Glomerular Regeneration Is Delayed in Nephritic α_8 -Integrin-Deficient Mice: Contribution of α_8 -Integrin to the Regulation of Mesangial Cell Apoptosis

Andrea Hartner^a Ines Marek^a Nada Cordasic^b Christian Haas^b
Harald Schocklmann^d Gudrun Hulsmann-Volkert^b Isabel Plasa^b
Wolfgang Rascher^a Karl F. Hilgers^b Kerstin Amann^c

^aHospital for Children and Adolescents, ^bDepartment of Nephrology and Hypertension, and ^cInstitute for Pathology, University of Erlangen, Erlangen, and ^dDepartment of Nephrology, University of Kiel, Kiel, Germany

Key Words

α_8 -Integrin • Cell-matrix interactions • Glomerulonephritis • Glomerular injury • Habu nephritis • Mesangiolysis

Abstract

Background/Aims: $\alpha_8\beta_1$ -Integrin is expressed in mesangial cells. In vitro studies suggest a role for α_8 -integrin in the regulation of cell proliferation and apoptosis. We tested the hypothesis that α_8 -integrin is essential for the healing process after mesangioproliferative glomerulonephritis. **Methods:** Mice homozygous for a deletion of the α_8 -integrin chain were compared with wild-type mice. To study glomerular healing, we used the habu toxin model of reversible mesangioproliferative glomerulonephritis. Animals received 6 mg/kg habu toxin intravenously; controls received saline only. **Results:** Early mesangiolysis occurred in wild-type and α_8 -integrin-deficient mice. However, mesangiolysis was no longer detectable after 7 days in wild types but persisted after 14 days in α_8 -integrin-deficient animals. Mesangial activation marker α -smooth muscle actin was detectable only at day 7 in wild-type mice but persisted until day 14 in α_8 -integrin-deficient mice. In wild types, glomerular cell proliferation and apoptosis peaked at day 7 and decreased thereafter

but remained elevated in α_8 -integrin-deficient mice until day 28. In cultivated mesangial cells, α_8 -integrin expression was associated with increased cell survival. **Conclusion:** Interactions between α_8 -integrin and the mesangial matrix may contribute to healing of glomerular injury by influencing cell proliferation and apoptosis.

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Introduction

Integrins are cell-matrix receptors consisting of one α - and one β -subunit [1]. Altered expression of β_1 -integrins as well as their matrix ligands have been described in human renal inflammatory diseases, like in immunoglobulin (Ig) A nephritis [2] or other forms of glomerulonephritis [3]. Moreover, upregulation of β_1 -integrins, like $\alpha_1\beta_1$ or $\alpha_5\beta_1$, was shown in animal models of glomerular disease [4, 5]. The role of integrins in the severity or progression of glomerular disease is still a matter of investigation and in some cases controversial: $\alpha_1\beta_1$ -integrin seemed to have profibrotic effects in the Thy1.1 model of glomerulonephritis in the rat [6], where administration of an antibody to $\alpha_1\beta_1$ resulted in a reduction of

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Andrea Hartner, PhD
Hospital for Children and Adolescents
Loschgestrasse 15, DE-91054 Erlangen (Germany)
Tel. +49 9131 853 3978, Fax +49 9131 853 5821
E-Mail andrea.hartner@rzmail.uni-erlangen.de

glomerulosclerosis. On the other hand, lack of $\alpha_1\beta_1$ led to severe glomerulosclerosis following glomerular injury induced by adriamycin [7].

The α_8 -integrin chain was described to be expressed in mesenchymal and neuronal cells and to heterodimerize solely the β_1 -integrin chain [8, 9]. Integrin $\alpha_8\beta_1$ is a receptor for fibronectin, vitronectin, tenascin C, osteopontin and nephronectin [10–12]. In vitro studies suggest a role for α_8 -integrin in the regulation of cell proliferation and cell survival [13]. In the glomerulus of the kidney the α_8 -integrin chain is exclusively expressed in mesangial cells and is induced in the Thy1.1 rat model of glomerulonephritis [14]. Mice with a homozygous deletion of the α_8 -integrin chain have a reduced renal mass due to impaired epithelial-mesenchymal interactions in early stages of kidney development [15]. However, glomeruli are apparently normal with only subtle morphological alterations and no functional abnormalities [16]. Studies of the sequelae of glomerular hypertension in these mice revealed a role for integrin $\alpha_8\beta_1$ in maintaining the integrity of the glomerular capillary tuft during mechanical stress [17]. The role of integrin $\alpha_8\beta_1$ in the development or progression of mesangioproliferative glomerular injury is not clear. Given the increase in α_8 -integrin chain expression during experimental Thy1.1 glomerulonephritis [14] and its assumed influence on mesangial cell proliferation and survival, it is tempting to speculate that lack of α_8 -integrin would alter the course of mesangioproliferative glomerulonephritis. In mice, it is not feasible to induce Thy1.1 glomerulonephritis because the Thy1.1 antigen is not present on mouse mesangial cells. We therefore evaluated glomerular changes in α_8 -integrin-deficient mice after induction of habu glomerulonephritis.

