Beneficial effects of integrin αvβ3-blocking RGD peptides in early but not late phase of experimental glomerulonephritis

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Abstract

Background. Integrin αvβ3 plays an important role in the regulation of cell proliferation and neoangiogenesis. We found mesangial de novo expression of integrin αvβ3 in mesangio-proliferative glomerulonephritis (MesGN). The aim of the study was to clarify if blockade of αvβ3 integrin with the specific αvβ3-blocking cyclic peptide RGDdFV (cRGD) has beneficial effects on the course of this disease.

Methods. Habu snake venom (Habu) GN was induced in male C57BL/6 mice 1 week after uninephrectomy (6 mg Habu toxin/kg body weight intravenously). After 24 h, nephritic animals received αvβ3-inhibitory cRGD or cRAD control peptides for 3 or 7 days, respectively. The kidneys were investigated using morphometry, immuno-histochemistry and TaqMan polymerase chain reaction.

Results. At Day 3, serum creatinine and albuminuria were lower after cRGD compared to cRAD treatment. At Day 3, glomerulosclerosis index, percentage of glomerular injury, mesangial cell (MC) number and volume density of mesangial matrix were significantly lower (P < 0.05) in cRGD-treated mice than in cRAD-treated controls. At Day 7, only a mild effect of cRGD on mesangial matrix expansion and fibronectin messenger RNA was still detectable (P < 0.05). Complementary in vitro studies in MCs revealed that inhibition of αvβ3 by cRGD-blocked adhesion, reduced proliferation and increased apoptosis of MCs.

Conclusion. Habu GN inhibition of integrin αvβ3 by cRGD partly ameliorates early injury but has no or only mild effects on late glomerular lesions.

Keywords: Habu snake venom GN; inflammation; αvβ3 integrin; mesangial cell proliferation; neoangiogenesis

Introduction

Glomerular cell proliferation is tightly regulated in the normal kidney. An imbalance in the control of glomerular mesangial cell (MC) proliferation resulting in MC hyperplasia is a histopathological hallmark in many forms of glomerulonephritis (GN), including IgA GN, membranoproliferative GN, post-infectious endocapillary proliferative GN and lupus GN. Studies in experimental models of GN supported the pathogenetical concept that MC hyperplasia frequently precedes and is linked to increased extracellular matrix (ECM) expression and progressive glomerular scarring [1, 2]. These studies elucidated the effects of cytokine-mediated signalling in experimental GN. However, whether and how MC growth is modulated by ECM receptor signalling is still unclear.

Integrins are heterodimeric transmembrane glycoproteins, which belong to the group of cell adhesion molecules. They function as ECM protein receptors playing an important role in mediating cell–matrix interactions [3]. Until today >20 different integrins have been reported consisting of an α and a β chain which are non-covalently linked and only functionally active as complete dimers. For the αv sub-group, five heterodimers are known, i.e. αvβ1, αvβ3, αvβ5, αvβ6 and αvβ8. Integrins can bind to
one or various ECM ligands. Integrin-mediated adhesion initiates a signalling cascade that regulates cell proliferation, migration, survival and apoptosis [4].

For integrins of the α sub-group, specific roles in angiogenesis and proliferative inflammatory disease are postulated. αvβ3 integrins are specifically expressed on endothelial cells (ECs) during growth and remodelling. A potential role of αvβ3 integrin as a negative regulator of angiogenesis had been reported [5]. In fact, results obtained from experiments employing cyclic RGDDFV peptides (cRGD) seemingly did not show compatible outcome compared with experiments utilizing genetic mice models lacking β3 integrin in case of angiogenesis studies. Several ECM ligands including vitronectin, fibronectin, thrombospondin, fibrinogen and osteopontin attach to αvβ3 integrin via the RGD (arginine–glycine–aspartate acid) tripeptide sequence [6]. αvβ3 integrin mediates EC binding to the ECM and transduces an intracellular signal prompting survival of EC and various tumour cells [7]. Thus, αvβ3 integrin is involved in fibrogenesis [8], vasculogenesis [9] or vascular disease [10], respectively. Consequently, inhibitors of αvβ3 integrin are used as antifibrotic or anti-angiogenic agents [11, 12] and recently also for cancer therapy [13].

Integrins also play an important role in kidney development, function and disease [14–18]. In the normal kidney, αvβ3 integrin is expressed only in podocytes [19]. This expression pattern can vary, however, in glomerular disease [19, 20]. We have shown transient de novo expression of αvβ3 integrin in MCs during rat anti-Thy 1.1 GN [21]. αvβ3 integrin-mediated actions can be inhibited in vivo and in vitro by specific peptides containing the RGD sequence [22, 23]. Moreover, the 49-amino acid peptide echistatin belonging to the family of disintegrins and binds αvβ3 integrin with high affinity via its RGD sequence [24]. Inhibition of αvβ3 integrin-mediated signalling by echistatin effectively blocked the proliferative response of cultured smooth muscle cells to insulin-like growth factor-1 [25]. cRGD selectively antagonized αvβ3 integrins, but not the related αvβ5 integrin, and reduced angiogenesis and cartilage destruction in an arthritis model [26]. Furthermore, αvβ3 integrin-inhibitory RGD-containing peptides have been shown to induce apoptosis and expression of interleukin-1β-converting enzyme in cultured human MCs [27]. Thus, cRGD may be a candidate for regulating apoptosis in vivo. Of note, RGD-binding sites were identified in the ischaemic rat kidney [28] and RGD peptides have been used to prevent tubular obstruction and to ameliorate ischaemic acute renal failure in rats [29]. The effect of therapeutic RGD treatment on GN, however, has not been investigated.

