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Biglycan, a Nitric Oxide-regulated Gene, Affects Adhesion, Growth, and Survival of Mesangial Cells*

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During glomerular inflammation mesangial cells are the major source and target of nitric oxide that profoundly influences proliferation, adhesion, and death of mesangial cells. The effect of nitric oxide on the mRNA expression pattern of cultured rat mesangial cells was therefore investigated by RNA-arbitrarily-primed polymerase chain reaction. Employing this approach, biglycan expression turned out to be down-regulated time- and dose-dependently either by interleukin-1β-stimulated endogenous nitric oxide production or by direct application of the exogenous nitric oxide donor, diethylenetriamine nitric oxide. There was a corresponding decline in the rate of biglycan biosynthesis and in the steady state level of this proteoglycan. *In vivo*, in a model of mesangioproliferative glomerulonephritis upregulation of inducible nitric-oxide synthase mRNA was associated with reduced expression of biglycan in isolated glomeruli. Biglycan expression could be normalized, both *in vitro* and *in vivo*, by using a specific inhibitor of the inducible nitric-oxide synthase, l-N^6-(l-iminoethyl)-l-lysine dihydrochloride. Further studies showed that biglycan inhibited cell adhesion on type I collagen and fibronectin because of its binding to these substrates. More importantly, biglycan protected mesangial cells from apoptosis by decreasing caspase-3 activity, and it counteracted the proliferative effects of platelet-derived growth factor-BB. These findings indicate a signaling role of biglycan and describe a novel pathomechanism by which nitric oxide modulates the course of renal glomerular disease through regulation of biglycan expression.

Biglycan (BGN)[1] belongs to the family of small, leucine-rich repeat glycoproteins/proteoglycans (SLRPs), which are characterized by core proteins with centrally located leucine-rich repeat motifs flanked by cysteine clusters. Near the *N*-terminal end BGN carries two (or sometimes only one) chondroitin/dermatan sulfate chain(s). Despite its abundance in different tissues, the precise biological role of BGN is still a matter of debate. SLRPs are primarily considered to play a role as organizers of extracellular matrices. BGN, decorin, and other members of the family of SLRPs interact with fibrillar collagens, thereby modulating fibril formation and stability (reviewed in Refs. 1–3). Targeted disruption of the BGN gene resulted in abnormal collagen fibril morphology (4) and in an osteoporosis-like phenotype (5), possibly because of defects in bone marrow stromal cells (6). Additionally, BGN, as well as decorin, modulates adhesion of cells to matrix glycoproteins like fibronectin and thrombospondin (7, 8). Interactions with cell membrane components have also to be taken into account, because BGN is most abundant near the cell surface (9). Several SLRPs were shown to form complexes with TGF-β (10), which in the case of decorin affects the biological activity of the cytokine (11, 12). No such properties have been reported so far for BGN·TGF-β complexes (12).

It is becoming increasingly clear that, in addition to their interaction with TGF-β, small proteoglycans are also directly involved in cell signaling (3). Decorin has been shown to interact with members of the ErbB receptor family (13), which leads to the induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ (14) and growth arrest of certain tumor cells. In endothelial cells (15), renal tubular epithelial cells (16), and macrophages (17) decorin protects against apoptosis and induces $p27^{KIP1}$. BGN seems to be required for endothelial cell migration (18). It may also affect signal transduction during growth and differentiation via induction of $p27^{KIP1}$ (19). Further signaling functions may be deduced from the capability of BGN to interact via its glycosaminoglycan chains with dystroglycan (20). BGN has been shown to stimulate growth and differentiation of monocytic lineage cells from various lymphatic organs (21).