Methods

Induction of Habu Glomerulonephritis

Mice were housed in a room maintained at $22 \pm 2^\circ\text{C}$, exposed to a 12-hour dark/light cycle. The animals were allowed unlimited access to chow (No. 1320, Altromin, Lage, Germany) and tap water (or 1% saline, see below). Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed. All procedures performed on animals were done in accordance with guidelines of the American Physiological Society and were approved by the local government authorities (Regierung von Mittelfranken, AZ No. 621-2531.31-1/01).

Male homozygous α_8 -integrin-deficient mice were a gift from U. Muller (Scripps Institute, LaJolla, Calif., USA) and kept as a homozygous breeding line on a mixed 129 $\times$ C57BL/6 background. These mice generally develop only one kidney or two smaller kidneys, leading to a reduced total renal mass of approx. 50%. Age- and weight-matched male C57BL/6 mice (Charles River, Sulzfeld,

Germany) were used as the primary wild-type controls: all mice used for experiments were between 70 and 80 days of age and 20–24 g of weight. Being aware that C57BL/6 mice are not the ideal wild-type control for α_8 -integrin-deficient mice, we additionally investigated another wild-type strain: 129 $\times$ C57BL/6 intercrosses. The results from our histologic and immunohistochemical studies did not differ in both wild-type controls. For this reason, we did not distinguish between both controls in our 'Results' section.

At an average weight of 16–18 g, wild-type mice underwent right unilateral nephrectomy to adjust for the reduced kidney mass in α_8 -integrin-deficient mice. After 2 weeks of recovery, 25 α_8 -integrin-deficient and 35 wild-type animals received 6 mg/kg habu toxin (venom of the habu snake *Trimeresurus flavoviridis*, Sigma, Deisenhofen, Germany) intravenously via the tail vein; 10 α_8 -integrin-deficient and 10 wild-type animals received saline as control. Habu glomerulonephritis is a well-established model of acute glomerulonephritis and leads to a self-limited transient glomerular injury [18]. It is characterized by an initial capillary widening due to mesangiolysis and rupture of the capillary tuft in approx. 20–30% of the glomeruli 24 h after induction of disease. This is followed by compensatory mesangial cell proliferation and matrix expansion which can be detected at days 4–10. At day 7, an increase in apoptotic glomerular cells leads to resolution of glomerular hypercellularity some days later. After 20 days, normal glomerular architecture is restored [19, 20]. Mice were sacrificed 1, 7, 14 or 28 days after injection and kidneys were harvested for histologic and immunohistochemical studies. Parts of the kidneys were put in methyl-Carnoy solution (60% methanol, 30% chloroform and 10% glacial acetic acid) for fixation. Other parts were snap frozen in liquid nitrogen for mRNA analyses, or were embedded in TissueTek (Sakura, Zoeterwoude, The Netherlands) and frozen in liquid nitrogen for cryostat sections.

Renal Histology

After overnight fixation in methyl-Carnoy solution, tissues were dehydrated by bathing in increasing concentrations of methanol, followed by 100% isopropanol. After embedding in paraffin, 2- μm sections were cut with a Leitz SM 2000 R microtome (Leica Instruments, Nussloch, Germany). Before any staining procedure, sections were deparaffinized by bathing in xylol and rehydrated in decreasing concentrations of alcohol. For evaluation of general renal histology, kidney sections were PAS stained. Glomerular apoptotic nuclei were counted in all glomeruli of a kidney cryostat section (approx. 60–80) after Hoechst stain.