Therefore, in the present study, we used the well-established Habu snake venom model of acute mesangioproliferative GN (Habu GN) which is characterized by an initial capillary widening due to mesangioysis and rupture of the capillary tuft [30], leading to compensatory MC proliferation and matrix expansion which in turn is followed by increased glomerular cell apoptosis and resolution of glomerular hypercellularity.

We hypothesized that inhibition of αvβ3 integrin signalling may prove beneficial for the course of mesangioproliferative glomerulonephritis (MesGN) by interfering with MC proliferation, apoptosis rate and mesangial matrix production. In vivo, we investigated the effects of αvβ3 integrin-inhibitory cRGD on renal structure and function in experimental Habu GN. Complementary in vitro studies examined how αvβ3 inhibitory peptides affect adhesion, proliferation and apoptosis rate in cultured MCs.

**Materials and methods**

In vivo studies

**Animals.** Habu GN was induced in 18–20 g male C57BL/6 mice (Charles River, Sulzfeld, Germany) by intravenous (i.v.) injection of the toxin from the Habu snake Trimeresurus flavoviridis (6 mg/kg body weight; Sigma–Aldrich Chemicals, Deisenhofen, Germany) 1 week after unilateral nephrectomy (UNX). One day after Habu injection, 12 mice per group were either injected with cRGD or the control peptide cRAD, respectively (2 × 10 μg subcutaneously per mouse per day; Bachem Co., Heidelberg, Germany). Peptide doses were chosen according to previous studies in a model of proliferative retinopathy in C57BL/6 mice, where two injections per day were applied [23, 31]. Two groups of animals were treated for 2 days with cRGD and cRAD to investigate the effects on initial glomerular damage (Day 3). Two other groups were treated for 6 days with cRGD and cRAD to analyse the effect on repair of glomerular lesions (Day 7). Six uninjected mice receiving phosphate-buffered saline (PBS) intravenously served as controls (Day 0). For some comparisons, historical data and paraffin sections from untreated Habu snake venom GN at Day 3 were used. Successful induction of GN was assessed by detection of haematuria with the dipstick method (Multistix 10 SG; Bayer Vital GmbH, Fernwald, Germany). All animals were housed in cages at constant room temperature (20°C) and humidity (70%) under a controlled light–dark cycle. Animals were fed a standard diet (Altromin C 1324; Altromin Co., Lage, Germany). For urine collection, animals were placed in metabolic cages for 24 h. At Days 3 and 7 post-induction of Habu GN, mice (n = 6 per group) were perfusion fixed [32]. All animal procedures were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local government authorities (Regierung von Mittelfranken, AZ 621-2531.31-19/98).

**Tissue preparation.** After determination of total organ weight and volume, kidneys were dissected in 1 mm thick slices perpendicular to the longitudinal axis. Tissue sampling for morphometric and stereological analysis was performed using the area weighted sampling technique [32]. Paraffin and semi-thin sections (five blocks per animal) were prepared and stained as described [32]. For qualitative electron microscopical investigations, several ultrathin sections (0.08 µm) per animal were prepared and stained with uranyl acetate/lead citrate. Sections were investigated using a Zeiss electron microscope EM 10 (Zeiss Co., Oberkochen, Germany) at various magnifications.

**Semi-quantitative analysis of mesangiocytic glomerular damage.** To analyse the extent of mesangioysis, a semi-quantitative score (0–4) was used on Periodic acid Schiff (PAS)-stained paraffin sections (100 systematically sub-sampled glomeruli per animal; magnification ×400) as described previously [32]. Paraffin and semi-thin sections (five blocks per animal) were prepared and stained as described [32]. For qualitative electron microscopical investigations, several ultrathin sections (0.08 µm) per animal were prepared and stained with uranyl acetate/lead citrate. Sections were investigated using a Zeiss electron microscope EM 10 (Zeiss Co., Oberkochen, Germany) at various magnifications.

**Semi-quantitative analysis of mesangial matrix expansion.** The degree of mesangial matrix expansion and glomerular sclerosis was determined on Periodic acid Schiff (PAS)-stained paraffin sections (100 randomly sampled glomeruli per animal; magnification ×400) adopting a semi-quantitative scoring system (Scores 0–4) as described before [33].

**Immunohistochemistry.** The rabbit polyclonal antiserum to αv integrin was a gift from U. Muller (Scripps Institute, La Jolla, 1:200) [21]. Staining for integrin αv chain was performed using a polyclonal goat antibody (1:100; Santa Cruz, Heidelberg, Germany). A monoclonal antibody to rat anti-F4/80 (1:100; Serotec, Oxford, UK) was used for macrophage and a polyclonal antibody to rat CD3 for T-cell staining (1:100; neo-Markers/Dunn, Fremont, USA). Antibodies against Collagen IV (1:100; SouthernBiotech, USA) and fibronectin (1:100; Gibco, USA) were used to detect differences in ECM production. Quantification of proliferating glomerular cells was performed by immunohistochemistry with antibodies...
against PCNA (1:500) and Ki-67 (1:100; DAKO, Hamburg, Germany). Deparaffinized kidney sections were incubated with the primary antibody overnight at 4°C after blocking of endogenous peroxidase activity. After addition of the secondary antibody (dilution 1:500; biotin conjugated, goat anti-rabbit immunoglobulin G or rabbit anti-mouse immunoglobulin G; all Dianova, Hamburg, Germany), the sections were incubated with avidin–horseradish peroxidase complex and exposed to diaminobenzidine tetrahydrochloride using the Vectastain DAB kit (Vector Lab, Burlingame, CA). Slides were counterstained with haematoxylin. As a negative control, equimolar concentrations of preimmune rabbit or mouse immunoglobulin G were used. Staining localization and intensity of integrin αv and α8 was qualitatively analysed. Staining intensity of fibronectin and Collagen IV (%) was assessed using a semi-automated image analysing system (Analysis; SIS, Münster, Germany). The number of PCNA-, Ki-67-, CD3- and F4/80-positive cells per glomerulus and glomerular area was counted.

