Regulation of Fibrillin-1 by Biglycan and Decorin Is Important for Tissue Preservation in the Kidney During Pressure-Induced Injury

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There is growing evidence that the two small leucine-rich proteoglycans biglycan and decorin regulate the assembly of connective tissues and alter cell behavior during development and pathological processes. In this study, we have used an experimental animal model of unilateral ureteral ligation and mice deficient in either biglycan or decorin. We discovered that pressure-induced injury to the wild-type kidneys led to overexpression of decorin, biglycan, fibrillin-1, and fibrillin-2. In contrast, in biglycan-deficient kidneys the overexpression of fibrillin-1 was markedly attenuated and this was associated with cystic dilatation of Bowman's capsule and proximal tubules. Notably, we found that in ligated kidneys from decorin-null mice, fibrillin-1 expression was initially enhanced to the same extent as in wild-type animals. However, long-term obstruction resulted in down-regulation of fibrillin-1 and concurrent cystic dilatation of Bowman's capsule in 33% of kidneys at 5 months after obstruction. In all of the genotypes, no differences in fibrillin-2 expression were observed. These *in vivo* data correlated with a significant induction of fibrillin-1 expression in renal fibroblasts and mesangial cells by recombinant biglycan and decorin. Our results indicate a novel role for decorin and biglycan during pressure-induced renal injury by stimulating fibrillin-1 expression. (*Am J Pathol* 2004, 165:383–396)

Biglycan and decorin belong to the family of small leucine-rich proteoglycans (SLRPs), which are characterized by core proteins with centrally located leucine-rich motifs flanked by cysteine clusters and by one (decorin) or two (biglycan) chondroitin/dermatan-sulfate side chains. SLRPs are primarily considered to play a role in collagen fibril formation and stability.^{1–3} Ablation of each SLRP gene is associated with disturbed type I collagen-containing fibrils resulting in enhanced skin fragility in decorin-deficient mice.^{4,5} Decorin and biglycan have been shown to form complexes with transforming growth factor (TGF)- β ⁶ leading, in the case of decorin, to inhibition and/or sequestration of this cytokine in the extracellular matrix.^{7,8} Beside the modulation of cytokine activities both SLRPs are directly involved in cell signaling, thereby regulating proliferation and apoptosis of various cell types *in vitro* and *in vivo*.^{3,9–12}

In the normal kidney, distinct and spatially different expression patterns of decorin and biglycan have been described.^{13,14} Studies in animal models of renal disease and human kidney biopsies have shown that decorin, endogenously produced by mesangial or interstitial cells, exerts anti-fibrotic effects by blocking TGF- β activity and by regulating apoptosis of tubular epithelial cells.^{7,8,10} Biglycan, is expressed in the tubulointerstitium and in all cell types of the glomerulus, including Bowman's capsule.^{13,14}

There is growing evidence that SLRPs may also be involved in elastic fiber biology.^{15–18} Mature elastic fibers, consisting of an elastin core surrounded by microfibrils, complement the function of collagen fibrils and are essential for the resilience of connective tissues. Microfibrils can also be found in elastin-free bundles associated with basement membranes.¹⁹ The main constituents of microfibrils are fibrillin-1 and -2, two structurally related glycoproteins with distinct temporal and spatial expression patterns.^{20,21} Notably, mutations in any of the elastic proteins cause human diseases. For instance, fibrillin-1 mutations cause Marfan syndrome; mutations of fibrillin-2

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cause congenital contractural arachnodactyly; and elastin mutations cause Williams syndrome, supraaortic stenosis, and cutis laxa.¹⁹ Besides fibrillins, a number of other proteins have been reported to be associated with microfibrillar structures.¹⁹ For example, microfibril-associated glycoproteins 1 and 2 as well as latent TGF- β -binding proteins are either integral or peripherally associated components of microfibrils.^{17,22,23} Recent studies have shown that fibrillin binds to latent TGF- β -binding protein-1 and by sequestering TGF- β controls the activation of this cytokine.^{23,24} Because of the ability of decorin to form complexes with fibrillin-1, tropoelastin (the soluble precursor of mature elastin) and MAGP-1^{15–17} and of biglycan to bind to the latter two components,¹⁸ both proteoglycans were classified as microfibril- and elastic fiber-associated molecules.¹⁹ However, the relevance of these interactions for elastic fiber biology is not yet clear, especially as neither decorin nor biglycan-null mice have so far been shown to display any elastic fiber abnormalities.^{4,25}

Treatment of rat embryonic metanephroi with fibrillin-1 anti-sense oligonucleotides results in renal dysmorphogenesis.²⁶ In the adult kidney, fibrillin-1 was found mainly in the mesangial matrix and the glomerular basement membrane (GBM) as well as in the vasculature suggesting its contribution to the mechanical stability of the glomerular capillary tuft.^{20,26,27}

In this study, we used an animal model of tubulointerstitial injury of the kidney in which renal pathology is induced by ligating one ureter. We discovered that pressure-induced injury in WT animals was associated with a marked up-regulation of biglycan, decorin, fibrillin-1, and fibrillin-2 expression. In contrast, the absence of biglycan was associated with a blunted up-regulation of fibrillin-1 and a concurrent loss of elastic properties of renal tissue, as evidenced by cystic dilatation of Bowman's capsule and proximal tubules as well as hemorrhaging into the renal pelvis. In decorin-null mice, the compensatory overexpression of biglycan induced fibrillin-1 and preserved renal morphology to some extent. Moreover, we show that decorin and biglycan specifically induced the expression of fibrillin-1, but not of fibrillin-2, in renal cells. Thus, biglycan and decorin regulate the expression of fibrillin-1 in the renal parenchyma during pressure-induced injury in obstructive nephropathy.

Materials and Methods

Animals

All animal experimentation was conducted in accordance with the German Animal Protection Act and were approved by the Ethics Review Committee for laboratory animals of the District Government of Muenster, Germany. The kidneys studied were from wild-type (WT), *biglycan*-0 (*Bgn*-0, as the biglycan gene is located on the X chromosome, male animals have only one allele) and *decorin*-/- (*Dcn*-/-) mice.^{4,25} Obstruction of the left ureter was performed in 2-month-old male animals. The contralateral and sham-operated kidney served as a

control. Kidneys (at least $n = 4$ per group) were analyzed up to 150 days after ligation.

