Role of fibrillin-1 in hypertensive and diabetic glomerular disease

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Submitted 11 July 2005; accepted in final form 19 December 2005


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THE MICROFIBRIL-ASSOCIATED protein fibrillin-1 is abundant in elastic and nonelastic tissues of many organs. Fibrillin-1 is often associated with elastin but can also be found in elastin-free microfibrillar bundles (4). Mutations in the fibrillin-1 gene lead to Marfan syndrome. In addition to skeletal and eye abnormalities, patients with Marfan syndrome suffer from severe dilatation or even rupture of the aorta, which is the most common cause of premature death in Marfan patients (16). Fibrillin-1 is abundantly expressed in the wall of large arterial vessels. Recent studies revealed that the protein is also present in the extracellular matrix produced by mesangial cells in vitro and in the mesangium of the glomerular capillary tuft in vivo (26). The function of fibrillin-1 in the glomerular mesangium, however, is not known. Several case reports describing different types of glomerular disease in patients with Marfan syndrome have been published (1, 11, 17, 18, 22), but there is no evidence that this is more than a chance association. No systematic study on renal sequelae of Marfan syndrome has been performed so far.

A mutant mouse model that exhibits a fivefold underexpression of fibrillin-1 was established by Pereira et al. (15). These mice showed some hallmarks of Marfan syndrome, including cardiovascular complications due to mechanical collapse and dissecting aneurysms of the aortic wall. However, no obvious abnormal renal phenotype was observed. Moreover, previous studies from our laboratory revealed neither functional nor major structural alterations in the kidneys from these mice (8). A low expression of fibrillin-1 apparently does not disturb the development and function of a healthy kidney. This observation does not rule out the possibility that fibrillin-1 may affect the progression of glomerular injury. Here, we show that fibrillin-1 was more abundant in glomeruli of animals with hypertensive or diabetic nephropathy than in healthy glomeruli. To test the hypothesis that fibrillin-1 contributes to renal disease, we induced hypertensive or diabetic glomerular damage in fibrillin-1-underexpressing mice and their wild-type littermates.

MATERIALS AND METHODS

Animal Procedures

Male Sprague-Dawley rats were obtained from Charles River (Sulzfeld, Germany). Heterozygous fibrillin-1-underexpressing mice (strain mgR) were a generous gift from Dr. F. Ramirez (Hospital for Special Surgery, New York, NY), were kept on a mixed genetic background (129SV in C57BL/6), and were bred at our animal facilities. Fibrillin-1-underexpressing mice were genotyped by PCR primers for fibrillin-1 (forward: 5′-TG-3′-CCA GGT GTG TTT CGA CAT TG-5′; reverse: 5′-TG-3′-GTC CTG ATA GCG GTC CGC CA-5′) and neomycin (forward: 5′-TG-3′-TTC CGG CTG TCA GCG CA-5′; reverse: 5′-TG-3′-TTC CGG CTG TCA GCG CA-5′), detecting the native fibrillin-1 gene or the introduced neomycin cassette, respectively. Mice were used for experiments at the age of 10 wk. Animals were housed in a room maintained at 22 ± 2°C, exposed to a 12:12-h dark-light cycle with free access to standard chow (no. 1320, Altromin, Lage, Germany) and tap water (or 1% saline; see below). All procedures performed using animals were done in accordance with guidelines of the American Physiological Society and were approved by local government authorities (Regierung von Mittelfranken, AZ 621–2531.31–1/01 and AZ 621–2531.3–18/94).

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To determine glomerular fibrillin-1 expression patterns in diseases associated with glomerular injury, we performed a study, investigating fibrillin-1 mRNA and protein expression in glomeruli of rats with experimental DOCA-induced glomerular hypertension (DOCA hypertension) and in glomeruli of rats with streptozotocin (STZ)-induced diabetic nephropathy (STZ diabetes). Disease models were induced as described elsewhere (10, 30).