For detection of apoptotic cells, the In Situ Cell Death Detection Kit’ (TUNEL staining; Roche Diagnostics GmbH, Penzberg, Germany) as well as immunohistochemistry for activated (cleaved) caspase-3 (1:50; Chemicon International Inc., Temecula, USA) was performed according to the manufacturer’s instructions. After counterstaining with 4’,6-diamino-2-phenylindol (DAPI), glomerular TUNEL and caspase-3 staining was analysed in 100 glomeruli per animal using fluorescence technique and a semi-quantitative scoring system (0: no expression; 1: expression <30% of glomerular cells; 2: expression >30% of glomerular cells).

**Detection of αvβ3 integrin in cultured MCs.** For flow cytometric detection of αvβ3 expression, MCs grown for 12 h on vitronectin were incubated with rabbit polyclonal antibody against αvβ3 (1: 200; Telios, San Diego, CA), followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:100; Vector). Flow cytometric analysis was performed using a Beckman Coulter Epics XL cytometer and the System II software (Beckman Coulter, Krefeld, Germany). For immunocytochemical detection of αvβ3 integrin, quiescent MCs were seeded on D0.1 on vitronectin. After 6 h, MCs were fixed, blocked with 2% BSA and incubated with a polyclonal antibody against αvβ3 integrin followed by incubation with Cy2-labelled goat anti-rabbit antibody (Dianova, 2 h).

**MC adhesion assay.** The MC adhesion assay was performed as described before [37]. In brief, trypsinized MCs were re-suspended in assay buffer (DMEM, 0.5% BSA, 20 mL HEPES, pH 7.4). Next, 8 × 106 MCs per well were seeded onto 96-well culture plates coated with matrix proteins and blocked with BSA in the presence of indicated concentrations of peptides. Three hours after seeding, non-adherent MCs were washed off in a standardized fashion with a 96-well plate washer (Tipette; ICN, UK), allowing highly reproducible determination of the percentage of adherent cells. Numbers of adherent cells were determined by measuring hexosaminidase activity with the substrate p-nitrophenyl-N-acetyl-b-d-glucosamine [37]. Values for adherent MCs were compared to a standard curve, prepared from the MC suspension used for plating. The eRGD, cRGD, control peptide CRAD and αvβ3 integrin-inhibitory disintegrin echistatin were purchased from Bachem Co.

**Determination of MC proliferation.** About 2 × 104 quiescent MCs were seeded in D0.1 on eight-well chamber slides coated with a mixture of vitronectin (10 µg/mL) and PLL (100 µg/mL), or PLL (100 µg/mL) alone to allow integrin-independent attachment in the presence of inhibitory peptides. Three hours after plating, 10 µL bromodeoxyuridine (BrDU) (Boehringer, Mannheim, Germany) was added and MCs were stimulated with 25 mg/mL PDGF in the presence of peptides as indicated. Eighteen hours later, MCs were fixed by incubation in 70% ethanol/glycine ph 2.5 at −20°C. Staining of BrDU-positive MCs was performed with a BrDU-labelling kit (Boehringer). The percentages of BrDU-positive MCs were determined by analysing 25 independent microscopic fields of 100 MCs each.

**Protein extraction and western blot analysis.** Proteins from quiescent MCs were extracted using radioimmunoprecipitation assay solution (50 mM Tris–HCl, pH 7.2, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulphate (SDS), 1% sodium deoxycholate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 µg/mL leupeptin, 50 µM sodium orthovanadate). Denatured 20 µg total protein samples were separated on a 10% SDS–polyacrylamide gel electrophoresis. After electrophoresis, the proteins were transferred onto nitrocellulose membranes (Amersham Life Science, Little Chalfont, UK). Primary antibodies were directed against: p42/p44 ERK, 1:1000 (New England Biolabs, Beverly, MA, USA). The secondary antibody was horseradish-conjugated donkey anti-goat IgG, 1:10000 (Gibco). For visualization, the ECL system (Amersham Life Science) was used.

**Assessment of MC apoptosis.** For evaluation of apoptosis, quiescent MCs were exposed to eRGD or CRAD for 24 h. MCs were then fixed and stained with Hoechst dye 33258 (1 µg/mL). Nuclear chromatin condensation in apoptotic MCs was visualized using fluorescence microscopy.
Percentages of apoptotic MCs were determined by analysing 10 independent microscopic fields of 100 MCs each.

For detection of activated caspase-3 reactivity, fixed MCs were incubated with anti-caspase-3 (cleaved) antibody, 1:100 (4°C overnight; Cell Signaling Technology, Danvers, MA) followed by incubation with the secondary biotinylated goat anti-rabbit antibody (1:500). Next, slides were incubated with peroxidase-conjugated avidin D followed by development of the peroxidase reaction using the DAB Substrate Kit. Percentages of MCs with a positive staining reaction for activated caspase-3 were determined by analysing 20 independent microscopic fields of 100 MCs each.