Human Tissues for Histological Investigations

Samples representing normal human kidneys were obtained from patients undergoing nephrectomy for renal cell carcinoma. Only tumor-free samples were used for the present investigation. Human kidneys were used because several species of appropriate primary antibodies were available in hosts of different origin allowing double staining for both biglycan and fibrillin-1. The samples were fixed immediately with 4% formaldehyde in 18 mmol/L sodium phosphate, pH 7.4, containing 0.15 mol/L NaCl [phosphate-buffered saline (PBS)], and embedded in paraffin. The use of human tissue was approved by the Ethics Review Committee of the University of Muenster.

Reagents

Radiochemicals, hyperfilms, nitrocellulose membranes, and the enhanced chemiluminescence reagents were acquired from Amersham Pharmacia Biotech Europe GmbH (Freiburg, Germany). The BCA protein assay reagent was purchased from Pierce (Rockford, IL). TRIzol, reagents for reverse transcriptase-polymerase chain reaction media, and sera were from Invitrogen (Groningen, The Netherlands). Tissue culture plastic was from Falcon (Becton-Dickinson, Heidelberg, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

Production and Purification of Biglycan and Decorin

Expression of human biglycan or decorin in 293 HEK cells and purification of the native proteoglycans were performed as described previously.^{28,29} Briefly, for purification the conditioned medium was supplemented with proteinase inhibitors (0.1 mol/L ϵ -amino-*n*-caproic acid, 10 mmol/L ethylenediaminetetraacetic acid, 5 mmol/L benzamidine, 10 mmol/L *N*-ethyl-maleimide, 1 mmol/L phenylmethyl sulfonyl fluoride) and passed over a DEAE-Tris-Acryl M (Serva, Heidelberg, Germany) column followed by elution with 20 mmol/L Tris/HCl, pH 7.4, containing 1 mol/L NaCl. After concentration of the relevant fractions with Aquacide I as instructed by the manufacturer (Calbiochem, Bad Soden, Germany), the proteoglycans were dialyzed for 2 hours against 20 mmol/L Tris-HCl, pH 7.4, containing 150 mmol/L NaCl and separated by high performance liquid chromatography on a SEC-DEAE column (Phenomenex, Aschaffenburg, Germany) by a discontinuous NaCl gradient. The purity of the proteoglycans was verified by silver staining after sodium dodecyl sulfate gel electrophoresis. For some experiments the proteoglycans were digested with chondroitin ABCase (Seikagaku Kogyo, Tokyo, Japan) to remove chondroitin sulfate and dermatan sulfate chains.¹¹

Cell Culture and Stimulation

The rat mesangial cell (MC) line (kindly provided by Dr. J. Pfeilschifter, University of Frankfurt, Frankfurt/Main, Germany)³⁰ and normal rat kidney (NRK) fibroblasts (catalog no. 6509; American Type Culture Collection, Rockville, MD) were cultured as described previously.^{11,31} MCs were grown in RPMI 1640 and NRK fibroblasts in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mmol/L glutamine, 5 ng/ml insulin, 100 U/ml penicillin, and 100 μ g/ml streptomycin for 8 to 19 passages. To obtain quiescent MCs or NRK fibroblasts, cells were maintained in serum-free Dulbecco's modified Eagle's minimum supplemented with 0.1 mg/ml of fatty acid-free bovine serum albumin for 24 hours before the addition of recombinant human biglycan or decorin for 1 to 24 hours. As both cell types display concentration-dependent responses at concentrations between 1 to 10 μ g/ml of recombinant proteoglycans, we used biglycan and decorin in concentrations of 4 μ g/ml for all further experiments. Intact proteoglycans were used in the majority of experiments because this is their presumed natural form *in vivo*. Viability of cells was not altered under these conditions, as determined by lactate dehydrogenase release into the culture medium using a cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany).

Morphological and Immunohistochemical Studies

Serial sections (4 μ m) of paraffin-embedded samples were stained with periodic acid-Schiff reaction as well as a modified Hart's stain for elastin and processed for immunohistochemical studies by immunoperoxidase or alkaline phosphatase anti-alkaline phosphatase techniques.³¹ Primary antibodies included rabbit anti-human fibrillin-1³² and anti-human fibrillin-2 antisera³³ that extensively cross-react with mouse fibrillin-1 and -2, LF-113,³⁴ a rabbit anti-murine decorin and LF-106,³⁴ a rabbit anti-murine biglycan antiserum, chicken anti-rat biglycan cross-reacting with human biglycan,³¹ rabbit anti-collagen-I (Biogenesis, Berlin, Germany), rabbit anti-collagen-II (Chemicon, Hofheim, Germany) and goat anti-collagen-V, -VI (both from Southern Biotechnology Associates, Birmingham, AL). Rabbit antisera against the fibromodulin peptide Asn255-Gly267 and lumican peptide Pro300-Leu311, which are conserved in rat, mouse, and human, were raised after conjugating the peptide with keyhole limpet hemocyanin. After blocking endogenous peroxidase and incubation with the respective antibodies, sections were developed with the diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). Counterstaining was with methyl green or with Mayer's hemalaun. The specificity of immunostaining was tested by omitting the primary antibody and by using nonimmune serum/unspecific IgG.

To evaluate individual kidneys, 10 randomly selected nonoverlapping fields were examined by a blinded observer as described previously.¹⁰ In brief, a grid containing 117 (13 $\times$ 9) sampling points was superimposed on

images of cortical high-power fields ($\times$ 400) and deposits of elastin and collagen-I, -II, -V, and -VI were counted and expressed as a percentage of all sampling points. Mean values of at least four kidneys per group were averaged.

