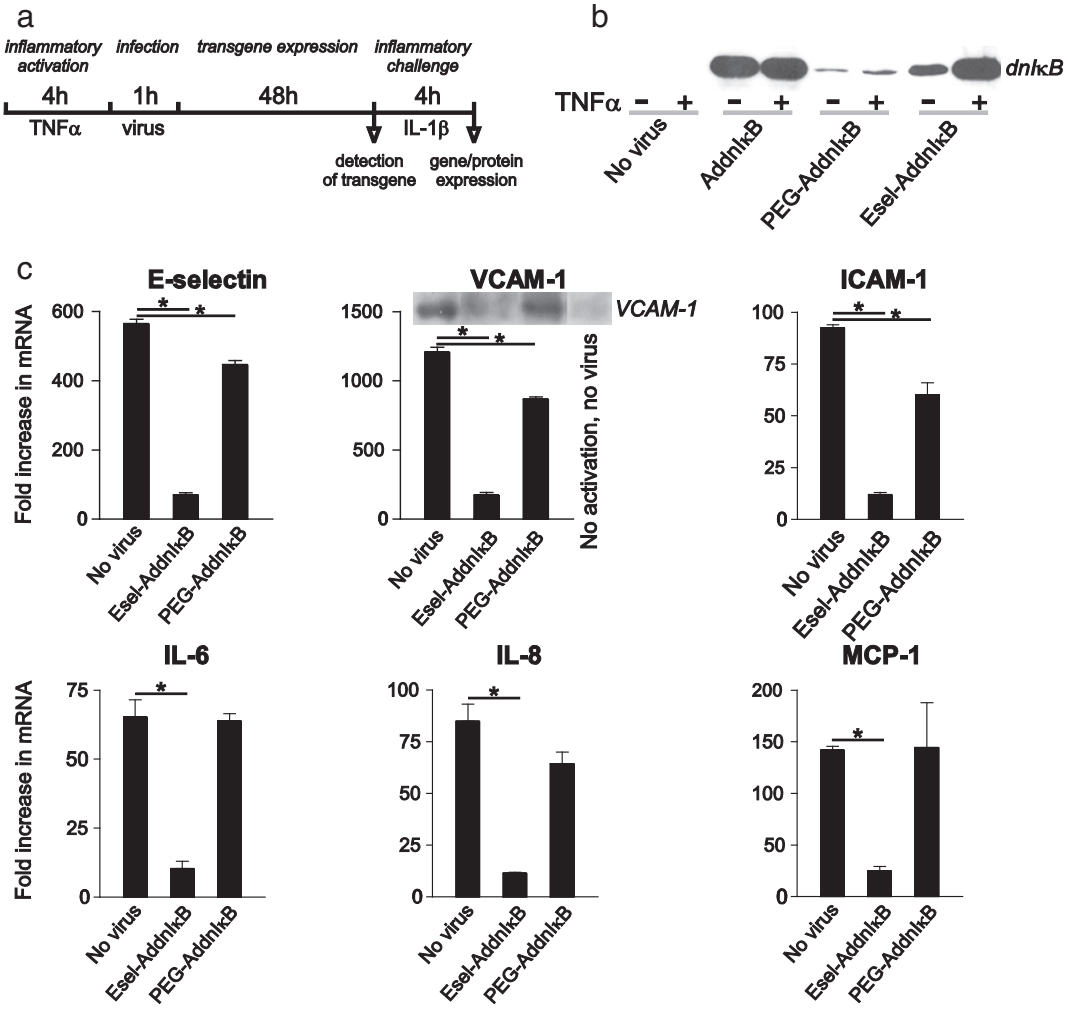

Fig. 4. Retargeting of AddnIκB enabled functional expression of the therapeutic transgene. (a) Schematic representation of the experimental set-up employed. Resting or TNFα-activated (4 h, 10 ng/ml) HUVEC were incubated with viruses at 10^4 vp/cell for 1 h. Cells were subsequently washed and incubated for another 48 h during which time the transgene could be expressed. Cells were next activated with 10 ng/ml IL-1β as a re-challenge to induce an inflammatory response. (b) Western blot of HA-tagged dnIκB transgene expressed in cells transfected with unmodified and modified viruses. (c) Inflammatory gene expression in HUVEC incubated with 10^4 vp/cell Ab_{EseI}-PEG-AddnIκB or PEG-AddnIκB. Gene expression was adjusted for the expression of the housekeeping gene GAPDH and calculated as fold increase in mRNA over basal gene expression in resting HUVEC set at 1. Mean values of triplicate ± SD ($n=3$). *$p<0.05$. Insert: VCAM-1 protein assessed by Western blot analysis.