Statistical analysis. Statistical significance between different groups was tested using one-way analysis of variance (ANOVA) followed by post-hoc tests. Individual groups were subsequently tested using Wilcoxon–Mann–Whitney test. P < 0.05 was considered significant. For statistical analysis of in vitro data, two-way ANOVA, followed by post-hoc Scheffe test, was used. Values of P < 0.05 were considered significant. The procedures were carried out using the SPSS software (SPSS, Chicago, IL, USA). Values are displayed as means ± standard deviation (SD).

Results

In vivo studies

cRGD treatment lowered serum creatinine and albuminuria at Day 3. At the end of the study, body weight and kidney weight were not significantly different between the groups (Table 1). Albuminuria was significantly higher in all Habu groups than in controls. On Day 3, S-creatinine and albuminuria were lower after cRGD treatment, the difference, however, failed to reach statistical significance due to large standard deviation (Table 1). On Day 7, values for S-creatinine and albuminuria did not differ in both treated Habu groups. Of note, treatment with the control peptide cRAD alone had no effect on renal function compared to untreated Habu GN [38].

cRGD treatment prevented early glomerular injury but had only mild effects on tubulointerstitial and vascular damage indices. At Days 3 and 7, semi-quantitative tubulointerstitial damage index and vascular damage index (VSI) as well as mesangial index were higher in all Habu-treated groups compared to controls. Due to intragroup variability, differences between the groups did not reach statistical significance (Table 2).

At Day 3, the percentage of injured glomeruli was significantly (P < 0.01) lower in cRGD (12.1 ± 3.4%) compared to cRAD (45.8 ± 11.8%) treated Habu indicating glomerular protection by cRGD. In contrast, at Day 7, there was no difference between both groups (5.5 ± 1.0% versus 4.2 ± 1.2%, not significant (n.s.)).

Table 1. Animal data—effects of cRGD treatment (n = 6 animals per group)*

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Kidney weight (mg)</th>
<th>Albuminuria (µg/mL)</th>
<th>S-creatinine (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22.8 ± 0.5</td>
<td>139.3 ± 8.7</td>
<td>0.05 ± 0.07</td>
<td>0.17 ± 0.002</td>
</tr>
<tr>
<td>Habu + cRAD Day 3</td>
<td>23.8 ± 0.6</td>
<td>151 ± 6.8</td>
<td>0.35 ± 20.4*</td>
<td>0.30 ± 0.002</td>
</tr>
<tr>
<td>Habu + cRGD Day 3</td>
<td>23.9 ± 1.2</td>
<td>145.7 ± 9.6</td>
<td>0.18 ± 14.3*</td>
<td>0.19 ± 0.001</td>
</tr>
<tr>
<td>Habu + cRAD Day 7</td>
<td>23.7 ± 1.4</td>
<td>137.2 ± 18.4</td>
<td>0.78 ± 3.77*</td>
<td>0.20 ± 0.005</td>
</tr>
<tr>
<td>Habu + cRGD Day 7</td>
<td>23.8 ± 1.5</td>
<td>147.5 ± 18.8</td>
<td>7.63 ± 3.12*</td>
<td>0.17 ± 0.002</td>
</tr>
<tr>
<td>ANOVA</td>
<td>n.s.</td>
<td>n.s.</td>
<td>P &lt; 0.05</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

*n.s., non significant.

*P < 0.05 versus control.

Mesangial matrix expansion assessed as glomerulosclerosis score (GSI) was also significantly lower in the cRGD than in the cRAD-treated group at Day 3 (Table 2 and Figure 1A and B), whereas on Day 7 (Figure 1C and D), there was no difference. Of note, in the cRGD-treated group, GSI on Day 3 was not different from PBS controls (Table 2). Lower MC proliferation and matrix expansion after cRGD (Figure 1F) compared to cRAD (Figure 1E) treatment on Day 3 was even more pronounced in semi-thin sections. Again, on Day 7, there was no visible difference between cRGD (Figure 1H) and cRAD (Figure 1G) treatment.

cRGD treatment lowered glomerular expression of some ECM components. Compared to healthy control animals (Figure 2A), mice with untreated Habu GN showed an up-regulation of the integrin αv chain on protein level in podocytes and MCs (Figure 2B). At a higher magnification, lower integrin αv chain expression after treatment with cRGD (Figure 2D) compared to cRAD treatment (Figure 2C) is visible. In parallel with mesangial matrix expansion, increased glomerular integrin αvβ3 protein expression was seen in untreated Habu GN (Figure 2F) compared to PBS controls (Figure 2E). Lower matrix expansion and MC number were accompanied by lower integrin αvβ3 chain staining after cRGD (Figure 2H) compared to cRAD (Figure 2G) treatment. At Day 3, mesangial fibronectin protein expression was also significantly (P < 0.05) lower after cRGD (5.1 ± 2.4%) compared to cRAD (10.4 ± 4.6%) treatment. At Day 7, Collagen IV protein expression was significantly (P < 0.05) lower in the cRGD (22.8 ± 4.3%) than in the cRAD (28 ± 3.4%) treated group.

On the mRNA level (Figure 3A–D), cRGD treatment was associated with a tendency to lower values for CTGF, TGF-β1, fibronectin and Collagen IV at Days 3 and 7; the difference, however, was only statistically significant for fibronectin at Day 7 (Figure 3D, P < 0.05).

Of note, in Habu GN, there is only a very mild glomerular inflammatory cell infiltrate; our detailed immunohistochemical analyses showed no difference in either interstitial or glomerular macrophage or T-cell infiltration at Days 3 or 7.
Table 2. Effects of cRGD treatment on semi-quantitative index of renal damage (Scores 0–4) and inflammatory cells (*n = 6 animals per group).  