**DOCA hypertension in mice.** At an average weight of 25- to 30-g male mice with a homozygous (mgR/mgR) or heterozygous (mgR/+) gene defect for fibrillin-1 and their wild-type (+/+) littersmates underwent a right unilateral nephrectomy in deep ketamine/xylazine anesthesia. After 2 wk of recovery, 21-day-release DOCA pellets containing 50 mg DOCA (Innitiative Research of America, Sarasota, FL) were implanted subcutaneously by incision of the right flank under ether anesthesia. Control animals were sham operated. After 21 days, DOCA-treated animals received a replacement pellet. All animals (DOCA and control) received isotonic saline (10 g NaCl/l) for drinking over a period of 6 wk, starting with the first day of DOCA treatment. Mice of the different genotypes were distributed randomly to control and total RNA was extracted with RNeasy Mini columns (Qiagen) according to the standard protocol provided by the manufacturer. First-strand cDNA was synthesized with TaqMan RT reagents (Applied Biosystems, Darmstadt, Germany) using random hexamers as primers. Final RNA concentration in the reaction mixture was adjusted to 0.1 ng/µl. Reactions without RT were used as negative controls to test for genomic DNA contamination. RT products were diluted 1:1 with dH2O before the PCR procedure. PCR was performed with the ABI PRISM 7000 Sequence Detector System and SYBR Green reagents (Applied Biosystems) according to the manufacturer’s instructions. For amplification of the rat fibrillin-1 cDNA, the forward primer was 5'-TGCTCTGAAAGGACCCATTG-3' and the reverse primer was 5'-CGGGGACACGATGCCTTACAC-3'. The relative amount of the specific mRNA was normalized with respect to 18S rRNA. Primers used for amplification of 18S cDNA were the following: forward primer 5'-TTGATTAGTCTCGGTCCCTTTGT-3' and reverse primer 5'-CGATCCGAAGGGCCTACCTA-3'. All samples were analyzed in triplicate.

**Immunohistochemistry**

Staining of cryostat sections was performed as described elsewhere (9). The primary rabbit antibody to fibrillin-1 (28) was used at a dilution 1:500. CY3-labeled anti-rabbit IgG (DAKO Diagnostica, Hamburg, Germany) was used as a secondary antibody.

For detection of glomerular collagen IV, renal sections were deparaffinized, and endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 20 min at room temperature. Sections were then layered with an antibody to collagen IV (1:1,000; Southern Biotechnology Associates, Birmingham, AL) and incubated at 4°C overnight. After addition of the secondary antibody (dilution 1:500; biotin-conjugated goat anti-rabbit IgG, Dianova, Hamburg, Germany), the staining procedure was performed as described before (7). Decorin was detected with LF-113 (kindly provided by Dr. L. W. Fisher, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD), a rabbit anti-murine decorin antibody using an alkaline phosphatase anti-alkaline phosphatase technique (19).

Expansion of glomerular fibrillin-1 or collagen IV staining was evaluated in a Leitz Aristoplan microscope (Leica Instruments) by Metaview software (Visiion Systems, Puchheim, Germany) in every third glomerulus per cross section, and the stained area was expressed as the percentage of the total area of the glomerular tuft.