Confocal and Electron Microscopy

For localization of fibrillin-1 or fibrillin-2 and co-localization of biglycan and fibrillin-1 in the normal kidney paraffin sections of human kidneys were incubated with a chicken antibody against biglycan,³¹ and rabbit antibodies against fibrillin-1 or fibrillin-2, respectively. Unspecific staining was blocked with PBS/1% (w/v) bovine serum albumin/20% (v/v) goat serum. For visualization fluorescein-conjugated donkey anti-chicken IgG and Texas Red-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) were used. Laser scan microscopy was performed with a Nikon confocal microscope PCM 2000 (Nikon, Duesseldorf, Germany). Nonspecific staining was determined by the use of secondary antibodies alone. For electron microscopical analysis a small piece of cortical tissue from the lower kidney pole was fixed in glutaraldehyde and embedded in araldite as described.³⁵

Northern Blot Analysis

Total RNA was extracted as described.³¹ To prepare the probes specific for mouse fibrillin-1 and fibrillin-2, cDNA was prepared by reverse transcription of total RNA from mouse embryonic fibroblasts using oligo dT primer and Superscript II as instructed by the manufacturer (Invitrogen). The cDNA was amplified by polymerase chain reaction with oligo nucleotides 5'-GCTCTGTCCCTCTGGTAATTC-3' and 5'-GGACCACTGGCAGTAATCAG-3' resulting in a 204-bp fragment, which spans the coding sequence for the unique mouse proline-rich sequence of fibrillin-1 (nucleotides 1166 to 1369), and with 5'-TGGATGGTCTTCCGATGGGTG-3' and 5'-GGCATGGTGCTTACAGATGTC-3' resulting in a 233-bp fragment coding for the unique mouse glycine-rich sequence of fibrillin-2 (nucleotides 1253 to 1485). Although the overall homology between the coding sequences of mouse fibrillin-1 and fibrillin-2 is high (~67%), these sequences show the most divergent homologies (~12%). The amplified DNA fragments were cloned into the pCRII-Topo vector as instructed by the supplier (Invitrogen). The cDNA probes for decorin, biglycan, fibromodulin, and lumican were those used previously.¹⁰ The cDNA probe for GAPDH was from American Type Culture Collection. Northern blots were performed and analyzed as described previously.⁸ For quantification, the Phosphor screens were analyzed by a STORM860 Phosphor Imager using IQ Solutions Image Quant software (both from Molecular Dynamics, Uppsala, Sweden). Each individual mRNA band was normalized for GAPDH to correct for the difference in RNA loading and/or transfer. Values are given as means $\pm$ SEM from three Northern blots. *In situ* hybridization of renal sections from WT and *Bgn*^{-/-} mice was performed with the sense and anti-sense probes for biglycan and decorin in parallel and under the same conditions.¹⁰

Western Blot Analysis

Western blots were performed and quantified as described previously⁸ using rabbit anti-fibrillin-1, anti-fibrillin-2, and anti- β -tubulin (Santa Cruz, Heidelberg, Germany) as primary antibodies. The probes were visualized by using the enhanced chemiluminescence Western blotting reagent kit (Amersham Pharmacia Biotech, Freiburg, Germany) and quantified with IQ Solutions Image Quant software (Molecular Dynamics). Results from kidney samples were expressed as optical density (OD) of the whole kidney or were normalized to protein content or OD of β -tubulin. Results from cell culture media or homogenates were normalized to cell number, protein content, or OD of β -tubulin in homogenized cells. For quantification the results of three samples per group were averaged.

Determination of Decorin in Kidney Homogenates

Decorin in homogenates from whole contralateral and ligated kidneys ($n = 3$ in each group) was extracted, semipurified, and quantified as described previously.^{8,11} Briefly, kidney homogenates and appropriate standard solutions were supplemented with 10 mmol/L Tris/HCl, pH 7.4, 0.1% Triton X-100, and protease inhibitors.¹¹ Subsequently, samples were mixed with DEAE Trisacryl M (100 mg wet weight), equilibrated with 20 mmol/L Tris/HCl, pH 7.4, containing 0.15 mol/L NaCl, 0.1% Triton X-100, and protease inhibitors (buffer 1) and mixed by rotation for 1 hour at 4°C. The samples were washed sequentially with 3 ml of buffer 1 containing 7 mol/L urea, 3 ml of urea-free buffer 1, and 3 ml of buffer 1 containing 0.3 mol/L NaCl. Elution was achieved with 1.5 ml of buffer 1 containing 1 mol/L NaCl. On adding 5 vol of methanol and 1 vol of chloroform, proteoglycans were collected at the interphase between chloroform and aqueous methanol and washed with methanol. The proteoglycans were then digested with chondroitin ABCase (Seikagaku Kogyo) to remove glycosaminoglycan chains, and subjected to polyacrylamide gel electrophoresis followed by Western blotting with subsequent quantification exactly as described previously.⁸

Other Procedures

Serum urea and creatinine levels were measured using a Hitachi 747 autoanalyzer and urinary and tissue protein by the BCA protein assay reagent. Type I, II, V, and VI collagens were determined after exhaustive pepsin digestion of whole minced kidneys followed by 4 to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions and quantification (IQ Solutions Image Quant software) of Coomassie Blue-stained bands.¹⁰ A high- and low-molecular weight mixture of proteins (Invitrogen) and pepsin-digested purified type I, II, V, and VI collagens were used as standards. For the quantification of collagens results from three kidneys per group were averaged.

Statistics

Results are expressed as means $\pm$ SEM. Statistical analysis was performed by the unpaired Student's *t*-test. Significance was accepted at the 5% level.

Results

Morphology of Obstructed Kidneys from Biglycan-Deficient Mice

Unilateral ureteral obstruction (UUO) was performed in WT ($n = 38$) and *Bgn*⁻⁰ ($n = 40$) animals, and the development of hydronephrosis was followed throughout a period of 70 days. Three weeks after ligation, the *Bgn*⁻⁰ mice developed pelvic hemorrhages in the majority of cases (63%) in contrast to WT animals in which no hemorrhages were observed (Figure 1A). After day 3, light microscopy in periodic acid-Schiff-stained sections showed that tubuli were more dilated in ligated kidneys from *Bgn*⁻⁰ mice (Figure 1B) than in obstructed kidneys from WT animals (Figure 1C). More pronounced thickening of Bowman's capsule and occasionally of the tubular basement membrane (TBM) was detected in ligated kidneys from *Bgn*⁻⁰ mice (Figure 1D) as compared to WT mice (Figure 1E). We noticed that, although the size of the glomerular tuft was unchanged, cystic dilatations of the Bowman's capsule were easily detected in the ligated kidneys from *Bgn*⁻⁰ mice as early as 2 weeks after ligation, affecting 87% of glomeruli at day 70 (Figure 1, F and H). Occasionally, cysts were also observed in proximal tubules (Figure 1I). Ligated kidneys from WT mice displayed typical changes in renal morphology (dilated tubular lumina) without any cystic dilatation of Bowman's space (Figure 1G). Nonligated kidneys from WT and *Bgn*⁻⁰ mice were similar in terms of renal morphology (data not shown).