<table>
<thead>
<tr>
<th>Group</th>
<th>Glomerulosclerosis index</th>
<th>Tubulointerstitial damage index</th>
<th>Mesangiolysis index</th>
<th>T cells (cells/mm² of glomerulus)</th>
<th>Macrophages (cells/mm² of interstitial area)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.43 ± 0.11</td>
<td>0.2 ± 0.18</td>
<td>0.18 ± 0.07</td>
<td>0</td>
<td>0</td>
<td>n.s.</td>
</tr>
<tr>
<td>Habu + cRAD Day 3</td>
<td>0.86 ± 0.20</td>
<td>0.2 ± 0.18</td>
<td>0.18 ± 0.07</td>
<td>0.21 ± 0.02</td>
<td>0.02 ± 0.04</td>
<td>*P &lt; 0.05</td>
</tr>
<tr>
<td>Habu + cRGD Day 3</td>
<td>0.86 ± 0.20</td>
<td>0.2 ± 0.18</td>
<td>0.18 ± 0.07</td>
<td>0.21 ± 0.02</td>
<td>0.02 ± 0.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>Habu + cRGD Day 7</td>
<td>0.86 ± 0.20</td>
<td>0.2 ± 0.18</td>
<td>0.18 ± 0.07</td>
<td>0.21 ± 0.02</td>
<td>0.02 ± 0.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>ANOVA</td>
<td>-</td>
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</tbody>
</table>

n.s., non significant.  
*P < 0.05 versus control, **P < 0.05 versus cRAD treatment Day 3.

Animals on Day 3 but not on Day 7, whereas in PBS-treated controls, no apoptotic cells at all were detected (Figure 4A). Increased TUNEL positivity on Day 3 was paralleled by a significantly higher immunohistochemical staining score for activated caspase-3 in the cRGD treatment group (Figure 4B). Of note and in contrast to the TUNEL stain, this was also the case on Day 7. Moreover, proliferation of glomerular cells on Day 3, i.e. PCNA + cells per glomerular area (mm²), was higher in the cRAD group (0.081 ± 0.057) compared to controls (0.005 ± 0.009) and was significantly (P < 0.05) lower in the cRGD group (0.026 ± 0.008). On Day 7, however, there was no significant difference in the number of glomerular proliferating cells (0.013 ± 0.003 in cRAD versus 0.023 ± 0.020 in cRD, n.s.).

cRGD treatment lowered MC number on Day 3 and restored glomerular capillaries on Day 7. Effects of cRGD on glomerular ultrastructure was analysed using semi-thin and ultrathin sections. Mean glomerular volume was significantly higher in both treated Habu GN groups than in PBS controls with no differences between cRAD and cRGD treatment (Table 3). The detailed analysis of semi-thin sections revealed a significantly higher number of MCs per glomerulus in all treated Habu GN animals compared to PBS controls (P < 0.01). Mean MC number was significantly lower, however, in the cRGD compared to the cRAD-treated group on Day 3 but not on Day 7 (Figure 5A). In parallel, increased volume density of mesangial matrix in Habu GN was significantly lower after cRGD treatment on Days 3 and 7 compared to cRAD groups (Figure 5B).

Mean volume of the capillary tuft was significantly higher in all Habu GN groups compared to controls (0.15 ± 0.01 mm³ × 10⁻³) with no effect of cRGD treatment (Day 3: 0.21 ± 0.04 versus 0.23 ± 0.06 in cRAD, Day 7: 0.19 ± 0.04 mm³ × 10⁻³ versus 0.19 ± 0.05 mm³ × 10⁻³ in cRAD). Length density of glomerular capillaries was lower in both cRGD- and cRAD-treated Habu GN groups on Day 3 than in controls indicating destruction and loss of capillaries due to mesangiolysis and aneurysm formation (Table 3). Due to the large standard deviation in the cRAD-treated group, the difference was only statistically significant in the cRGD-treated group. In contrast, on Day 7, capillary length density had returned to normal levels in the cRAD-treated Habu GN group, whereas it remained significantly lower in the cRAD group compared to controls (P < 0.05) (Table 3).

Volume density of glomerular capillaries, a parameter of glomerular dilatation that describes remodelling of the capillary tuft architecture, was significantly lower in all Habu GN groups than in controls with a tendency to higher values in the cRGD treatment group on Day 3 and a significantly higher value on Day 7 compared to the cRAD-treated groups (Table 3). Mean EC number per glomerulus was significantly lower in all Habu GN groups compared to controls apart from the cRGD-treated group on Day 3 (Table 3). On Days 3 and 7, EC number was slightly higher in the cRGD-treated groups than in the cRAD groups. Mean podocyte number per glomerulus was not significantly different in all groups (Table 3).

Qualitative analysis of glomerular structure (Figure 1A–D) and ultrastructure (Figure 1E–H, Figure 6), respectively, confirmed lower MC number and mesangial...
matrix in the cRGD-treated Habu GN animals on Day 3 compared to cRAD treatment. Using electron microscopy on higher magnification (Figure 6C, D, G and H), no alterations of either glomerular basement membrane thickness or morphology of podocytes in Habu GN were detected and consequently, no effect of either cRGD or cRAD treatment was noted.

Of note, in all investigations, no negative effects of cRGD treatment at all were observed.