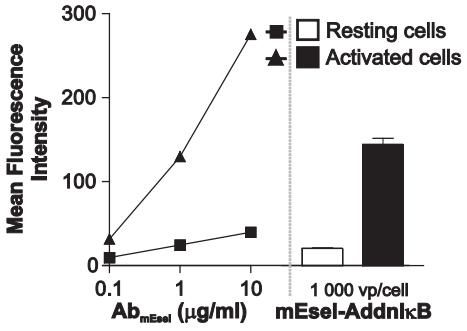

Fig. 5. Ab_{mEseI}-PEG-AddnIκB binds to cytokine-activated mouse endothelial cells. Binding of 10^3 vp/cell Ab_{mEseI}-PEG-AddnIκB to the surface of resting and cytokine-activated H5V cells assessed by flow cytometry, with mouse Ab_{mEseI} binding as positive control. H5V cells were either resting or activated for 4 h with mouse TNFα and IL-1β, both at 100 ng/ml, for induction of E-selectin protein expression prior to antibody or Ab_{mEseI}-PEG-AddnIκB virus incubation. Mean fluorescence intensity for each sample ± SD ($n=3$).

We then investigated the effects of targeted dnIκB gene delivery on the expression of a series of inflammatory genes in an early phase of glomerulonephritis development as a measure of pharmacological activity. Laser microdissection was applied to isolate glomeruli from kidney biopsies prior to quantitative analysis of RNA levels to circumvent masking of effects by gene expression in neighboring, non-targeted cells. E-selectin and VCAM-1 were significantly down regulated in glomeruli of Ab_{mEseI}-PEG-AddnIκB treated mice to 35 and 40% of untreated control, respectively (Fig. 6c). In case of P-selectin and intracellular adhesion molecule (ICAM)-1, we observed no significant inhibition of expression. In contrast to *in vitro* observations, IL-6 and monocyte chemotactic protein (MCP)-1 were not inhibited. The expression of endothelial markers CD31 and VE-cadherin, which are not controlled by NF-κB, was not influenced by virus treatment (data not shown). Overall, these data demonstrated that E-selectin-retargeted adenovirus encoding dnIκB transgene could inhibit endothelial proinflammatory activation *in vivo* in the early phase of disease development.

Histological analysis of kidneys revealed that PAS-positive thrombi were formed in the glomerular intracapillary space, and that intratubular protein casts were observed already at the second day of disease