Next, we evaluated the ultrastructure of the glomerular tuft, Bowman's capsule, as well as TBM and peritubular capillaries in ligated kidneys from WT and *Bgn*⁻⁰ mice by electron microscopy. No abnormalities were detectable in Bowman's capsule and in the glomerular tuft in *Bgn*⁻⁰ mice, which would have explained the presence of erythrocytes in the renal pelvis. Additionally, peritubular capillaries appeared normal without any ruptures. However, the TBM in ligated kidneys from *Bgn*⁻⁰ mice was clearly abnormal displaying an irregular structure and considerable variations in width (Figure 2A). In contrast, the TBM from WT animals had a much more homogeneous appearance (Figure 2D). Using a higher magnification the irregularities of the TBM in ligated kidneys from *Bgn*⁻⁰ mice, such as abrupt changes (Figure 2B) and widening of basement membrane sections and focal fibrillary alterations, were more clearly visible (Figure 2C). In contrast, even at higher magnifications, such as those in Figure 2, B and C, the TBM in WT mice was of normal width and displayed a homogeneous structure (Figure 2, E and F). There were no genotype-specific differences in survival, kidney/body weight ratio, serum urea and creatinine levels, or urinary protein excretion both in mice with normal kidneys as well as in animals after UUO. In

Figure 1. Renal phenotype in WT and *Bgn*^{-/-} mice following UUU. **A:** After unilateral ureteral ligation (UUO) both WT and *Bgn*^{-/-} mice developed hydronephrosis with hemorrhage into the renal pelvis of *Bgn*^{-/-} mice, shown here at day 35 (arrow marks the kidney from a *Bgn*-deficient mouse), whereas in WT kidneys bleeding was not observed. There were no genotype-specific differences in the size of kidneys. The scale is given in cm. Periodic acid-Schiff-stained sections from ligated kidneys from *Bgn*^{-/-} mice (**B**, white arrowhead) demonstrated more severe dilatation of tubuli compared to those from obstructed WT kidneys (**C**, white arrowhead) 7 days after UUO. At day 14 after UUO, a more pronounced thickening of Bowman's capsule (arrow) and occasionally of the TBM (black arrowhead) was detected in ligated kidneys from *Bgn*-deficient (**D**) compared to WT mice (**E**). At day 35, cystic dilatation of Bowman's capsule (**F**, arrow) without changes in the size of the glomerular convolute (**H**) as well as cystic dilatations of proximal tubules (**I**, black arrowhead) occurred. No such changes were observed in WT kidneys (**G**).

Bgn^{-/-} mice no hemorrhaging was observed in other organs except in the ligated kidneys.

Immunostaining for Fibrillin-1, Fibrillin-2, and Biglycan in the Normal Kidney

In kidneys from different species biglycan is expressed in Bowman's capsule, in endothelial cells, smooth muscle

cells and in the adventitia of blood vessels, in the interstitial and mesangial matrix, and in association with tubular epithelial cells.^{10,13,14,31} Based on the phenotype and the morphological changes in ligated kidneys from *Bgn*^{-/-} mice, we postulated that a lack of biglycan might affect the assembly and composition of elastic fibers, resulting in altered biomechanical properties of blood vessels, Bowman's capsule, and the TBM. At first, we

Figure 2. Electronmicroscopy of TBMs in ligated kidneys from *Bgn*⁰ (A–C) and WT (D–F) mice after 56 days of UUO. **A:** Overview of a collapsed tubule with surrounding interstitium with many mononuclear cells. TBMs vary considerably in width and display an irregular structure. Tubular epithelial cells appear dedifferentiated. **D:** In contrast, in WT animals the TBM has a much more homogenous appearance. Again the tubular epithelial cells are severely damaged and do not display a segment-specific differentiation pattern. **B** and **C:** At a higher magnification the irregularities of the TBM are more clearly visible with thinning in **B**, widening and thinning in **C**, and with small fibrillary changes of some sections of the TBM. **E** and **F:** In contrast, in WT animals even at magnifications comparable to those in **B** and **C** the TBM displays a homogeneous inner structure and is relatively broad. **Arrowheads** indicate the TBM. Original magnifications: $\times 4970$ (A, D); $\times 24,000$ (B, C, E, F).

compared the immunolocalization of elastic fiber components, such as fibrillin-1, fibrillin-2, and elastin, in the normal kidney^{20,21,27} with the known location of biglycan.^{10,13,14,31} Human tissue had to be used because several species of appropriate primary antibodies were available in hosts of different origin allowing double staining for both biglycan and fibrillin. Fibrillin-1 was observed in mesangial areas of the glomerulus, in the GBM, in Bowman's capsule, and in the TBM. In the peritubular interstitium fibrillin-1 appeared to form microfibrillar networks (Figure 3A). Fibrillin-2 was detected in the mesangial matrix, in the GBM and in afferent and efferent arterioles, whereas only trace amounts were present in Bowman's capsule and in the TBM (Figure 3B). Double staining for fibrillin-1 and biglycan showed that biglycan, which does not co-localize with fibrillin-1, was present in Bowman's capsule and tubular epithelial cells in addition to some weak staining in the interstitial matrix (Figure 3C). The inset, representing a higher magnification of Bowman's capsule, shows an inner positive lining by biglycan and a positive stain for fibrillin-1 in the immediate interstitial vicinity (Figure 3C). Figure 3D shows a preglomerular artery staining positive for fibrillin-1 in the lamina elas-

tica interna and externa, in the media, and in the transition zone between adventitia and the surrounding interstitium. Fibrillin-1 showed again a cribriform-staining pattern in the expanded interstitium. Biglycan stained positive in the newly formed elastica interna, the media, and in the adventitia as well as in epithelial cells and to some extent in the interstitium, where it was localized in close proximity to fibrillin-1 without direct co-localization (Figure 3D). The upper inset represents the negative control by omitting the primary antibodies (Figure 3D). The lower inset shows the morphology of the preglomerular artery used for double staining (Figure 3D).