**In Situ Hybridization**

In situ hybridization for decorin was performed as described previously (19). Briefly, deparaffinized paraffin sections were rehydrated, treated with 5 µg/ml proteinase K (Roche Diagnostics), refixed with 4% paraformaldehyde/PBS, acetylated, and incubated with prehybridization buffer in a humid chamber at 50°C for 2 h. Hybridization was performed for 16 h at 50°C in the same buffer containing a riboprobe (7.5 ng/µl). Specimens were washed twice with 2× SSC containing 50% formamide and 1% 2-mercaptoethanol followed by 2× and 0.1× SSC at 50°C. The sections were blocked with 2% normal sheep serum and 0.05% Triton X-100 in 2× SSC, washed twice with buffer 1 (50 mM Tris-HCl, pH 7.5, 225 mM NaCl), and incubated with alkaline phosphatase (AP)- conjugated anti-digoxigenin Fab in a humid chamber for 2 h at 37°C. Slides were washed twice with buffer 1 and equilibrated with 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl and 50 mM MgCl2. Endogenous AP was blocked with 5 mM levamisole. The digoxigenin/anti-digoxigenin conjugates were visualized by reaction with 0.15 mg/ml 5-bromo-4- chloro-3-indolyl phosphate/0.3 mg/ml nitroblue tetrazolium (FAST BCIP/NBT, Sigma) in 100 mM Tris-HCl, pH 9.5, containing 5 mM MgCl2, for 16 h at 4°C. Slides were then dehydrated and analyzed without counterstaining. Sections were hybridized with the sense and antisense probes in parallel using identical conditions.

**Real-Time RT-PCR**

Renal cortical tissue (10 mg) was homogenized in 500 µl of RLT buffer reagent (Qiagen, Hilden, Germany) with an ultratrarrax for 30 s, and total RNA was extracted with RNasey Mini columns (Qiagen)
Indexes of Renal Damage

Paraffin-embedded sections were stained with periodic acid-Schiff’s reagent (PAS) and investigated using light microscopy. The degree of sclerosis within the glomerular tuft was determined on PAS-stained paraffin sections using a semiquantitative scoring system as described before in detail (10). The size of the glomerular area was evaluated in PAS-stained renal sections with a Leitz Aristoplan microscope (Leica Instruments) and Metaview software (Visitron Systems).

Analysis of Data

Two-way ANOVA, followed by a post hoc Newman-Keuls test with adjustment for multiple comparisons, was used to test the significance of differences between groups. A P value <0.05 was considered significant. The procedures were carried out using SPSS software (SPSS, Chicago, IL). All data are expressed as means ± SE.

RESULTS

Induction of Fibrillin-1 Expression and Deposition in Glomeruli of Kidneys with Hypertensive or Diabetic Renal Disease in Rats and Mice

DOCA-salt-treated rats had significantly higher mean arterial blood pressure than controls (154.6 ± 11.2 vs. 98.4 ± 1.7 mmHg in controls, P < 0.05). Moreover, proteinuria was pronounced in DOCA-salt-treated rats compared with controls (739 ± 119 vs. 46 ± 4 µg/day in controls, P < 0.05). Glomerular fibrillin-1 deposition was significantly increased in DOCA-salt-treated rats compared with controls (35.0 ± 4.0 vs. 16.7 ± 3.3% glomerular area stained in controls, P < 0.05). There was also a significant induction of renal fibrillin-1 mRNA expression detected in DOCA-salt treated rats (491.5 ± 199.8 vs. 62.8 ± 11.0 relative copy numbers in

Fig. 1. Induction of renal cortical fibrillin-1 in response to DOCA-induced hypertension (A) or streptozotocin (STZ)-induced diabetes (B). A: immunofluorescent detection of fibrillin-1 in the renal cortex of control (co) and DOCA-treated mice. Right: densitometric evaluation of glomerular fibrillin-1 staining. B: immunofluorescent detection of fibrillin-1 in the renal cortex of control and STZ-diabetic mice. Right: densitometric evaluation of glomerular fibrillin-1 staining. *P < 0.05 DOCA- or STZ-treated mice vs. co.

Fig. 2. Expression levels of fibrillin-1 mRNA in wild-type mice (WT) and mice with a heterozygous (mgR+/−) or homozygous (mgR/mgR) mutation in the fibrillin-1 gene after 5 wk of treatment with STZ. *P < 0.05 vs. STZ-treated WT.