In vitro studies

Cultured rat MCs express $\alpha\beta_3$ integrin. Flow cytometric analysis of cultured MCs with a polyclonal antibody against the vitronectin receptor $\alpha\beta_3$ confirmed a strong cell surface expression of $\alpha\beta_3$ integrin (Figure 7A). In addition, immunocytochemical staining of MCs adherent to vitronectin confirmed expression of $\alpha\beta_3$ integrin and showed an integrin-specific distribution pattern consistent with its recruitment into focal contacts (Figure 7B).

$\alpha\beta_3$ integrin-inhibitory peptides dose dependently block MC adhesion onto vitronectin. Next, we investigated how $\alpha\beta_3$ integrin-inhibitory peptides modulate adhesion, proliferation and apoptosis in cultured MCs. To confirm that the cellular effects induced by cRGD were due to its
αvβ3 integrin-inhibitory action, we also studied the effects of the αvβ3 integrin-inhibitory peptide echistatin in these experiments. To examine the influence of αvβ3 integrin-inhibitory peptides on MC adhesion, MCs were seeded on vitronectin or fibronectin in the presence of increasing concentrations of echistatin, cRGD or cRAD. Both vitronectin and fibronectin are ligands for αvβ3 integrin. However, αvβ3 integrin serves as the primary receptor for vitronectin, while MCs are still able to bind fibronectin via the α5β1 integrin [39]. Echistatin dose dependently blocked MC adhesion onto vitronectin (Figure 8A). In contrast, MC adhesion onto fibronectin was not affected by echistatin (Figure 8A) or by cRGD (data not shown), suggesting that α5β1-mediated binding was not affected by these peptides. Also, cRGD dose dependently blocked adhesion of MCs onto vitronectin, while the control peptide cRAD did not influence MC adhesion (Figure 8B). Taken together, these data functionally demonstrate the potency of echistatin and cRGD to antagonize the function of αvβ3 in cultured MCs.

αvβ3 integrin-inhibitory peptides dose dependently reduce PDGF-induced MC proliferation. Since our in vivo data suggested that growth-inhibitory effects of αvβ3 integrin-inhibitory peptides are able to reduce MC hyperplasia, we examined their effects on S-phase entry of MCs stimulated with the potent MC mitogen PDGF as measured by BrdU uptake. To enable adherence of MCs in the presence of αvβ3 integrin-inhibitory peptides, we seeded quiescent MCs onto a mixture of vitronectin and poly-L-lysine (vitronectin/PLL) to allow integrin-independent attachment to PLL. Attachment assays of MCs plated onto this matrix confirmed that incubation with αvβ3 integrin-inhibitory peptides did not reduce MC adhesion (data not shown). Eighteen hours after mitogenic stimulation with 25 ng/mL PDGF, BrdU-positive MCs were quantified by counting MCs stained with an anti-BrdU-antibody. Baseline proliferation rates of quiescent MCs plated on vitronectin/PLL were higher than in MCs plated on PLL alone, indicating that adhesion to vitronectin mediates pro-mitotic signals in MCs (Figure 8A). Also, PDGF had only a weak mitogenic

Fig. 2. Expression of αv (A–D) and α8 (E–H) integrin chains. Glomerular expression of both integrins is increased in Habu GN (B and F) compared to controls (A and E); expression is lower; however, after 3 days of cRGD (D and H), but not of cRAD (C and G) treatment.
Fig. 3. Results of TaqMan PCR for CTGF, TGF-β1, fibronectin and Collagen IV. Boxplot analysis. (A–D) cRGD treatment led to lower expression mRNA values at Days 3 and 7. The difference, however, was only statistically significant for fibronectin mRNA expression at Day 7 (C). *P < 0.05.

Fig. 4. Apoptosis of glomerular cells assessed as score of TUNEL-positive cells (A) and by activated caspase-3 staining (B). Boxplot analysis. (A) Please note the significantly higher percentage of glomerular apoptosis score in the cRGD-treated group on Day 3. (B) Staining of activated caspase-3 confirmed the data shown in A. In addition, there was also a significantly higher staining score in the cRGD-treated group on Day 7 indicating a higher percentage of apoptosis after cRGD treatment. *P < 0.05, **P < 0.01.

Table 3. Effects of cRGD treatment on glomerular capillaries and cells in Habu Snake Venom GN (n = 6 animals per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean glomerular volume (mm³ × 10⁻³)</th>
<th>Length density of capillaries (L_v) (mm/mm³)</th>
<th>Volume density of capillaries (VV) (%)</th>
<th>Number of ECs per glomerulus</th>
<th>Number of podocytes per glomerulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.15 ± 0.01</td>
<td>15810 ± 1958</td>
<td>53.6 ± 3</td>
<td>61.7 ± 10.5</td>
<td>57.9 ± 7.2</td>
</tr>
<tr>
<td>Habu + cRAD Day 3</td>
<td>0.23 ± 0.06*</td>
<td>13280 ± 2992</td>
<td>34.5 ± 5.5*</td>
<td>42.3 ± 7.8*</td>
<td>74.4 ± 9.7</td>
</tr>
<tr>
<td>Habu + cRAD Day 7</td>
<td>0.21 ± 0.04*</td>
<td>13338 ± 766*</td>
<td>42.2 ± 7.5*</td>
<td>48.8 ± 15.7</td>
<td>66.8 ± 6.75</td>
</tr>
<tr>
<td>Habu + cRAD Day 7</td>
<td>0.19 ± 0.05*</td>
<td>12580 ± 1413*</td>
<td>24.8 ± 5.8*</td>
<td>36.5 ± 17.6*</td>
<td>54.2 ± 14.4</td>
</tr>
<tr>
<td>Habu + cRAD Day 7</td>
<td>0.19 ± 0.04*</td>
<td>14581 ± 2065</td>
<td>34.7 ± 5.9***</td>
<td>45.4 ± 12.9*</td>
<td>64.7 ± 12.9</td>
</tr>
<tr>
<td>ANOVA</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>P &lt; 0.05</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

*a.n.s., non significant.
*P < 0.05 versus control, **P < 0.05 versus cRAD treatment Day 7.
Fig. 5. Changes of MC numbers (A) and mesangial matrix volume fraction (B). Boxplot analysis. (A) On Day 3, a significantly lower number of MCs is found in the cRGD-treated group compared to cRAD treatment, whereas at Day 7, there is only a tendency to lower values. (B) In addition, volume fraction (in %) of the mesangial matrix is significantly lower in both cRGD-treated groups compared to cRAD treatment. *P < 0.02.