In the normal kidney BGN is found primarily in the tubulointerstitium. The normal glomerulus contains trace amounts of BGN produced by mesangial and endothelial cells, as well as podocytes (22). In contrast, in advanced stages of glomerulo-

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[1] The abbreviations used are: BGN, biglycan; NO, nitric oxide; DETA-NO, diethylenetriamine-NO; FACS, fluorescence-activated cell scanning; IL-1β, interleukin-1β; iNOS, inducible NO synthase; MC, mesangial cell; l-NIL, l-N^6-(l-iminoethyl)-l-lysine dihydrochloride; l-NMMA, N^G-monomethyl-l-arginine; PDGF-BB, platelet-derived growth factor-BB; RAP-PCR, RNA-arbitrarily-primed PCR; SLRPs, small leucine-rich proteoglycans; SNAP, *S*-nitroso-*N*-acetyl-*dl*-penicillamine; TGF-β, transforming growth factor-β; TNFα, tumor necrosis factor-α; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GN, glomerulonephritis; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling; ELISA, enzyme-linked immunosorbent assay.

sclerosis high amounts of BGN are deposited in the mesangial matrix (23, 24). In obstructive nephropathy BGN becomes upregulated, an effect that is dramatically enhanced in decorin-deficient mice. This increase is primarily because of the appearance of BGN-expressing macrophages (16). Taken together, these data suggest a regulatory role of BGN during the development of renal diseases (23, 24).

Nitric oxide, either produced in physiological amounts by endothelial cells and macrophages or overproduced by the inducible isoform of NO synthase (iNOS), has been shown to be an important regulatory factor in a number of inflammatory diseases (reviewed in Refs. 25 and 26). In the kidney, NO triggers the expression of proinflammatory and protective gene products in various types of glomerulonephritis (27, 28). Besides infiltrating cells, renal mesangial cells (MCs), exposed to inflammatory cytokines such as interleukin-1β (IL-1β) or tumor necrosis factor-α (TNFα), start to express iNOS followed by enhanced generation of NO (29). NO exerts complex regulatory actions on proliferation (30, 31), adhesion (32), and death of MCs (33, 34).

Here we show for the first time, using PCR-based analysis of differential mRNA expression patterns of MCs exposed to exogenously or endogenously produced NO, that BGN is an NO-regulated gene in MCs both *in vitro* and *in vivo* and that it is involved in the modulation of the extent of adhesion, proliferation, and survival of MCs.

MATERIALS AND METHODS

Reagents—Radiochemicals and a Recliprime DNA Labeling System were obtained from Amersham Biosciences. Nylon blotting membranes were from Schleicher & Schüll or Millipore. Tissue culture plastic was from Falcon (BD Biosciences), and media and sera were from Invitrogen. The NO donors DETA-NO and SNAP, as well as the inhibitors of iNOS, l-NMMA and l-NIL, were from Alexis (Grünberg, Germany). Chemicals for reverse transcriptase-PCR were obtained from Stratagene. All other chemicals were purchased from Sigma.

Cell Culture and Stimulation—Rat MCs were cultured as described previously (35). They were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 5 ng/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin for eight to 19 passages. To obtain quiescent cells, MCs were maintained in serum-free Dulbecco's modified Eagle's medium supplemented with 0.1 mg/ml fatty acid-free bovine serum albumin for 24 h prior to the addition of buffer, DETA-NO (0.063–1 mM), l-NIL (0.3–3 mM), or IL-1β (2 nM). Viability of MCs was not altered under the conditions used for the experiments, as determined by lactate dehydrogenase release into the culture medium using a cytotoxicity detection kit (Roche Applied Science).