In agreement with earlier observations,²⁷ elastin was localized in the renal vasculature, in the glomerular vascular pole, and in the afferent and efferent arterioles extending to Bowman's capsule, but was absent in the peritubular space and in the glomerular capillary tuft (data not shown). Immunostaining for fibrillin-1 (Figure 4A) or fibrillin-2 and Hart's staining for elastin (not shown) demonstrated unequivocally that there were no differences in localization of both fibrillins and elastin between human and murine kidneys.

Figure 3. Immunofluorescence staining for fibrillin-1 (**A**) and fibrillin-2 (**B**) and double staining for fibrillin-1 (red) and biglycan (green) in the human kidney (**C**, **D**). **A:** Fibrillin-1 was located in the mesangial extracellular matrix and the GBM, in Bowman's capsule and in the TBM. **B:** Fibrillin-2 was strongly expressed in the mesangial matrix, in the GBM and in the afferent and efferent arterioles, whereas only trace amounts were present in Bowman's capsule and TBM. **C:** Double immunostaining for fibrillin-1 in red and biglycan in green demonstrated that biglycan, which does not co-localize with fibrillin-1, is present in Bowman's capsule, tubular epithelial cells, and in the interstitial matrix. The **inset** corresponding to a higher magnification of Bowman's capsule showed an inner positive lining by biglycan and a positive stain for fibrillin-1 in the immediate interstitial vicinity. **D:** A preglomerular artery being positive for fibrillin-1 in the lamina elastica interna (**arrowhead**) and externa (**arrow**) as well as in the transition zone between the adventitia (**a**) and the surrounding interstitium. Biglycan was observed in newly formed elastica interna (**small double arrows**), in the media (**m**), and in surrounding epithelial cells and partly in interstitium. The area of subintimal fibrosis is labeled (**s**). The **top inset** represents a negative control by omitting the primary antibodies. The **bottom inset** shows the morphology of the preglomerular artery used for double staining. The kidney sections shown in **A–D** were from a 52-year-old patient who had undergone nephrectomy because of a renal cell carcinoma.

Reduced Expression of Fibrillin-1 in Ligated Kidneys from *Bgn*-Deficient Mice

In a further step we investigated whether *Bgn* deficiency would influence the expression of fibrillin-1 and/or fibrillin-2 in the mouse kidney. Immunostaining for fibrillin-1 in nonligated kidneys (control, contralateral, or sham-operated) did not reveal any differences either in localization nor in the intensity of staining between WT and *Bgn*^{-/-}

mice (Figure 4, A and B; *n* = 6 in each group). Seven days after UUO, there was a marked increase in immunostaining for fibrillin-1 in ligated kidneys from both WT and *Bgn*^{-/-} mice, which persisted throughout the whole course of the experiment (Figure 4, C and D; renal sections at day 35 after UUO). This was because of a pronounced accumulation of fibrillin-1 mainly at its typical locations, such as Bowman's capsule and TBM. In addition, fibrillin-1 was also prevalent in the peritubular inter-

Figure 4. Decreased expression of fibrillin-1 in ligated kidneys from *Bgn*^{-/-} mice after 35 days of UUU. **A** and **B**: In normal kidneys from WT (**A**) and *Bgn*^{-/-} (**B**) mice the pattern of immunostaining for fibrillin-1 (brown color) was comparable with what was observed in the human kidney. There were no differences in the localization and/or intensity of staining between WT (**A**) and *Bgn*^{-/-} (**B**) in normal kidneys. **C** and **D**: In ligated kidneys from both WT (**C**) and *Bgn*^{-/-} (**D**) mice expression of fibrillin-1 in the Bowman's capsule and in the tubulointerstitium was markedly enhanced as compared to normal kidneys. In obstructed kidneys from *Bgn*^{-/-} (**D**), staining for fibrillin-1, while still being more intense than in unligated kidneys, was reduced as compared to ligated WT kidneys (**C**). **E** and **F**: A peritubular arteriole in normal kidneys of WT (**E**) and *Bgn*^{-/-} (**F**) mice staining positive for fibrillin-1 in the lamina elastica interna (arrowhead), externa (arrow), and in the media (m). No differences were observed in the intensity and staining pattern between WT and *Bgn*^{-/-} mice. **G** and **H**: Immunostaining for fibrillin-1 in a ligated kidney from WT (**G**) and *Bgn*^{-/-} (**H**) mice labeling the lamina elastica interna (arrowhead), externa (arrow), and the adventitia (a). Weak positivity was also present in periphery of vascular smooth muscles (small double arrows). No differences were observed in the intensity and staining pattern between WT and *Bgn*^{-/-} mice. Western blots for fibrillin-1 (**I**) and fibrillin-2 (**J**) were performed using homogenates of whole ligated and contralateral (denoted as "C" in **I** and **J**) kidneys from WT and *Bgn*^{-/-} mice. Semiquantification of Western blots for fibrillin-1 and -2 in ligated kidneys from WT and *Bgn*^{-/-} mice (**K**). Data are given as means of the OD calculated per whole kidney. The asterisk indicates statistical differences, *n* = 3, *P* < 0.05. **L** and **M**: Hart's staining for elastin showing no differences in the localization and expression of elastin in ligated kidneys from WT (**L**) and *Bgn*^{-/-} (**M**) mice.

stitial matrix (Figure 4, C and D). However, after day 35, staining for fibrillin-1 in obstructed kidneys from *Bgn*⁻⁰ (Figure 4D), while still being more intensive than in unligated kidneys, was reduced as compared to ligated WT kidneys (Figure 4C). This relative reduction in the intensity of staining for fibrillin-1 in obstructed kidneys from *Bgn*⁻⁰ mice was accompanied by progressive dilations of Bowman's space and tubular lumina and affected Bowman's capsule, TBM, and the peritubular interstitial matrix (Figure 4D). Arteries of normal kidneys of WT (Figure 4E) and *Bgn*⁻⁰ (Figure 4F) mice and of the respective ligated kidneys (WT, Figure 4G; *Bgn*⁻⁰, Figure 4H) stained positive for fibrillin-1 in the lamina elastica interna, externa, and in the media as well as in the adventitia. Weak positivity was also present in the periphery of vascular smooth muscles (Figure 4, G and H). However, there were no differences in the intensity and/or staining pattern for fibrillin-1 in renal blood vessels between contralateral or ligated kidneys from WT and *Bgn*⁻⁰ mice. These observations suggest that the relative reduction of fibrillin-1 in ligated kidneys from *Bgn*⁻⁰ mice occurred primarily in the tubulointerstitium (the renal compartment that is predominantly exposed to the increased intraluminal pressure in UUO) and Bowman's capsule and did not involve the renal vasculature.