Fig. 6. Ultrastructural changes detected by electron microscopy (original magnification ×3000 A, B, E, F and ×12 000 C, D, G, H) The increase in MCs and mesangial matrix in cRAD (A) treated Habu GN on Day 3 is prevented by cRGD treatment (B), whereas at Day 7, there is no significant difference in MC number (E–H). Of note, at higher magnifications (C, D, G, H), there is no evidence of differences in glomerular basement thickness or podocyte morphology, respectively, in cRAD- and cRGD-treated Habu GN on Days 3 or 7, respectively.
effect on MCs attached to PLL alone (Figure 8). However, PDGF markedly increased proliferation of MCs plated onto vitronectin/PLL (Figure 9A and B). This PDGF-induced proliferation was inhibited in a dose-dependent fashion by incubation with echistatin (Figure 9A) and cRGD (Figure 9B), while incubation with the control peptide cRAD did not affect MC mitogenesis (Figure 9B).

**αvβ3 integrin-inhibitory peptide cRGD interferes with PDGF-induced phosphorylation of ERK.** To further elucidate the functional involvement of cRGD in proliferation-related signalling pathways of MCs, we examined how cRGD affects a major mitogenic signalling step in MCs, i.e. phosphorylation of the p42/p44 ERK. Mitogenic stimulation of quiescent MCs adherent to vitronectin/PLL with 25 ng/mL PDGF led to an increase of phosphorylation of predominantly p42, which was detectable 10 min after addition of PDGF (Figure 9C, upper panel). However, presence of 200 μM cRGD clearly lowered phosphorylation of p42 ERK and this effect was not due to lower protein loads, as shown by western blot analysis of total p42/p44 (Figure 9C, lower panel). In contrast, incubation with the control peptide cRAD did not alter PDGF-induced phosphorylation of p42 ERK and this effect was not due to lower protein loads, as shown by western blot analysis of total p42/p44 (Figure 9C, lower panel). In contrast, incubation with the control peptide cRAD did not alter PDGF-induced phosphorylation of p42 ERK in MCs. Since ERK activation is a signalling step down-stream of PDGF receptor activation, we further investigated whether cRGD interferes with PDGF receptor phosphorylation. By immunoprecipitation of the PDGF β receptor sub-unit and western blot with anti-phosphotyrosine antibody, we found no influence of cRGD on phosphorylation of the PDGF receptor (data not shown).

**αvβ3 integrin-inhibitory peptides increase the apoptosis rate in MCs.** Besides inhibition of MC replication, increased MC apoptosis rate is another potential mechanism counteracting MC hyperplasia in vivo. Therefore, we studied the effects of αvβ3 integrin-inhibitory peptides on apoptosis rate in adherent MCs. Growth-arrested MCs were seeded onto a mixture of vitronectin and PLL to allow for integrin-independent MC adhesion in the presence or absence of αvβ3 integrin-inhibitory peptides. After a 24-h incubation period, apoptosis in adherent MCs was quantified by detection of nuclear chromatin condensation in apoptotic nuclei after Hoechst 33258 staining (Figure 10A and B) or by detection of a positive staining reaction for activated (cleaved)
caspase-3. Apoptosis rates in MCs plated on PLL alone and on vitronectin/PLL did not differ significantly. Echistatin and cRGD led to a moderate, but significant increase of MC apoptosis in quiescent, adherent MCs, as assessed by nuclear chromatin condensation. Moreover, incubation with cRGD significantly increased the percentage of MCs with activation of caspase-3 as assessed by immunocytochemical detection of the cleaved caspase-3 fragment (control: 3.5 ± 1.1%; 100 μM RGDdFV: 11.3 ± 2.3%; P < 0.05). Incubation with the control peptide cRAD did not significantly alter MC apoptosis rate or caspase-3 activation (100 μM cRAD: 5.8 ± 1.8%; n.s.).

Taken together, the in vitro data show that functional inhibition of αvβ3 integrin in cultured MCs which are adherent to the αvβ3 ligand vitronectin leads to a decrease of mitogenicity and to an increase of the apoptosis rate.

Discussion

The present study investigates whether inhibition of αvβ3 integrin-mediated actions by cRGD affects the course of MesGN in the Habu snake venom disease model of the mouse (Habu GN). The main finding is a marginal
beneficial effect of cRGD peptides in the early but not late phase of this model on some histopathological parameters, i.e. MC hypercellularity, mesangial matrix expansion and glomerular capillarisation, whereas glomerular size, number of ECs or podocytes and renal function were not positively affected. The data indicate a pro-apoptotic and anti-proliferative role for integrin αvβ3 in the early course of inflammatory glomerular disease due to damage of the mesangium.