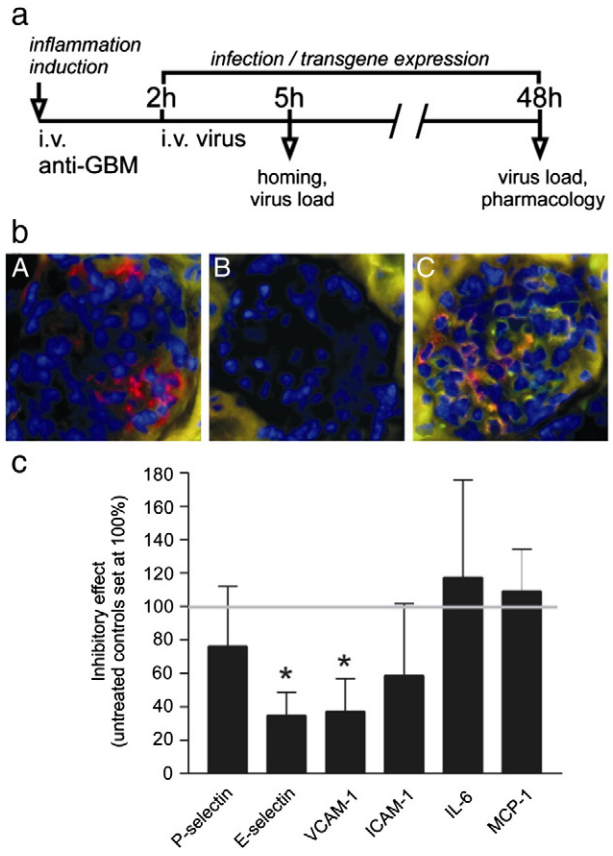

Fig. 6. Homing and short-term effect of Ab_{mEsel}-PEG-AddnIκB on inflammatory gene expression in mouse glomerulonephritis. (a) Schematic representation of the experimental protocol employed. Viruses were i.v. injected 2 h after induction of glomerulonephritis. After another 3 h, mice were sacrificed to study virus homing to the kidney and virus load in the main organs. At 2 days of inflammation progression, mice were sacrificed to study the effects of transgene expression in the kidney, and virus load in the organs. (b) Immunofluorescent detection (red) of Ab_{mEsel}-PEG-AddnIκB (A) and Ab_{IgG}-PEG-AddnIκB (B) localization in glomeruli in the kidney 3 h after i.v. administration of the viruses, as well as colocalization (yellow) of Ab_{mEsel}-PEG-AddnIκB (red) with endothelial cell marker protein CD31 (green) (C). Pictures show a representative glomerulus at 400× magnification. (c) Quantitative RT-PCR analysis of P- and E-selectin, VCAM-1, ICAM-1, IL-6, and MCP-1 performed on RNA isolated from glomeruli as described in Materials and methods. Ct values of the respective gene were adjusted for the expression of the housekeeping gene GAPDH, and values obtained from non-treated diseased mice (48 h) were set at 100%. Mean values of 3 (non-treated glomerulonephritis controls) and 4 (Ab_{mEsel}-PEG-AddnIκB-treated glomerulonephritis) mice ±SD. *$p<0.05$: significant difference in gene expression between the two groups.

progression, both indicative of glomerulonephritis development (Fig. 7). There was no significant difference of Ab_{mEsel}-PEG-AddnIκB treatment on PAS-positive material deposition compared to non-treated control or treatment with Ab_{IgGl}-PEG-AddnIκB.

We established the viral load in the kidneys of mice sacrificed at 3 h and 46 h after virus administration by quantitative PCR analysis (Table 1). At 3 h no differentiation in distribution between Ab_{mEsel}-PEG-AddnIκB and Ab_{IgG}PEG-AddnIκB could be made, implying that adenoviral vector distribution and accumulation directly after injection was independent of the Ab specificity attached. At 46 h the levels of Ab_{mEsel}-PEG-AddnIκB were significantly higher in the target organ compared to the levels of Ab_{IgG}-PEG-AddnIκB. As it is likely that 2 days after virus administration the viral genome copies reflect the actual content of virus in the cells, these results indicate that E-selectin-retargeted virus was able to accumulate in the kidney, while control modified virus was not.

4. Discussion

In the current study, we investigated whether adenoviral gene therapy vectors could be designed to selectively deliver a functional transgene into endothelial cells engaged in inflammation. The introduction of endothelial cell-specific anti-E-selectin or anti-VCAM-1 antibody-PEG modification resulted in transfer of the transgene into cytokine activated endothelial cells only. Moreover, we showed that both *in vitro* and *in vivo*, retargeted dnIκB transgene expression in endothelial cells interfered with the expression of a series of inflammatory genes. Our data imply that antibody-PEG modification of adenoviral vectors for gene therapy can be modified on demand to loose intrinsic CAR recognition and to gain target cell specificity.