To quantify the amount of fibrillin-1 deposited in obstructed kidneys, fibrillin-1 was extracted from whole kidney homogenates and was determined by Western blot analysis. The amount of fibrillin-1 in ligated kidneys from *Bgn*⁻⁰ mice was more than threefold lower compared to obstructed kidneys from WT animals (Figure 4, I and K; data shown at day 35 after UUO). Similar results were obtained when the amount of fibrillin-1 was recalculated either per mg of protein or per OD of β -tubulin (data not shown). Because the amount of fibrillin-1 in contralateral kidneys (Figure 4I, labeled as "C") was much lower compared to ligated kidneys, a double volume of tissue homogenates from unligated kidneys had to be used to quantify Western blots. Quantification of Western blots showed that there were no differences in the fibrillin-1 contents of contralateral kidneys from WT and *Bgn*⁻⁰ mice (WT, $0.92 \pm 0.19 \text{ OD} \times 10^6/\text{contralateral kidney}$ versus *Bgn*⁻⁰, $0.86 \pm 0.14 \text{ OD} \times 10^6/\text{contralateral kidney}$, $n = 5$; $P > 0.05$).

The observed differences were specific for fibrillin-1, because fibrillin-2, being markedly up-regulated in ligated kidneys, did not reveal any differences between obstructed kidneys from WT and *Bgn*⁻⁰ mice. There was no genotype-specific difference in the amount of fibrillin-2 in nonligated kidneys as well. These results were confirmed by Western blot analysis (Figure 4, J and K) and immunohistochemistry (not shown).

Semiquantification of elastin by Hart's staining did not reveal any differences between obstructed kidneys from WT and *Bgn*⁻⁰ mice (Figure 4, L and M). Comparison of the deposition of type I, II, V, and VI collagens (binding partners of biglycan) either by immunostaining or after extensive pepsin digestion of whole minced kidneys followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed only a slight increase in the

amount of type VI collagen in obstructed kidneys from *Bgn*-deficient animals (data not shown).

Compensatory Enhancement of Decorin Deposition in Ligated Kidneys from Bgn-Deficient Mice

We also investigated the mechanism by which fibrillin-1 was up-regulated in ligated kidneys and why this up-regulation was less pronounced in obstructed kidneys from *Bgn*-deficient mice. Our previous studies in UUO from WT and *Dcn*^{-/-} mice provided evidence that biglycan mRNA and protein are overexpressed in ligated kidneys for up to 70 days with a peak at day 7 after ligation.¹⁰ In analogy with these findings, UUO in WT mice (C57/BL6) resulted in the most pronounced up-regulation of biglycan mRNA at day 7 after ligation ($4.2 \pm 0.8\text{-fold}$; $n = 4$; $P < 0.05$; quantification of Northern blots) and persisted to a lesser degree throughout the whole experimental period (up to 70 days). The expression pattern of biglycan mRNA and protein detected by *in situ* hybridization and immunohistochemistry was identical in WT (C57/BL6) kidneys to that previously observed,¹⁰ suggesting that biglycan might be responsible for the up-regulation of fibrillin-1 in UUO.

In mice lacking individual SLRP genes partial compensation by related members of the SLRP family has been reported.^{10,25,36,37} We compared, therefore, the expression of decorin, lumican, and fibromodulin in whole kidneys before and after ligation. In analogy with previous studies¹⁰ decorin was up-regulated $5.3 \pm 0.7\text{-fold}$ ($n = 4$; $P < 0.05$; quantification of Northern blots) in ligated kidneys from WT mice at day 7 after UUO compared to the respective contralateral kidneys. However, the absence of biglycan resulted only in a slight enhancement of decorin mRNA in obstructed kidneys from *Bgn*-deficient mice ($6.1 \pm 0.9\text{-fold}$ more than the contralateral kidney; $n = 4$; $P < 0.05$; quantification of Northern blots) in comparison to ligated WT kidneys. Decorin mRNA remained up-regulated throughout the experiment (up to 70 days). The overexpression of decorin mRNA in ligated kidneys from WT mice was followed by a strong accumulation of the respective protein mainly in the peritubular matrix (Figure 5A). Surprisingly, the deposition of decorin protein in obstructed kidneys from *Bgn*-deficient mice was much higher compared to the kidneys from WT animals from day 4 up to 35 days of UUO (Figure 5B). For the quantification of renal decorin from WT and *Bgn*⁻⁰ mice the proteoglycan was semipurified from whole obstructed and contralateral kidneys. After digestion of the glycosaminoglycan chains the decorin core protein was quantified by Western blot analysis (Figure 5C). There was a $3.4 \pm 0.6\text{-fold}$ higher accumulation of decorin core protein in ligated kidneys from *Bgn*⁻⁰ mice as compared to ligated kidneys from WT animals ($n = 3$; $P < 0.05$). Fibromodulin and lumican were unchanged during the course of UUO (not shown). These data suggest that decorin is involved in the up-regulation of fibrillin-1 in obstructed kidneys from WT animals. Despite considerable deposition in *Bgn*⁻⁰ mice, decorin was not capable

Figure 5. Compensatory overexpression of decorin in ligated kidneys from *Bgn*⁻⁰ mice after 7 days of UUO. Immunostaining for decorin in ligated kidneys from WT (A) and *Bgn*⁻⁰ (B) mice shown at day 7 of UUO (peak of decorin expression). C: Western blot for decorin after semipurification from whole ligated (L) and contralateral (C) kidneys with subsequent digestion of the side chain. Decorin standard (St. DCN) represents 500 ng of decorin core protein.

to compensate for the lack of biglycan with respect to fibrillin-1 expression.