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controls, \( P < 0.001 \). In STZ-treated rats, blood glucose was significantly elevated \( (433.7 \pm 22.3 \text{ vs. } 116 \pm 7.1 \text{ ng/100 ml in controls, } P < 0.05) \). After 5 wk, diabetic animals had developed marked albuminuria \( (1.11 \pm 0.08 \text{ vs. } 0.22 \pm 0.05 \mu g \text{ albumin/creatinine in controls, } P < 0.05) \). Glomerular fibrillin-1 deposition was more pronounced in the kidneys of diabetic rats than in controls \( (21.3 \pm 1.5 \text{ vs. } 12.4 \pm 0.6 \text{ in controls, } P < 0.05) \). Fibrillin-1 mRNA expression in the renal cortex tended to be higher in diabetic rats \( (2.222.5 \pm 776.3 \text{ vs. } 1.571.2 \pm 332.5 \text{ relative copy numbers in controls, not significant}) \).

Similar results were obtained in DOCA-salt- and STZ-treated wild-type mice. DOCA-salt treatment led to an induction of fibrillin-1 mRNA expression \( (421.2 \pm 49.1 \text{ vs. } 258.8 \pm 45.7 \text{ relative copy numbers in controls, } P < 0.05) \) and in glomerular fibrillin-1 protein deposition (Fig. 1A). STZ treatment led to an induction of fibrillin-1 mRNA expression \( (146.4 \pm 22.8 \text{ vs. } 98.0 \pm 9.9 \text{ relative copy numbers in controls, } P < 0.05) \) and in glomerular fibrillin-1 protein deposition (Fig. 1B).

To study the role of elevated fibrillin-1 expression during glomerular disease, we then induced both DOCA-salt hypertension and STZ diabetes in mice underexpressing fibrillin-1. Fibrillin-1 expression levels were first evaluated in untreated mice with a homozygous \( (\text{mgR/mgR}) \) or heterozygous \( (\text{mgR/mgR}^+/) \) mutation in the fibrillin-1 gene vs. their wild-type siblings. In \( \text{mgR/mgR} \) mice, fibrillin-1 expression levels were about fivefold lower than in wild-type \( (22.5 \pm 9.0\% \text{ compared with } 100 \pm 23.7\% \text{ in wild-type}) \). In contrast, fibrillin-1 expression levels in \( \text{mgR/mgR}^+/ \) mice were not significantly reduced compared with wild-type \( (87.4 \pm 21.0\% \text{). After induction of STZ diabetes, however, the increase in fibrillin-1 expression was significantly reduced in \( \text{mgR/mgR}^+/ \) mice compared with wild-type (Fig. 2). Similar results were obtained in DOCA-salt hypertensive \( \text{mgR/mgR}^+/ \) mice. Fibrillin-1 expression was only 63% of the fibrillin-1 expression of DOCA-salt hypertensive wild-type mice.

Effects on Survival and Extent of Renal Damage after Induction of DOCA-Salt Hypertension in Fibrillin-1 Gene-Mutated Mice

Induction of DOCA-salt hypertension in \( \text{mgR/mgR} \) mice led to the death of most animals after implantation of the first DOCA pellet (Fig. 3). Only 2 of 11 mice survived for 3 wk.
until implantation of the second pellet, but they died soon afterward, whereas wild-type mice and mgR/+ mice survived DOCA-salt treatment until the end of the experiment. Systolic blood pressure was not different in wild-type and mgR/+ mice, neither in the control nor in the DOCA-salt-treated groups after 6 wk (Fig. 4A). However, albumin excretion in DOCA-salt-treated mgR/+ mice was significantly lower than in DOCA-salt-treated wild-type litters (Fig. 4B). DOCA-salt treatment led to an increase in urine volume and relative kidney weights in both genotypes (Table 1). Moreover, sclerotic lesions were less severe in DOCA-salt-treated mgR/+ mice compared with DOCA-salt-treated wild-type mice (Fig. 4D), and the increase in glomerular collagen IV expansion detected in wild-type mice after DOCA-salt treatment is blunted in mgR/+ mice (Fig. 4C).