Analysis of the mRNA Expression Pattern in MCs by RAP-PCR—In the present work BGN was identified as a nitric oxide-regulated gene in a set of experiments performed analogously as in a previous study on NO-mediated regulation of macrophage inflammatory protein 2, using the same conditions and primers (36). Briefly, mRNA from MCs was prepared using an mRNA isolation kit (Stratagene) followed by the low stringency RAP-PCR protocol provided by the manufacturer and using [α-^{33}P]dCTP as radioactive precursor. As an internal control the reverse transcription step was performed additionally using the primer G5 that matched with the reverse strand of rat GAPDH cDNA at position 705–724 (GenBank™ accession number M17701), whereas during the PCR steps primer G3, which matched with the coding strand of rat GAPDH cDNA at position 131–150, was also present. The PCR was performed for one cycle at an annealing temperature of 35 °C and for 40 further cycles at 53 °C. The products were separated on a 4% sequencing gel. After an overnight exposition to x-ray film, bands of interest were excised, reamplified, and blunted using *Pfu* polymerase (Promega) according to the high stringency protocol mentioned above and sequenced. For further analysis, the PCR fragments were cloned into *Eco*RV sites of pBluescript KS$^+$ (Stratagene).

Nitrite Analysis—To verify endogenous NO production, nitrite as a stable end product of NO metabolism was measured routinely in culture media using the Griess reagent (Merck).

Rat Model of Glomerulonephritis—The anti-Thy 1-glomerulonephritis (anti-Thy 1-GN) was induced as described before (36). l-NIL, a selective inhibitor of iNOS, was administered intravenously at a dose of 5 mg/kg body weight to control and nephritic rats 45 min before and 8 h after anti-Thy 1 injection. Kidneys were harvested 16 h (n = 5 animals per group) after injection of the anti-Thy 1.1 antibody. Monitoring of systolic blood pressure and isolation of glomeruli were performed as described (36, 37).

Northern Blot Analysis and In Situ Hybridization—Total RNA was extracted from isolated glomeruli using TRIzol (Invitrogen). Northern blots were performed and analyzed as described previously (37). *In situ* hybridization of rat renal sections was performed in parallel with the sense and antisense probes for rat BGN (24, 37).

Determination of BGN in Isolated Rat Glomeruli and MC Cultures—Cell culture supernatants from stimulated (30 h) and control MCs were collected. MCs were washed three times with Hanks' solution before cell protein was extracted with 50 mM sodium acetate, pH 6.0, 4 M guanidinium chloride, 0.1% Triton X-100, and protease inhibitors (38). Five percent of the total volume of each cellular or glomerular extract was collected for Western blot analysis of β-tubulin and for the determination of protein content. After centrifugation, cell lysates (diluted to give 0.2 M guanidinium chloride as final concentration and made 7 M with respect to urea by adding solid substance), cell culture supernatants, and the appropriate standard solutions were loaded on 0.5 ml columns of DEAE-Trisacryl M (Invitrogen), prepared in Pasteur pipettes, and equilibrated with Buffer 1 (20 mM Tris/HCl, pH 7.4, containing 0.15 M NaCl, 0.1% Triton X-100, 7 M urea, and protease inhibitors) and processed as described (24). Glomerular homogenates containing equal amounts of glomeruli were mixed with DEAE-Trisacryl M (100 mg wet weight), equilibrated with Buffer 1, and mixed by rotation for 1 h at 4 °C. The samples were washed sequentially with 3 ml of Buffer 1, 3 ml of urea-free Buffer 1, and 3 ml of urea-free Buffer 1 containing 0.3 M NaCl. Elution was achieved with 1.5 ml of urea-free Buffer 1 containing 1 M NaCl. Upon adding 5 volumes of methanol and 1 volume of chloroform followed by freezing on dry ice, proteoglycans were collected after thawing at the interphase between chloroform and aqueous methanol. The upper phase was removed, and proteoglycans were precipitated to the bottom of the tube by adding again 5 volumes of methanol. The proteoglycans from cell culture supernatants, MCs, or glomerular homogenates were digested with chondroitin ABC lyase (Seikagaku Kogyo, Tokyo, Japan) to remove chondroitin sulfate and dermatan sulfate chains. BGN from plasma or urine samples was semipurified as described previously (24). According to the analysis of [^{35}S]sulfate-labeled biglycan from fibroblast secretions as an internal standard, the recovery after the ion exchange chromatography step varied by 85 ± 10%. The presence of 0.1% Triton X-100 was the critical component for achieving good recovery. Additional control experiments, yielding the expected results, were performed by adding known quantities of BGN to the culture medium prior to loading on the DEAE column.