To quantify glomerulosclerosis, a score of 0–4 was used as described before [17]: score 0 = normal glomerulus; score 1 = mesangial expansion or sclerosis involving up to 25% of the glomerular tuft; score 2 = sclerosis 25–50%; score 3 = sclerosis 50–75% and/or segmental extracapillary fibrosis or proliferation; score 4 = global sclerosis (>75%) or global extracapillary fibrosis or proliferation, or complete collapse of the glomerular tuft. To assess the extent of the formation of microaneurysms, capillary widening was scored [16]. Similarly to the score for sclerosis, a score of 0–4 was applied: score 0 = no capillary widening in the glomerular tuft; score 1 = capillary widening involving up to 25% of the glomerular tuft; score 2 = capillary widening 25–50%; score 3 = capillary widening 50–75%; score 4 = capillary widening involving more than 75% of the glomerular tuft. Scoring was performed on blinded sections and all glomeruli per section were scored.

Cell Isolation and Cultivation

Mesangial cells were isolated from kidneys by the sieving method, similar to the method described previously [21] using 63-, 75- and 38- μ m grid sieves. Cultured wild-type and α_8 -integrin-deficient mesangial cells were characterized as described [21]. Mesangial cells were grown in Dulbecco's modified Eagle's medium (PAA Laboratories GmbH, Linz, Austria) containing 10% fetal calf serum, 5 μ g/ml insulin, 5 μ g/ml Plasmocin (TEBU, Frankfurt, Germany) and 2 mM L-glutamine (Sigma) in a 95% air/5% CO₂ humidified atmosphere at 37°C. Mesangial cells were used for experiments in passages 5–10.

Silencing of the α_8 -Integrin Gene

Gene silencing of α_8 -integrin was achieved by transfection of mesangial cells with α_8 -integrin siRNA according to the traditional protocol from the HiPerFect Transfection Reagent Handbook (Qiagen, Hilden, Germany). Mesangial cells were grown to subconfluence in petri dishes and cells were incubated with 100 nM α_8 -integrin siRNA (sense: GAC CUC CUC AGG AUG AAA UdT dT, antisense: AUU UCA UCC UGA GGA GGU CdT dT) for 24, 48 and 72 h. P8 (nuclear protein 1) siRNA (sense: GGA CGU ACC AAG AGA GAA GdT dT, antisense: CUU CUC UCU UGG UAC GUC CdT dT) was used as control.

Expression of α_8 -Integrin cDNA in 293 Human Embryonic Kidney Cells

Full-length α_8 -integrin cDNA was a gift from Dr. Muller, Basel (described in Bieritz et al. [21]), and was cloned into the expression vector pcDNA3.1+/Neo. To obtain a functional deletion in the cytoplasmic domain of the α_8 -integrin chain, a 65-bp fragment at the 3' end of the α_8 -integrin cDNA was removed as described before [21].

Determination of Apoptosis Rates

For caspase-3 assays, cells were grown in petri dishes to 80% confluency and apoptosis was induced by incubation with 50 μ M cisplatin (Sigma) or serum deprivation for 16–24 h. Cells were washed, harvested and resuspended in lysis buffer (100 mmol/l Hepes, pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 1 mmol/l EDTA). After sonification (15 s with 40% output), cell lysates were incubated on ice for 30 min and centrifuged (10,000 g at 4°C for 10 min). Protein concentration of cell lysates was determined using a protein assay kit (Pierce, Rockford, Ill., USA); 30 μ g protein of cell lysates were incubated in lysis buffer with 10 mmol/l dithiothreitol at 30°C with 12 μ mol/l of the caspase-3 substrate N-Acetyl-Asp-Glu-His-Asp-7-amido-4-methylcoumarin trifluoroacetate (Ac-DEVD-AMC; Biomol, Hamburg, Germany). Substrate cleavage and accumulation of AMC were followed fluorometrically (Spectra Fluor, TECAN) with excitation at 360 nm and emission at 465 nm during an incubation period of 120 min.

For Hoechst assays, cells were plated on petri dishes, grown to 80% confluency and apoptosis was induced by incubation with 50 μ M cisplatin (Sigma) or serum deprivation for 24 h. Hoechst 33342 was added (5 μ g/ml) and then the cells were incubated at 37°C for 15 min. Cell nuclei were visualized in fluorescent microscopy. Apoptotic nuclei were then identified as showing characteristic signs of chromatin condensation and fragmentation. Five hundred cells/petri dish in 3 petri dishes were counted and the percentage of Hoechst-positive cells was assessed.