**Effects of STZ-Induced Diabetes on Renal Function in Fibrillin-1 Gene-Mutated Mice**

Treatment with STZ led to an increase in food intake and urine excretion in all genotypes (Table 2). In STZ-treated mice, blood pressure was not different compared with control mice after 5 wk (Fig. 5A). Furthermore, no genotype differences in blood pressure were detected (Fig. 5A). Blood glucose was elevated in all genotypes 5 wk after injection of STZ, in mgR/+ or mgR/mgR mice even more prominently than in wild-type mice (Fig. 5B). Significant increases in matrix deposition or sclerotic lesions in the glomerulus, however, were not detected at this early time point investigated (data not shown). On the other hand, the size of the glomerular area was increased in renal sections of STZ-treated wild-type mice, whereas no increase in glomerular size was detected in STZ-treated mgR/+ or mgR/mgR mice compared with controls (Fig. 5C). Albuminuria was clearly induced in wild-type mice (Fig. 5B), whereas wild-type mice and mgR/+ mice after STZ treatment. In mgR/+ or mgR/mgR mice, the increase in albumin excretion was significantly lower after STZ treatment than in wild-type mice (Fig. 5D). Staining for decorin in renal sections of diabetic kidneys revealed immunoreactivity in podocytes of mgR/+ or mgR/mgR mice but not in podocytes of wild-type mice (Fig. 6, A–C). To confirm these findings, in situ hybridization for decorin was performed, which showed expression of decorin mRNA in podocyte distribution in both mgR/+ and mgR/mgR mice (Fig. 6D).

**DISCUSSION**

Our findings indicate that in mgR/+ or mgR/mgR mice, the sequelae of glomerular hypertension or hyperperfusion are attenuated, resulting in an amelioration of renal damage. Following induction of glomerular hypertension with DOCA (5), most mgR/mgR mice died probably from aortic dissections, as internal bleeding was commonly detected after opening of the body cavity. Pereira et al. (15) described occasional death of vascular etiology including aortic dissections in mice underexpressing fibrillin-1, arguing for a decreased elasticity of the vasculature. It thus seems conceivable that an additional hypertensive mechanical strain will frequently result in disruption of the structural integrity of vessels exposed to high blood pressure in these mice. mgR/+ mice did not die of aortic dissections after treatment with DOCA. Despite comparable blood pressure levels in wild-type and mgR/+ mice after induction of hypertension, albuminuria and glomerulosclerosis in mgR/+ mice were significantly lower than the values reached by their wild-type littermates.

These findings are in accordance with the results from another model of fibrillin-1 gene deletion, which showed expression of decorin mRNA in podocyte distribution in both mgR/+ and mgR/mgR mice (Fig. 6D).
the 5 wk of observation. Although in mgR/+ or mgR/mgR mice blood glucose levels even tended to be higher after STZ treatment than in wild-type mice, the increase in albuminuria was attenuated compared with wild-type. Moreover, the increase in glomerular size observed in diabetic wild-type mice
compared with controls was blunted in diabetic mgR/+ and mgR/mgR mice, arguing for a protective role for fibrillin-1 in the maintenance of glomerular structure during diabetic nephropathy. However, a significant induction of glomerular matrix deposition in diabetic mice was not observed. This finding may be due to the short duration of diabetes, or to the fact that we used female mice, which are less susceptible to develop glomerulosclerosis than are male mice (2). As a consequence, differences in glomerular matrix expansion among the three genotypes investigated could not be detected. Why are mice with a mutation of the fibrillin-1 gene apparently protected from an increase in albuminuria following hypertension or diabetes? If the deposition of fibrillin-1 itself would contribute to glomerular damage, a reduced expression of fibrillin-1 might well be protective. mgR/mgR mice express fivefold less fibrillin-1 compared with wild-type litters, which is in accordance with previously published data (8, 15). mgR/+ mice do not display a significant underexpression of fibrillin-1 per se, but induction of cortical fibrillin-1 expression seems to be attenuated in mgR/+ mice after glomerular injury (DOCA glomerular hypertension or STZ diabetic nephropathy) compared with wild-type mice after glomerular injury. Alternatively, a compensatory upregulation of other matrix molecule(s) in mice with a mutation in the fibrillin-1 gene could exert protective effects after DOCA or STZ treatment.