Untreated and chondroitin ABC lyase-treated samples from MCs and their culture supernatants were subjected to SDS-PAGE followed by Western blotting (38). Plasma, urine, and glomerular samples were transferred to nitrocellulose membranes using the Bio-Dot microfiltration apparatus (Bio-Rad). The membranes were blocked with 3% casein, 1% goat serum, and 0.002% Tween 20 in 10 mM Tris/HCl, pH 7.4, 0.15 M NaCl. Western and dot blot membranes were incubated with chicken anti-rat BGN (37) or with rabbit anti-mouse BGN (LF-106) antibodies (dilution 1:500 with 10 mM Tris/HCl, pH 7.4, 0.15 M NaCl/1% bovine serum albumin) for 90 min at 37 °C, whereas the second antibody, horseradish peroxidase-coupled goat anti-rabbit (enzyme immunoassay grade; Bio-Rad) was applied for 90 min at ambient temperature. Additionally, β-tubulin content in cell extracts was quantified by Western blot analysis (rabbit anti-β-tubulin; 1:500; Santa Cruz, Biotechnology, Inc.) as a control for loading. The samples were visualized by using the ECL Western blotting reagent kit (Amersham Biosciences), and analysis was performed with IQ Solutions ImageQuant software (Amersham Biosciences).

Metabolic Labeling of MCs and Determination of Newly Synthesized BGN—Metabolic labeling of MCs was performed either with [4,5-^{3}H]leucine or with [^{35}S]sulfate. Quiescent MCs were treated in the presence and absence of DETA-NO (1.0 mM) for 24 h followed by preincubation with leucine-free Weymouth medium for 1 h (7 ml/75-cm^2 culture flask) and subsequently labeled with 40 μCi/ml [4,5-^{3}H]leucine for 5 h with and without NO donor, respectively. Metabolic labeling of MCs with [^{35}S]sulfate was performed as described for fibroblasts (38). The culture medium was supplemented with proteinase inhibitors and made 70% saturated with $(NH_4)_2SO_4$. After centrifugation, the pellet was dissolved in Buffer 1 and processed as described above. MCs were harvested in Buffer 1 and treated identically as the culture medium samples. Because an immunoprecipitating anti-rat BGN antibody was