Antibodies

The rabbit polyclonal antiserum to α_8 -integrin was a gift from U. Muller (Scripps Institute) and used at a dilution of 1:200 as described before [14]. A monoclonal antibody to smooth muscle actin (DAKO Diagnostika, Hamburg, Germany) was used at a dilution of 1:50 to stain activated myofibroblasts. Counting of proliferating glomerular cells was performed by immunohistochemistry with a 1:50 diluted monoclonal antibody detecting proliferating cells (PCNA, DAKO Diagnostika). For fluorescence-activated cell sorting (FACS) analysis, antibodies to the α_6 (Chemicon, Hofheim, Germany) and β_1 (SantaCruz Biotechnologies, Heidelberg, Germany) integrin chains were used at a dilution of 1:100. Blocking antibodies to α_6 - and α_2 -integrin (both BD Pharmingen, Heidelberg, Germany) were used at a concentration of 2 μ g/ml to block α_6 -integrin or α_2 -integrin activity.

FACS Analyses

A single cell suspension was prepared by trypsination. An aliquot was used for trypan blue staining to identify the fraction of dead cells. In all aliquots tested the fraction of dead cells was below 5% and not different between α_8 -deficient and wild-type cells. After centrifugation, cells were resuspended to 2×10^6 cells/ml with staining buffer (5% fetal calf serum, 0.02% azide in phosphate-buffered saline). Cell suspension was aliquoted in 500 μ l per sample. The samples were incubated with 1–2 μ g/ml of antibody against α_6 - or β_1 -integrin chains for 30 min on ice. Mesangial cells were then washed twice in staining buffer, incubated with 2 μ g/ml FITC-conjugated secondary antibody for 30 min on ice. After washing, labelled cells were analyzed with a Beckmann Coulter Epics XL cytometer and the System II software (Beckman Coulter, Krefeld, Germany).

Immunohistochemistry

In deparaffinized kidney sections, endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol for 20 min at room temperature.

Sections were then layered with the primary antibody, and incubated at 4°C overnight. After addition of the secondary antibody (dilution 1:500; biotin-conjugated, goat anti-rabbit IgG or rabbit anti-mouse IgG, all Dianova, Hamburg, Germany), the sections were incubated with avidin-horseradish peroxidase complex and exposed to 0.1% diaminobenzidine tetrahydrochloride and 0.02% H₂O₂ as a source of peroxidase substrate. The Vectastain DAB kit (Vector Lab, Burlingame, Calif., USA) was used as a chromogen. Each slide was counterstained with hematoxylin. As a negative control, we used equimolar concentrations of pre-immune rabbit or mouse IgG. For detection of glomerular α_8 -integrin, 3- μ m cryosections were prepared. Staining of cryostat sections was performed as described elsewhere [22]. CY3-labelled anti-rabbit IgG (DAKO Diagnostika) was used as secondary antibody. Stained sections were evaluated in a Leitz Aristoplan microscope (Leica Instruments).

Real-Time Reverse Transcription Polymerase Chain Reaction

Renal cortical tissue (10 mg) was homogenized in 500 μ l of RLT buffer reagent (Qiagen, Hilden, Germany) with an ultraturax for 30 s, and total RNA was extracted with RNeasy mini columns (Qiagen) according to the standard protocol. First-strand cDNA was synthesized with TaqMan reverse transcription (RT) reagents (Applied Biosystems, Darmstadt, Germany) using ran-

dom hexamers as primers. Final RNA concentration in the reaction mixture was adjusted to 0.1 ng/ μ l. Reactions without MultiScribe reverse transcriptase were used as negative controls for genomic DNA contamination. RT products were diluted 1:1 with dH₂O before polymerase chain reaction (PCR) procedure. PCR was performed with an ABI Prism 7000 Sequence Detector System and SYBR Green reagents (Applied Biosystems) according to the manufacturer's instructions.

For amplification of the mouse α_8 -integrin cDNA, the forward primer was 5'-TCA AGG CGA GGA ACA GCA A-3' and the reverse primer was 5'-CCT TGG GAA CCC GAT GGT-3'. The relative amount of the specific mRNA was normalized with respect to 18S rRNA. Primers used for amplification of 18S cDNA were: forward primer 5'-TTG ATT AAG TCC CTG CCC TTT GT-3' and reverse primer 5'-CGA TCC GAG GGC CTC ACT A-3'. All samples were run in triplicate.

Analysis of Data

Two-way analysis of variance, followed by post hoc least significant difference test were used to compare groups. A p value <0.05 was considered significant. The procedures were carried out using the SPSS version 13.0 software (SPSS Inc., Chicago, Ill., USA). Values are displayed as means $\pm$ standard error of the mean.