Decorin is a small dermatan-sulfate proteoglycan that can form complexes with fibrillin-1 (29). In decorin-deficient mice, an overexpression of fibrillin-1 was observed, which most likely contributed to the preservation of kidney morphology after pressure-induced renal injury (21). Moreover, decorin seems to have antifibrotic properties, as it has been successfully used as a therapeutic agent to reduce glomerulosclerosis in experimental glomerulonephritis (3). Conversely, the absence of decorin leads to enhanced tubulointerstitial fibrosis following obstruction of the kidney (20).

An increase in decorin expression in the mouse kidney cortex has been observed after the onset of STZ diabetes (13). To determine the source of cortical decorin expression, several renal cell types were studied regarding their ability to express decorin. In cultivated mesangial and proximal tubular epithelial cells, decorin is constitutively expressed and upregulated by high glucose, whereas glomerular epithelial and endothelial cells do not express decorin (13). In normal kidneys of adult rats, parietal epithelial cells and interstitial cells express decorin (19). Expression of decorin in mesangial cells in vivo was only detected in trace amounts (19). This is in accordance with our findings regarding decorin expression in the glomeruli of mice. In wild-type mice, no detectable glomerular decorin protein staining was observed. In contrast, glomeruli of mgR/+ and mgR/mgR mice showed decorin mRNA and protein expression in podocytes after STZ treatment. According to earlier reports, fibrillin-1 is not expressed in podocytes; therefore, the expression of decorin in podocytes from fibrillin-1-underexpressing mice seems not to represent a compensatory mechanism in which decorin as an inducer of fibrillin-1 becomes upregulated (21). However, it is conceivable that reduced amounts of fibrillin-1 in the mesangial matrix may lead to changes in matrix assembly and composition, resulting in the release of growth factors and cytokines normally sequestered within the extracellular matrix. Thus transforming growth factor-β1, IL-6, or IL-10, factors that have been shown to regulate the expression of decorin (14, 27), once released from the mesangial matrix could pass through the glomerular base- 

membrane and induce decorin in podocytes.

Although the mechanisms of the antiproteinuric effects of decorin are still unknown, it is tempting to speculate that decorin, which has been shown to protect endothelial and tubular epithelial cells from apoptosis by binding to IGF-IR and inducing Akt/PKB (20, 23, 24), might have similar effects on podocytes. This in turn might lead to reduced podocyturia (31) and could explain how decorin facilitates the reduction of albuminuria described here and which has also been observed after administration of decorin (3) and by decorin gene transfer (12) in a model of mesangioproliferative glomerulonephritis.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of Mirosława Kuppusamy-Hultz and Rainer Wachtveitl. The authors thank Dr. Francesco Ramirez for kindly providing the fibrillin-1-underexpressing mice.

Parts of this study were presented in abstract form at the 36th Annual Meeting of the American Society of Nephrology, November 12–17, 2003, San Diego, CA, and in J Am Soc Nephrol 14: 137A, 2003.

GRANTS

This study was supported by grants from the Deutsche Forschungsgemein- schaft, Bonn, Germany (STE 196/4–1; SFB 423, Project A2 and SFB 492, Project B10), an ELAN grant from the Faculty of Medicine, University of Erlangen-Nürnberg, the Interdisciplinary Center for Clinical Research, University of Münster (Schae2026/06), and the Canadian Institutes of Health Research (MOP-68836).

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