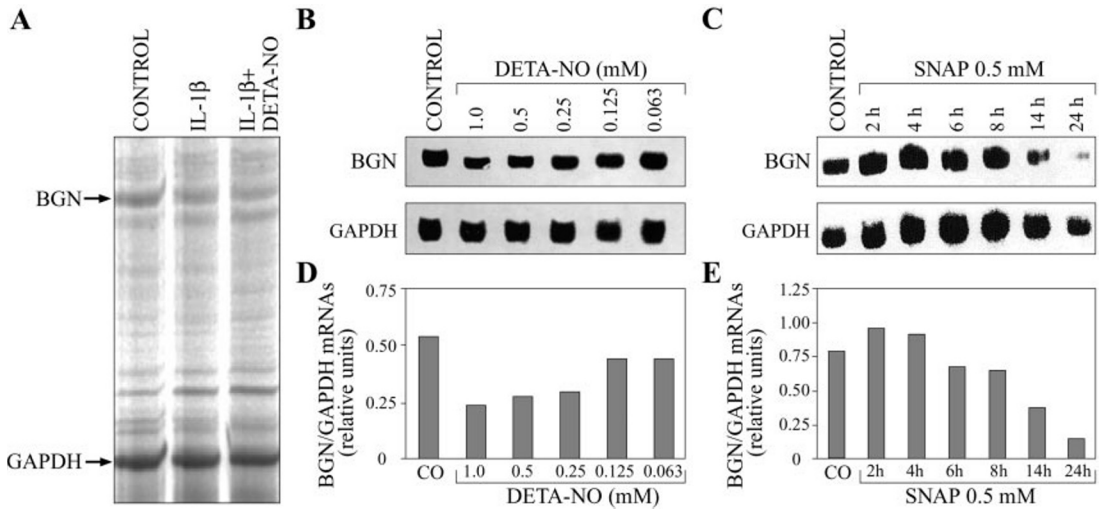

FIG. 1. **IL-1β and IL-1β + DETA-NO-induced mRNA expression.** 100 ng of isolated mRNA from quiescent MCs, pretreated as indicated, was subjected to RAP-PCR. A part of the sequencing gel is shown in *A*. BGN appeared as one of the NO-regulated genes. To analyze dose and time dependence of NO on BGN mRNA steady state levels, quiescent MCs were treated for 24 h with various concentrations of DETA-NO (*B*) or with 0.5 mM SNAP for the indicated time periods (*C*). Total RNA (20 μg each) was subjected to Northern blotting with probes specific for BGN and GAPDH. A densitometric evaluation of BGN/GAPDH values is shown in *D* and *E*, respectively. *CO*, controls.

not available, and because neither intact decorin and BGN nor their respective core proteins can reliably be separated by gel filtration, decorin was first removed by immunoprecipitation with an immobilized rabbit antibody against rat decorin (37). After immunoprecipitation, supernatants of the samples labeled with [^{35}S]sulfate were concentrated and then fractionated on a Superose 6 column (Amersham Biosciences) equilibrated with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 6.0, 1% Triton X-100, and proteinase inhibitors at a flow rate of 300 μl/min. [^{35}S]Sulfate-labeled human BGN from stably transfected 293 cells was used as a control (39). Appropriate radioactive fractions were tested by a dot blot assay with the anti-biglycan antibody (LF-106) as described above. Biglycan was eluted at a K_{av} value of 0.27. Supernatants of the samples labeled with [4,5-^{3}H]leucine were purified on a concanavalin A-Sepharose column (Fluka) and treated with chondroitin ABC lyase prior to SDS-PAGE (12.5% total acrylamide in the separation gel) and fluorography (38).

Assessment of Adhesion, Proliferation, and Survival of Cultured MCs—For determination of cell adhesion MCs were prepared using enzyme-free cell dissociation buffer (Invitrogen). Quantitative determination of adhesion was performed by using 96-well CytoMatrix cell adhesion strips (Chemicon, Hofheim, Germany) coated with fibronectin, acid-solubilized type I collagen, or bovine serum albumin, respectively. Briefly, 2×10^6 MCs/ml were seeded on the coated substrates under serum-free conditions with or without recombinant BGN (2.5–50 μg/ml) from 293 cells (39) for 1 h. Adherent cells were fixed and stained, and the relative attachment was calculated from the absorbance at 540 nm according to the manufacturer's protocol. The percentage of non-adherent MCs cultured in the absence of BGN was taken as baseline value. Additionally, 96-well culture plates, coated as described previously (7) with fibronectin peptides in concentrations equimolar to a fibronectin concentration of 10 μg/ml: F120 containing the cell-binding domain of fibronectin (FN CBD; Chemicon), F30 containing the N-terminal heparin-binding domain (FN N-term HBD; Sigma), F1977–1991 containing the C-terminal heparin-binding domain (FN C-term HBD; Sigma), or commercially coated wells with human fibronectin or type I collagen (BD Biosciences) dissolved in 7 mM acetic acid were used for the assessment of adhesion or in a solid-phase assay. The solid-phase assay was performed as described previously (40) using [^{35}S]sulfate- or [^{35}S]methionine-labeled human BGN from stably transfected 293 cells (39) in the presence or absence of heparin from porcine intestinal mucosa (Sigma) in a final concentration of 25 μg/ml. When required, the glycosaminoglycan chains of BGN were cleaved immediately before the experiment by chondroitin ABC lyase (Seikagaku Kogyo). Data are given as means of duplicates of three independent measurements in each group.