Results

Increase in α_8 -Integrin Expression during Habu Glomerulonephritis

Induction of habu glomerulonephritis in wild-type mice did not lead to an increase in renal α_8 -integrin chain mRNA expression at day 3 of disease, representing the early phase of disease (fig. 1a). However, 7 days after the onset of glomerulonephritis renal expression of the α_8 -integrin chain was about three times higher than in non-nephritic kidneys (fig. 1a). This increase was declining at a later time point (after 14 days) of disease (fig. 1a). In control mouse, glomeruli α_8 -integrin protein was localized in mesangial areas (fig. 1b). Together with the increase in mesangial area at day 7 of disease, the glomerular area staining positive for α_8 -integrin was expanded at this time point (fig. 1b).

Induction of Glomerular Injury Is Equally Severe in Wild-Type and α_8 -Integrin-Deficient Mice

Induction of habu glomerulonephritis resulted in destruction of the capillary tuft with mesangiolysis evaluated as capillary widening of glomeruli after 24 h in both wild-type and α_8 -integrin-deficient mice to a similar extent (fig. 2a, b). Following the initial injury, compensatory mesangial cell activation and increase in glomerular cell proliferation were observed at day 7 of disease (fig. 3a, b). However, there were no significant differences in the extent of mesangial cell activation and glomerular cell

Fig. 1. Expression of the α_8 -integrin chain in habu glomerulonephritis. **a** mRNA expression levels of α_8 -integrin in renal cortical tissue of control mice, and mice at days 3, 7 and 14 of habu glomerulonephritis as assessed by real-time RT-PCR. **b** Glomerular localization of α_8 -integrin in control mice (left panel) and mice at day 7 of habu glomerulonephritis (right panel). Data are means $\pm$ standard error of the mean. * p < 0.05, mice at day 7 versus mice at day 0.

proliferation at this time point between wild-type and α_8 -integrin-deficient mice (fig. 3a, b). Glomerular cell apoptosis as a means to resolve glomerular hypercellularity due to excess cell proliferation is also increased at day 7, and is comparable in both wild types and α_8 -integrin-deficient mice (fig. 3c). Glomerular matrix expansion peaked at day 14 of disease in both wild-type and α_8 -integrin-deficient mice to a similar degree (fig. 4).

Healing of Glomerular Injury Is Delayed in α_8 -Integrin-Deficient Mice

While in wild-type mice there was a persistent decline in mesangiolytic lesions from day 7 to day 28, in α_8 -integrin-deficient mice lesions did not resolve until day 28 (fig. 2a). At day 14, mesangial cell activation was reduced in wild-type mice to almost control levels, while in α_8 -integrin-deficient mice mesangial cells were still in an activated state (fig. 3a). Similarly, glomerular cell proliferation is reduced at days 14 and 28 in wild-type mice, but persistently increased at days 14 and 28 in α_8 -integrin-de-

Fig. 2. a Microaneurysm formation during the course of habu glomerulonephritis in α_8 -integrin-deficient ($\alpha_8^{-/-}$) and wild-type (WT) mice. Time points investigated are day 1, day 7, day 14 and day 28 after induction of disease. Data are means $\pm$ standard error of the mean. * $p < 0.05$, α_8 -integrin-deficient mice versus wild types of the same time point. **b** Representative photomicrographs of PAS-stained glomerular sections. Arrowheads mark area of capillary widening in the glomerular capillary tuft.

ficient mice (fig. 3b). The same pattern was observed for glomerular cell apoptosis: in α_8 -integrin-deficient mice apoptosis remained at a high level at days 14 and 28, which was not seen in wild-type mice (fig. 3c). At day 28, glomerular matrix expansion was reduced in both wild-type and α_8 -integrin-deficient mice (fig. 4). However, this reduction was more prominent in wild-type mice than in α_8 -integrin-deficient mice, leading to a significant difference in glomerulosclerosis levels at day 28 (fig. 4).

Mesangial Cell Apoptosis Rate Is Affected by α_8 -Integrin Expression

In our *in vivo* study described above, we observed prolonged glomerular cell proliferation and apoptosis dur-

ing mesangioproliferative glomerulonephritis in α_8 -integrin-deficient mice. Thus, we tested the hypothesis that α_8 -integrin is directly involved in these cellular processes. In view of the results from our *in vivo* study, we expected an antiproliferative and antiapoptotic effect of α_8 -integrin expression. In a recent study [21], we had already investigated the contribution of α_8 -integrin to mesangial cell proliferation. For this reason, we focused on studying the effect of α_8 -integrin expression on mesangial cell survival.