NF-κB involvement in kidney inflammation has been well described [2,23–25]. It controls endothelial cell activation, as it is crucial in inflammatory gene expression control and hence guidance of leukocyte recruitment. The number of studies aiming at selective delivery of NF-κB interference using (adenoviral) gene therapy to activated endothelium in inflammation is limited, which is rather surprising considering the efforts to develop chemical NF-κB inhibitors for clinical application. For glomerulonephritis, a few studies proposed targeted pharmacological interference with NF-κB aimed at glomerulus-associated mesangial cells, which are also actively involved in development and progression of the disease [26]. A glomerulus-specific NF-κB decoy oligodeoxynucleotide transfer method using virosomes substantially reduced albuminuria, tissue damage, and renal expression of inflammatory cytokines [27]. Another study employed macrophages, which accumulate in the kidney during disease progression. In these macrophages NF-κB signaling had been inactivated *ex vivo* and upon *in vivo* administration a significant reduction in macrophage glomerular infiltration leading to nephritis was seen [28]. Theoretically, a combination treatment approach to silence the undesired, excessive proinflammatory behavior of cells locally involved in disease progression will provide the strongest anti-inflammatory effect. We furthermore recently showed that simultaneous inhibition of NF-κB and p38MAPK resulted in much stronger blocking of the expression of E-selectin, IL-6, IL-8, and COX-2 than the effects observed after inhibition of each pathway separately [19]. Since p38MAPK is, together with NF-κB, a key regulator of glomerulonephritis [4], it is conceivable that blocking both transduction pathways simultaneously using a combination of adenoviral vectors encoding dnIκB or dnp38MAPK [29] would also present a superior pharmacological strategy.

For local therapy, a targeting approach is essential to direct the adenoviral vectors to the actual place of interference. To accomplish this the viral capsid can be modified either recombinantly or chemically with cell-specific ligands [30]. Pegylation of adenoviruses and coupling of antibodies to the distal end of the PEG chain has been shown increase blood circulation time and decrease uptake by the liver and spleen, indicating that the properties of modified virus are beneficial for targeting purposes [14]. Endothelial-specific adhesion molecules have been proposed as potential targets for delivery of therapeutics to activated endothelial cells [14,31,32]. Particularly E-selectin attracted attention due to its high, transient, and specific expression restricted to activated endothelial cells in the microvasculature engaged in the inflammatory process. Another essential component of the leukocyte-endothelial adhesion cascade, VCAM-1 was recently described as a possible target for specific drug delivery to activated endothelial cells [33]. We showed in the current study that both target epitopes are suitable for transgene delivery into activated HUVEC. However, delivery via E-selectin was more efficient than delivery via VCAM-1. Since *in vitro* both molecules are internalized [31,33], the difference in efficiency of cargo delivery cannot be explained by a different route of cellular handling. Possibly, different kinetics of endocytosis underlies the observations described here. Further studies are necessary to elucidate the relation between the

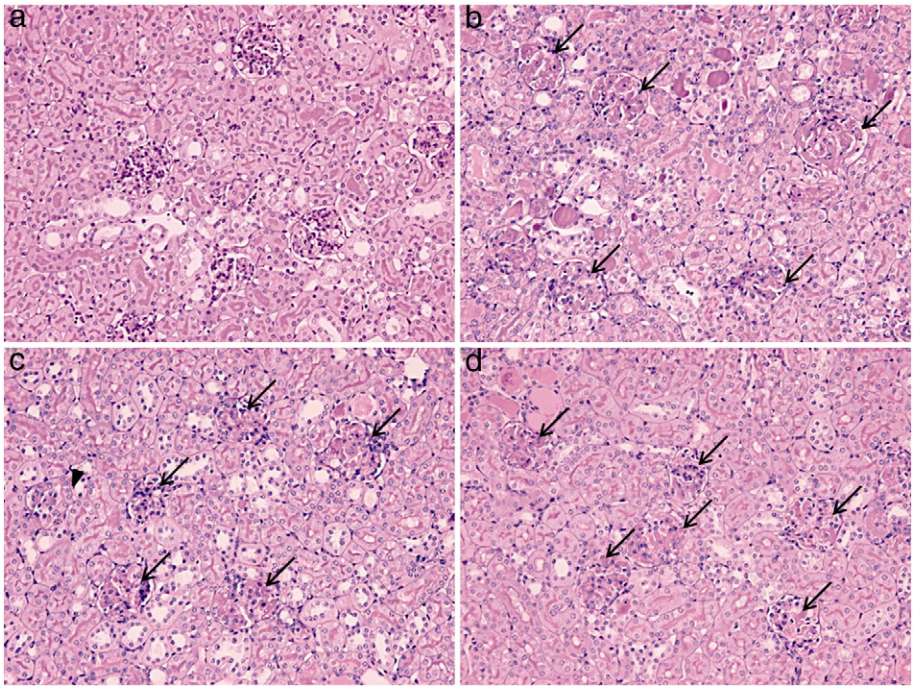

Fig. 7. PAS staining of the kidneys at 2 days after induction of glomerulonephritis. Periodic acid/Schiff (PAS) staining was performed on kidneys of healthy mice (a), diseased mice (b), and of mice treated with Ab_{mEsel}-PEG-AddnIκB (c), or Ab_{IgG}-PEG-AddnIκB (d). Arrows indicate glomeruli with PAS-positive thrombi, arrowhead indicates PAS-negative glomerulus.

amount of transgene delivered in the endothelial cells, the intracellular processing, the subsequent transcription and translation of the transgene and finally, the consequence for dnIκB functionality.