In our in vivo study, treatment with cRGD peptides prevented the increase in MC number and mesangial matrix volume fraction at Day 3 and partly also at Day 7 most likely by pro-apoptotic effects as shown by TUNEL and cleaved caspase-3 staining. This finding is in line with studies documenting a pro-apoptotic action of cRGD both in vitro [27, 40] and in vivo [26]. In cultured glomerular MCs from adult human kidneys, evidence was provided that cRGD peptides binding to αvβ1 and αvβ3 integrins induced apoptosis and expression of interleukin-1β-converting enzyme [27]. Also, anti-angiogenic effects of αvβ3 antagonism have been shown in a mouse model of proliferative retinopathy [23, 41] and a rabbit model of arthritis [26]. However, in our short-term study in Habu GN, cRGD treatment did not significantly affect EC numbers. This observation may be due to differences in the disease stimulus and the underlying pathomechanisms in GN as compared to retinopathy and arthritis, respectively.

In vivo and in complementary in vitro studies in primary MCs of the rat, expression of αvβ3 was confirmed, a finding which is in line with data in human MCs [42]. By immunocytochemistry in MCs adherent to the αvβ3 ligand vitronectin, a distribution pattern consistent with recruitment of αvβ3 into focal contacts was seen. Antagonism of αvβ3 by cRGD or the disintegrin echistatin dose dependently inhibited adhesion of MCs to vitronectin, thus demonstrating αvβ3 activity in MCs on a functional level. Notably, the control cRAD peptide did not affect MC adhesion to its ligand vitronectin. Furthermore, the αvβ3-inhibitory peptides did not block adhesion of MCs to fibronectin. While fibronectin is also a potential ligand of αvβ3, we assumed that MCs are still able to bind fibronectin via the αvβ1 integrin [39].

Besides mediating cell–matrix adhesion, integrins regulate a variety of cell functions, including proliferation, cell survival or differentiation by modulating signalling pathways downstream of other receptors. Indeed, we observed that antagonizing αvβ3 activity in cultured MCs by αvβ3-inhibitory peptides prevented PDGF-induced mitogenesis and increased apoptosis rate. An anti-proliferative and pro-apoptotic effect of αvβ3 blockade was also seen in other cell types, i.e. hepatic stellate cells [43].

Consistent with our observations of anti-mitogenic effects of αvβ3 inhibition in MCs, decreased PDGF-induced proliferation in ECs by αvβ3-blockade with a specific RNA aptamer was recently shown [44]. Our finding that cRGD reduced the PDGF-triggered phosphorylation of ERK in MCs may represent one potential mechanism of the anti-mitogenic effects of αvβ3-blockade in MCs. In tumour cell lines, αvβ3 interaction with vitronectin was found to induce ERK phosphorylation [45].

The finding that αvβ3 blockade increased the apoptosis rate in MCs suggests that αvβ3-mediated signalling contributes to MC survival. One potential mechanism how integrin antagonists may induce apoptosis in anchorage-dependent cells may be through interference with cell attachment, i.e. anoikis. However, in our in vitro experiments, αvβ3 inhibition clearly increased apoptosis rate of adherent MCs. In keeping with this observation, cRGD-induced apoptosis in microvascular ECs involves ceramide metabolic pathways indicating that detachment was not required for apoptosis [46].

Taken together, our in vitro data suggest two potential mechanisms how cRGD peptides might counteract MCs hyperplasia in experimental GN, i.e. reduced MC mitogeneity and increased MC apoptosis rate. Of note, our in vivo findings on Day 7 of the GN did not sustain a major additional anti-proliferative effect of cRGD. In view of the in vitro data, which clearly indicate a significant effect of cRGD on MC mitogenicity, this negative finding is most likely due to the late time point of the in vivo investigation in the mouse model.

Several methodological aspects of the in vivo study deserve comment: due to the high costs of the RGD peptides, the experiment could not be performed in the rat model of anti-Thy 1.1 MesGN. Unfortunately, the anti-Thy 1.1 model cannot be induced in mice, however, due to the fact that mouse MCs lack the Thy 1.1 antigen. Thus, the only widely used and well-established model of MesGN in mice is Habu GN. Although the course of the disease and the sequence of the various structural glomerular alterations has been characterized in detail [30, 47, 48], the reproducibility of glomerular damage is low and heterogeneity of the lesions is high. This problem of the model results in high standard deviations and may explain the observation that in contrast to the moderate effects of cRGD treatment on early glomerular damage, we saw no improvement of parameters of renal function, i.e. S-creatinine and albuminuria. Apart from the heterogeneity of the model, timing of the analyses may also be a problem since we investigated only two time points in the course of the GN. With respect to potential effects of cRAD treatment in Habu GN, we would like to emphasize, however, that the measured biochemical and morphological parameters of cRAD-treated mice did not differ from those of untreated Habu GN mice that were investigated in former studies of our group [38]. Thus, a disease-modifying effect of cRAD per se seems very unlikely. Moreover, it is noteworthy that no negative effect of cRGD has been observed.

In summary, our in vivo and in vitro data do not provide evidence for a major pathogenetic role of integrin αvβ3 in Habu GN since we found only evidence for a marginal effect in the early phase but not in the late course of the disease. These largely negative findings of αvβ3 integrin blockade may be due to only a minor role of αvβ3 integrin in this particular animal model of glomerular injury, inherent problems of this GN model or unspecific effects of the peptide itself.

Whether the finding of this experimental study in mice can be transferred to the human condition of MesGN remains to be proven. However, since no harmful side effects
of the cRGD therapy were observed our findings encourage testing of the potential therapeutic value of αvβ3 integrin blockade for treatment of human MesGN.

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Conflict of interest statement. None declared.

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