Mesangial cells isolated from wild-type or α_8 -integrin-deficient mice were compared with regard to their sensitivity to induction of apoptosis. Surprisingly, both serum deprivation and incubation with cisplatin in-

Fig. 3. Mesangial cell activation (**a**), glomerular cell proliferation (**b**) and glomerular cell apoptosis, assessed after Hoechst stain (**c**) during the course of habu glomerulonephritis in α_8 -integrin-deficient ($\alpha_8^{-/-}$) and wild-type (WT) mice. Time points investigated are day 1, day 7, day 14 and day 28 after induction of disease. **a** Representative photomicrographs of the immunodetection of smooth muscle actin in glomeruli of α_8 -integrin-deficient and wild-type mice at days 0, 7 and 14 of habu glomerulonephritis. $n = 7$ per group in wild types and $n = 5$ per group in α_8 -integrin-deficient mice. Data are means $\pm$ standard error of the mean. * $p < 0.05$, α_8 -integrin-deficient mice versus wild types of the same time point.

creased apoptosis in wild-type mesangial cells much more prominently than in α_8 -integrin-deficient mesangial cells as assessed by caspase-3 activity assay (fig. 5a). To confirm these findings, Hoechst staining was performed in cells incubated with cisplatin or after serum deprivation and Hoechst-positive cells were counted, yielding comparable results to those of the caspase-3 activity assay (fig. 5b). On the other hand, overexpression of a full-length α_8 -integrin chain in human embryonic kidney (HEK) cells resulted in a reduced apoptosis rate when compared to HEK cells overexpressing a truncated nonfunctional α_8 -integrin chain (fig. 6). Moreover, inhibition of α_8 -integrin expression in mesangial cells by treatment with siRNA for α_8 -integrin (fig. 7a) led to an

increase in apoptosis when compared to cells treated with control siRNA or untreated cells (fig. 7b).

In an attempt to explain the conflicting data from α_8 -integrin-deficient mesangial cells, we hypothesized that a compensatory induction of other integrin chains in these cells might alter cell survival. We detected an overexpression of α_6 -integrin in α_8 -integrin-deficient mesangial cells (fig. 8a). This overexpression was only detected in isolated α_8 -integrin-deficient cells, not in mesangial cells in vivo [16]. A blockade of α_6 -integrin in α_8 -integrin-deficient cells resulted in an increase in mesangial cell apoptosis after stimulation of cells with cisplatin when compared to untreated α_8 -integrin-deficient mesangial cells (fig. 8b). In contrast, no effects on apoptosis

Fig. 4. Glomerular matrix expansion during the course of habu glomerulonephritis in α_8 -integrin-deficient ($\alpha_8^{-/-}$) and wild-type (WT) mice. Time points investigated are day 1, day 7, day 14 and day 28 after induction of disease. Data are means $\pm$ standard error of the mean. * $p < 0.05$ α_8 -integrin-deficient mice versus wild types of the same time point.

Fig. 5. Induction of apoptosis in cultivated α_8 -integrin-deficient ($\alpha_8^{-/-}$) and wild-type (WT) mesangial cells. **a** Apoptosis as assessed by measurement of caspase-3 activity. **b** Apoptosis rates as assessed by counting of Hoechst-positive cells. Full medium = Cell culture medium containing 10% fetal calf serum; serum-free

medium = cell culture medium without fetal calf serum; Cis = cisplatin. Data are means $\pm$ standard error of the mean. * $p < 0.05$, apoptotic versus control; # $p < 0.05$, α_8 -integrin-deficient mice versus wild types.

Fig. 6. Induction of apoptosis in HEK cells transfected with a full length (HEKfull) or truncated (HEKtrunc) α_8 -integrin chain as assessed by measurement of caspase-3 activity. Solv = Solvent for cisplatin (dimethylformamide); Cis = cisplatin. Data are means $\pm$ standard error of the mean. * $p < 0.05$, apoptotic versus control; # $p < 0.05$, truncated α_8 -integrin versus full-length α_8 -integrin.