SLRPs Mediated Overexpression of Fibrillin-1 in Renal Fibroblasts and MCs

To investigate whether biglycan and decorin are involved in the regulation of fibrillin-1 expression, we performed *in vitro* experiments using renal fibroblasts (NRK) and MCs, which both produce fibrillin-1.^{18,27} Quiescent NRK fibroblasts and MCs were incubated with recombinant human biglycan or decorin, both purified from stably transfected 293 HEK cells, for 1 to 24 hours under serum-free conditions. Recombinant human SLRPs were used because of 1) the fact that the core proteins of biglycan and decorin are highly conserved across species,¹⁻³ with homologies of 89% and 81% between human and rat biglycan and decorin, respectively; 2) previously reported biological effects of human decorin and biglycan in the rat system both *in vitro* and *in vivo*;^{7,11,31} 3) availability of a well-established procedure for the production and purification of human decorin and biglycan.^{28,29}

After 2 hours of incubation of NRK fibroblasts with biglycan or decorin, there was an approximately twofold increase in fibrillin-1 mRNA (Figure 6A). The enhanced expression of fibrillin-1 mRNA returned to baseline within

6 hours. Enhanced amounts of fibrillin-1 were detected by Western blotting in culture media from NRK incubated with biglycan or decorin after 6 hours (Figure 6B), reaching a twofold increase at 24 hours over cells incubated without the proteoglycans (Figure 6C). In MCs the stimulatory effects of biglycan and decorin on the expression of fibrillin-1 mRNA were observed within 6 hours, returning to baseline after 12 hours (Figure 6D). The amount of secreted fibrillin-1 protein into the culture medium was increased between 6 to 24 hours (Figure 6, E and F), but to a lower degree than in the NRK (Figure 6, B and C). This same pattern of fibrillin-1 accumulation in the culture media from NRK fibroblasts and MCs was detected when the amount of fibrillin-1 was normalized by protein content of cultured cells (Figure 6, C and F) or by β -tubulin in the homogenate of cells as well as by the number of cells. Enhanced amounts of fibrillin-1 protein were detected by Western blot analysis in homogenates of NRK and MCs after incubation with SLRPs as well (not shown). This suggests that increased amounts of fibrillin-1 in culture media after incubation with biglycan and decorin were in fact because of higher synthesis rather than increased secretion of fibrillin-1. Moreover, the effects of biglycan and decorin were specific for fibrillin-1 because neither fibrillin-2 mRNA nor protein was affected by these proteoglycans in both cell types (data not shown).

Cystic Dilatation of Bowman's Capsular Space and Reduced Expression of Fibrillin-1 in Ligated Kidneys from *Dcn*-Deficient Mice (150 Days of UUO)

Based on the observation that both biglycan and decorin induce up-regulation of fibrillin-1 in renal cells, we hypothesized that lower expression of fibrillin-1 and subsequent loss of mechanical stability of the renal tissue could also occur in *Dcn*^{-/-} animals. However, previous studies for up to 70 days after ureteral ligation in *Dcn*^{-/-} mice did neither reveal any cystic dilatations of Bowman's space nor tubular cysts or hemorrhages into the renal pelvis.¹⁰ Immunostaining of ligated kidneys from WT and *Dcn*^{-/-} mice did not show any genotype-specific differences in the amount of fibrillin-1 between 4 to 70 days after UUO (Figure 7A). After 150 days, however, 33% of the ligated kidneys from *Dcn*^{-/-} mice displayed cystic dilatations of Bowman's capsular space and lower expression of fibrillin-1 (Figure 7B). In contrast, such changes did not occur in obstructed kidneys from WT animals (Figure 7B; *n* = 6 in each group). These data suggest that the observed overexpression of biglycan in obstructed kidneys from *Dcn*-deficient animals might substitute for decorin with respect to regulation of fibrillin-1 expression.

Discussion

The results of this study show that biglycan and decorin are capable of inducing expression of fibrillin-1 in renal cells both *in vitro* and *in vivo*. The biological importance of

Figure 6. Effects of recombinant biglycan (BGN) and decorin (DCN) on the expression of fibrillin-1 in rat kidney fibroblasts (A–C) and MCs (D–F). The relative changes of mRNA expression of fibrillin-1 in renal fibroblasts (A) and MCs (D), cultured in the presence and absence of 4 μg/ml of biglycan or decorin and quantitated by Northern blotting of three samples in each group after normalization to GAPDH (given as means ± SEM). B shows one example of a Western blot for fibrillin-1 in culture media from renal fibroblasts and MCs in E (C denotes control cells without exogenous SLRPs). Quantification of Western blots for fibrillin-1 in culture media from renal fibroblasts (C) and MCs (F) given as OD after normalization to the protein content in cell homogenates for three samples in each group (means ± SEM). Open bars represent control cells (cultured without recombinant SLRPs), gray bars represent cells cultured with biglycan, and black bars represent cells incubated with decorin. The asterisks indicate statistical differences between control cells and cells cultured with SLRPs, $P < 0.05$.

this regulation was clearly demonstrated *in vivo* in which pressure-induced injury of the kidney by UUO caused overexpression of decorin, biglycan, fibrillin-1, and fibrillin-2 in WT mice. Moreover, the absence of biglycan led to reduced up-regulation of fibrillin-1 in the obstructed

kidney. This was associated with more pronounced dilations of tubules and thickening of TBMs and Bowman's capsule. At later time points, cystic dilatation of Bowman's capsule and proximal tubules became apparent, the latter being associated in *Bgn*^{0/0} mice with abnormal-

Figure 7. Immunostaining for fibrillin-1 in ligated kidneys from WT and *Dcn*^{-/-} mice 35 (A) and 150 (B) days after UUO. There were no differences in the expression of fibrillin-1 in obstructed kidneys from WT and *Dcn*^{-/-} mice up to 150 days after UUO, shown in A at day 35 as an example. B: After 150 days, lower expression of fibrillin-1 was detected in ligated kidneys from *Dcn*^{-/-} animals and cystic dilatation of Bowman's capsular space of some glomeruli was observed. C: A negative control for the immunostaining of fibrillin-1 by omitting the primary antibody.