Our study shows that cell selective targeted inhibition of gene expression can be achieved both *in vitro* and *in vivo*, indicating that adenoviral tropism can be modified on the demand. However several issues require further attention. For example, we used a virus encoding a transgene under the control of a CMV promoter, a choice which for further studies should be discouraged because of its immunogenic properties. The use of endothelial-specific promoters such as VE-cadherin [34], preproendothelin-1 [35], or flt-1 [36] promoters would be optional, with the additional advantage that it increases the specificity of gene expression [36].

We were not able to detect dnIκB protein by Western blot or NF-κB inhibition by Electrophoretic Mobility Shift Assay, neither in whole kidney protein/nuclear extracts nor in microdissected glomeruli. Absence of signal in whole kidney protein extracts is most likely due to the fact that the effects of the targeted adenovirus are localized in the glomeruli, a minority of cell volume within the whole organ. Absence of the signal in laser dissected glomeruli is most likely due to the low yield of protein in relation to the detection limit of the assay. What we could show, though, was the downstream effect of NFκB blockade in the glomerular fraction, represented by inhibition of inflammatory genes upon targeted delivery of the dnIκB transgene. *In vivo* only the expression of E-selectin and VCAM-1 was significantly downregulated, while P-selectin and ICAM-1 were not affected. Investigation of the literature on the murine P-selectin promoter in endothelial cells revealed that it closely resembles the E-selectin gene promoter [37]. It is therefore unclear why P-selectin was not inhibited to similar extent as E-selectin. Absence of ICAM-1 response to targeted gene therapy treatment may be explained by the fact that in the glomeruli other cell types besides the endothelium can be the source of ICAM-1 [2]. Therefore, selective interference with the expression of ICAM-1 in glomerular endothelium may have been masked by the ICAM-1 expression in other cells. Another explanation for the limited effects of dnIκB on certain genes may be that *in vivo* the control of inflammatory gene expression in (specialized) subsets of microvascular endothelium is different from the ones described in *in vitro* cell culture systems [38].

Although we observed an inhibitory effect of the retargeted therapeutic adenovirus on inflammatory gene expression, we could not prevent glomerulonephritis from occurring. Future studies on kinetics of transgene expression in relation to pharmacological effects may disclose more details on the therapeutic potential of the strategy investigated here.

In summary, in this study we demonstrated the potential of tropism-modified adenovirus to deliver a functional therapeutic gene into microvascular endothelial cells in inflammation. The inhibition of the expression of multiple adhesion molecules, cytokines and chemokines may have an important pharmacological impact on disease initiation and progression. Future investigations regarding the

Table 1
Accumulation of modified viruses in the kidneys of mice with acute glomerulonephritis at 3 and 46 h after i.v. administration.

	3 h		46 h	
Treatment	Ab_{IgG}-PEG-AddnIκB	Ab_{mEsel}-PEG-AddnIκB	Ab_{IgG}-PEG-AddnIκB	Ab_{mEsel}-PEG-AddnIκB
No. mice /group	*2*	*3*	*3*	*4*
	Hexon copy numbers (copies/ng DNA)			
Kidney	217 ± 54	448 ± 421	46 ± 5	357 ± 49*

*$p<0.01$: significant difference between hexon copy numbers in mice treated with Ab_{mEsel}-PEG-AddnIκB and mice injected with control Ab_{IgG}-PEG-AddnIκB virus at the same time point (mean, ± SD).

functional consequences of NF-κB blockade on leukocyte trafficking and disease associated (patho)physiological parameters will enable a detailed characterization of the added value of the microvascular endothelium targeted gene therapy strategy presented here.

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