Fig. 7. Gene silencing of the α_8 -integrin chain by α_8 -integrin siRNA. **a** Real-time RT-PCR confirming downregulation of α_8 -integrin expression by α_8 -integrin siRNA. Cells were treated with siRNA for 24, 48 or 72 h. Co siRNA = Control siRNA (nuclear protein 1). **b** Apoptosis of mesangial cells after gene silencing with α_8 -integrin siRNA as assessed by counting Hoechst-positive cells.

Cells were treated with α_8 -integrin siRNA for 48 h prior to additional administration of 50 μ M cisplatin for 24 h. Co siRNA = Control siRNA (nuclear protein 1). Data are means $\pm$ standard error of the mean. * $p < 0.05$, treated with α_8 -integrin siRNA versus control siRNA.

Fig. 8. **a** FACS analysis of cell surface expression of α_6 -integrin, or β_1 -integrin as a positive control, in α_8 -integrin-deficient (α_8 -/-) and wild-type (WT) mesangial cells. Control = Negative control for background staining, without primary antibody. **b** Effects of blocking the function of α_6 -integrin and α_2 -integrin in α_8 -integrin-deficient (α_8 -/-) mesangial cells on apoptosis. Solv = Solvent for cisplatin (dimethylformamide); Cis = cisplatin. Data are means $\pm$ standard error of the mean. * $p < 0.05$, apoptotic versus control; # $p < 0.05$, α_6 -integrin antibody treated versus untreated.

rates were detected after blocking α_2 -integrin, which was described before to be induced in α_8 -integrin-deficient mesangial cells as well [16].

Discussion

Our studies show that glomerular injury following induction of habu glomerulonephritis is comparable in both wild-type and α_8 -integrin-deficient mice. However, the resolution of glomerular injury due to habu glomerulonephritis is delayed in α_8 -integrin-deficient mice, possibly due to prolonged glomerular cell cycling and apoptosis. These data do not suggest that α_8 -integrin plays an important role in the degree of injury in glomerulonephritis, but more readily contributes to the resolution of glomerular injury caused by habu glomerulonephritis by regulating mesangial cell proliferation and apoptosis.

This was somewhat surprising, because in a previous study we were able to show that mechanical strain in the glomerulus led to severer disruption of the glomerular capillary tuft in α_8 -integrin-deficient mice than in wild-type mice [17]. On the other hand, lack of α_8 -integrin may render the glomerulus more vulnerable to mechanical strain than to inflammatory injury. Similar observations were made in mice deficient for the matrix protein tenascin C, a ligand for α_8 -integrin. The initial glomerular injury following induction of habu glomerulonephritis was not different from wild-type mice [19]. However, subsequent restoration of the glomerular capillary tuft was impaired in tenascin-C-deficient mice [19].

The contribution of integrins to fibrosis is the subject of many investigations [5]. Some authors hypothesize a profibrotic action of α_8 -integrin [23, 24]. Our own data do not support this notion. In the present study, we did not detect major discrepancies in the degree of glomerulosclerosis in wild-type and α_8 -integrin-deficient mice, although there was a small difference at day 28. This is in accordance with our previous data from the DOCA model of glomerular hypertension induced in α_8 -integrin-deficient mice, where no differences in the development of glomerulosclerosis were detected compared to wild types [17]. Moreover, histologic evaluation of glomeruli of untreated α_8 -integrin-deficient mice revealed that matrix deposition even tended to be increased in α_8 -integrin-deficient mice compared to wild types, at least if wild-type mice were not uninephrectomized for adjustment of renal mass [16]. A tendency towards glomerular matrix expansion was observed in the present study as well and could be due to glomerular expression of collagen I or III

in α_8 -integrin-deficient mice, which is not seen in wild-type mice [16].

An increase in mesangial cell α -smooth muscle actin reactivity can be routinely detected in glomerular diseases and represents activation of mesangial cells to a myofibroblast phenotype [25, 26]. Habu glomerulonephritis led to induction of glomerular α -smooth muscle actin in both wild-type and α_8 -integrin-deficient mice to a similar degree, however, α -smooth muscle actin expression persisted on a high level in α_8 -integrin-deficient mice much longer. This finding supports the notion that α_8 -integrin contributes to timely healing of acute glomerular injury in habu glomerulonephritis. As described before, a basal expression of α -smooth muscle actin in α_8 -integrin-deficient mice was detected which was somewhat higher than in wild-type mice [16], without reaching statistical significance in the present study. The most consistent dysregulation of healing processes after habu glomerulonephritis was detected regarding glomerular cell proliferation and apoptosis. While in wild-type animals, both proliferation and apoptosis peak only transiently, in α_8 -integrin-deficient mice the proliferation and apoptosis rates remain increased for weeks, suggesting that the turnover of glomerular cells is continuously increased in α_8 -integrin-deficient mice.