ities of TBMs. Thus, the lack of biglycan with a subsequently reduced up-regulation of fibrillin-1 resulted in severe structural alterations of the renal parenchyma during the course of UUO. It is tempting to speculate that the lack of biglycan, which is usually associated with tubular epithelial cells^{10,14,31} and is overexpressed in tubular epithelial cells and accumulates in the tubulointerstitium already at early stages after ureter ligation,¹⁰ might be the reason for the enhanced dilatation of tubules. Similar changes were observed previously in ligated kidneys from *decorin*-deficient mice.¹⁰ The thickening of Bowman's capsule and of the TBM, observed at later stages, is probably because of compensatory mechanisms after obstruction of the ureter. Not only biglycan but also fibrillin-1 was markedly up-regulated in Bowman's capsule and in the peritubular space in WT-ligated kidneys. The lack of biglycan with a subsequently reduced expression of fibrillin-1 obviously seems to shut down an important compensatory mechanism, leading to severe structural damage of the kidney after unilateral obstruction.

Mutations in the gene encoding fibrillin-1 are found in Marfan syndrome and in a range of other connective tissue disorders collectively termed fibrillinopathies, which are associated with aneurysms and/or hemorrhaging.^{38,39} Moreover, aneurysms and enhanced tissue fragility do also occur in Ehlers-Danlos syndrome that to some extent is mimicked by the phenotype of *Bgn*-0 mice.^{40,41} In both syndromes the occurrence of renal cysts has been reported,^{42,43} which might be because of additional abnormalities in connective tissue based on the coincidence of other gene mutations. In addition, altered expression of decorin and biglycan has been described in patients with Marfan syndrome, suggesting a molecular relationship between these proteoglycans and fibrillin-1.^{44–46}

In keeping with this notion, 33% of obstructed kidneys from *Dcn*-/- mice displayed dilatation of Bowman's capsule and a less pronounced up-regulation of fibrillin-1 as compared to the WT after 150 days after UUO. The late appearance and low incidence of renal abnormalities could be explained by a compensatory up-regulation of biglycan in *Dcn*-/- mice¹⁰ that might have stimulated expression of fibrillin-1, thereby improving the biomechanical properties of tubules and Bowman's capsule in the obstructed kidney. In mice with ablated individual SLRP genes the possibility of a partial compensation by related SLRP has been raised before.^{4,10,36,37} However, a compensation for biglycan by decorin has not been shown previously. In *Bgn*-deficient mice after UUO we found enhanced accumulation of decorin, which, however, was not capable of stimulating fibrillin-1 expression and thereby preserving the integrity of the renal parenchyma in ureteral obstruction. This might have been because of different levels of expression or different temporal and/or spatial distributions of biglycan and decorin in the renal parenchyma.^{13,14,31}

This study confirms previous findings that fibrillin-1 and fibrillin-2, despite a high degree of structural homology, have different expression patterns in the kidney.^{20,21} The presence of fibrillin-1 and fibrillin-2 in the mesangium of the adult kidney as well as of fibrillin-1 in the TBM has

been shown previously,^{20,27} whereas we demonstrate additional expression of fibrillin-1 in Bowman's capsule. The biological relevance of this observation is highlighted by the fact that fibrillin-1 is overexpressed in Bowman's capsule during UUO. Moreover, when the up-regulation of fibrillin-1 was not sufficient, cystic dilatation of the Bowman's capsule occurred, suggesting an important contribution of fibrillin-1 to the biomechanical function of Bowman's capsule under circumstances of increased intracapsular pressure. Fibrillin-2, on the other hand, was detectable only in trace amounts in Bowman's capsule and in the peritubular space. This might explain that up to now the presence of fibrillin-2 at this particular localization in the adult kidney has not been described, even though it has been observed during renal development.^{20,21} After renal obstruction, fibrillin-2 expression was enhanced as well. However, despite considerable overexpression fibrillin-2 was not capable of compensating for fibrillin-1 in obstructed kidney from *Bgn*-0 mice.

Our findings for the first time provide evidence for a biglycan- and decorin-dependent regulation of fibrillin-1 both *in vitro* and *in vivo*. MCs and interstitial fibroblasts, both known to produce fibrillins, decorin, and biglycan, were chosen for their pathophysiological importance in the evolution of glomerular and interstitial disease.^{20,27,31} In both types of cells decorin and biglycan specifically induced fibrillin-1 mRNA and protein, without affecting the expression of fibrillin-2. Interestingly, a number of studies have shown that decorin expression is down-regulated in individuals with Marfan syndrome originating in a fibrillin-1 defect.^{44–46} These observations together with the results presented here suggest reciprocal mechanisms that regulate the expression of decorin and fibrillins. Despite the complexity of *in vivo* systems, deficiency of *Bgn* or *Dcn* clearly impacted on expression of fibrillin-1 in the obstructed kidney. At present, not much is known about the signaling pathways involved in the regulation of fibrillins. Recently, relaxin has been reported to regulate the expression of fibrillin-2 without affecting fibrillin-1.⁴⁷ Insulin-like growth factor-I and TGF- β have been shown to regulate fibrillin-1 expression among other factors in MCs.^{27,48} These observations support our present findings that, at least in the kidney, both fibrillins are differentially regulated.

The biological role of fibrillins in kidney disease is still not well defined. The present report demonstrates that fibrillin-1 considerably contributes to the biomechanical properties of Bowman's capsule and tubules in a model of obstructive nephropathy. Hartner and colleagues^{49,50} stressed the role of fibrillin-1 in regulation of MC attachment and proliferation in hypertensive and glomerular diseases. Because the mechanisms by which fibrillin-1 is involved in these processes is unknown, it is tempting to speculate that the interaction between fibrillin-1, TGF- β , and SLRPs might be biologically important. Thus, TGF- β -mediated stimulation of SLRPs^{7,10} would cause up-regulation of fibrillin-1, which because of its binding to latent TGF- β -binding protein-1 might sequester TGF- β thereby controlling activation of this cytokine.^{23,24} This negative feed-back loop might constitute a further mech-

anism by which SLRPs are involved in the regulation of TGF- β activity.

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