The contribution of α_8 -integrin to cell proliferation was described before in cell culture, where α_8 -integrin seemed to have antiproliferative actions [21]. Mesangial cells lacking α_8 -integrin proliferated more easily than wild-type mesangial cells and on the other hand, overexpression of α_8 -integrin reduced cell proliferation. Moreover, glomerular cell proliferation was somewhat higher in untreated α_8 -integrin-deficient mice than in wild types [16]. This observation can be confirmed with our present data: glomerular proliferation tends to be higher in α_8 -integrin-deficient mice at day 0 of disease than in wild-type mice. The fact that the differences in proliferation do not reach statistical significance in the present study is possibly due to methodical differences: i.e. to the use of uninephrectomized wild-type controls. Our finding that apoptosis remained increased longer in α_8 -integrin-deficient mice than in wild-type mice goes in line with in vitro data, which suggested that overexpression of α_8 -integrin promotes cell survival [13]. To gain more insight into the contribution of α_8 -integrin to the survival of mesangial cells, we performed additional in vitro apoptosis assays with mesangial cells isolated from α_8 -integrin-deficient and wild-type mice. Surprisingly, cultured mesangial cells lacking α_8 -integrin were not more susceptible to apoptosis than wild-type mesangial cells,

although in control experiments overexpression of α_8 -integrin protected HEK cells from apoptosis. We hypothesized that α_8 -integrin-deficient mesangial cells might have developed some compensation for the lack of α_8 -integrin during cultivation, and therefore repeated our apoptosis assays with wild-type mesangial cells with or without silencing of the α_8 -integrin gene by α_8 -integrin siRNA. This acute knockdown of α_8 -integrin expression led to the expected increase in apoptosis, arguing for a protective role of α_8 -integrin in cell survival. In an attempt to identify possible compensatory mechanisms which would protect cultivated α_8 -integrin-deficient mesangial cells from apoptosis, we screened for the expression of other integrin chains and found α_6 -integrin expressed in cultivated α_8 -integrin-deficient mesangial cells, while in wild-type mesangial cells it was barely detectable. The α_6 -integrin chain was described before to have antiapoptotic effects in epithelial cells [27, 28] and therefore could be a promising candidate for a compensatory integrin. Indeed, function blocking of α_6 -integrin in α_8 -integrin-deficient mesangial cells resulted in an increase in apoptosis. Thus, α_6 -integrin might contribute to the unexpectedly good survival of α_8 -integrin-deficient mesangial cells.

Several limitations of our study must be mentioned. To account for the reduced number of glomeruli in α_8 -integrin-deficient mice, we performed uninephrectomy in wild-type controls. However, this procedure does not really model the developmental mechanisms responsible for reduced renal mass in the α_8 -integrin-deficient mice. Applying other genetic models with reduced renal mass might also be problematic, because the genetic background might not be comparable with our genetic model. Further, we did not assess the blood pressure of nephritic mice. There is no significant blood pressure difference between nonnephritic α_8 -integrin-deficient mice and

wild-type controls (114.4 ± 12.3 vs. 110.6 ± 5.6 mm Hg in wild types), and the pattern of glomerular damage observed here was quite different from that previously observed in a model of hypertensive glomerulosclerosis [17]. Nevertheless, we cannot exclude a different response of blood pressure to nephritis in α_8 -integrin-deficient mice.

Taken together, the presence of α_8 -integrin seems to be protective for the glomerulus at least in situations where the glomerular capillary tuft is exposed to proliferative and apoptotic stimuli, like an immunologic injury following habu toxin administration. Similarly, α_3 -integrin promotes the maintenance of the integrity of the capillary tuft. In mice deficient for the α_3 -integrin chain widening of glomerular capillary loops was observed [29]. The upregulation of α_8 -integrin in different forms of glomerular disease like glomerular hypertension or glomerulonephritis [14, 17] could thus be a protective response of glomerular cells to prevent persisting damage after glomerular injury. In many glomerular diseases, inflammatory mediators and cytokines like TGF- β are induced, which stimulate the expression of integrins [19, 30]. Our studies in cultivated mesangial cells revealed that stimulation of these cells with TGF- β upregulated mRNA and protein expression of the α_8 -integrin chain [21].

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