Expression of BGN on the cell surface of MCs was examined by FACS analysis using rabbit anti-human BGN (24) and affinity-purified goat anti-rabbit, fluorescein isothiocyanate-labeled antibodies (Dianova, Hamburg, Germany). In brief, MCs were incubated for 30 min at 37 °C with or without 10 μg of BGN or BGN core protein. Thereafter, cells were washed with phosphate-buffered saline/1% FCS. Cells were resuspended in 100 μl phosphate-buffered saline/1% FCS, and the primary antibody (rabbit anti-human BGN; 1:200) was added and incubated for 30 min at room temperature. Cells were washed twice, and the fluorescein isothiocyanate-conjugated secondary antibody (goat anti-rabbit; 1:200) was added for another 30 min at room temperature. Subsequently cells were washed twice and resuspended in 500 μl of phosphate-buffered saline for analysis. Cells were evaluated with a FACScalibur flow cytometer using CellQuestPro software (BD Biosciences). For flow cytometric analysis 5×10^5 MCs were used, and all experiments were performed at least three times. Additionally, MCs were cultured for 18 h, plated on 8-well fibronectin-coated chamber slides (BD Biosciences) in the presence or absence of 10 μg of BGN, and subsequently immunostained for BGN with alkaline phosphatase anti-alkaline phosphatase (24).

To assess the effect of BGN on MC proliferation, subconfluent cells (2×10^4 MCs/well) were cultured in 96-well microtiter for 24 h under serum-free conditions in the presence or absence of recombinant BGN (2.5–25 μg/ml). Alternatively, MCs were deprived of serum for 24 h and then treated simultaneously with 10% FCS and BGN (5 μg/ml) or with rat recombinant PDGF-BB (20 ng/ml; Sigma) and BGN (5 μg/ml) for 24 h. Heparitinase-treated BGN (39) and heparan sulfate (Sigma) in concentrations equivalent to 5–15% of hexouronic acid content of BGN were used in the proliferation assays as controls. Subsequently, MC proliferation was quantified by [^{3}H]thymidine incorporation using 1 μCi/ml [^{3}H]thymidine (Amersham Biosciences) (38) or by a cell proliferation assay kit (Chemicon). Data are given in arbitrary units measured as counts per min for [^{3}H]thymidine incorporation or the absorbance of each sample at 450 nm with a reference wave length at 620 nm for the cell proliferation assay kit. Results are given as means of duplicates of three independent measurements in each group.

MC death was induced in quiescent cells by 2 nM TNFα and 100 nM cycloheximide under serum-free conditions in the presence or absence of recombinant BGN (10 μg/ml) for 2–24 h (41). Four different methods were used to study MC death. Histone-associated DNA fragments were assayed by using a cell death detection enzyme-linked immunosorbent assay (Roche Applied Science). Data are given in arbitrary units measured as the extinction of each sample in duplicate at 405 nm. Alternatively, MCs plated onto 8-well chamber slides were stained with 0.4% trypan blue (Sigma). For further differentiation between apoptosis and necrosis, MCs were stained with Cy3-labeled annexin V and 6-carboxyfluorescein diacetate using the annexin V-Cy3 apoptosis detection kit (Sigma) and analyzed with a PCM 2000 Nikon confocal microscope (Düsseldorf, Germany). Vital MCs show staining only with 6-carboxyfluorescein, whereas necrotic MCs can be stained only with annexin V-Cy3. Apoptotic MCs are stained with both annexin V-Cy3 and 6-carboxyfluorescein. In addition, MCs plated onto 8-well chamber slides were assayed by TUNEL staining (Roche Applied Science) as described before (16).

To exclude binding of TNFα to BGN, which might influence the

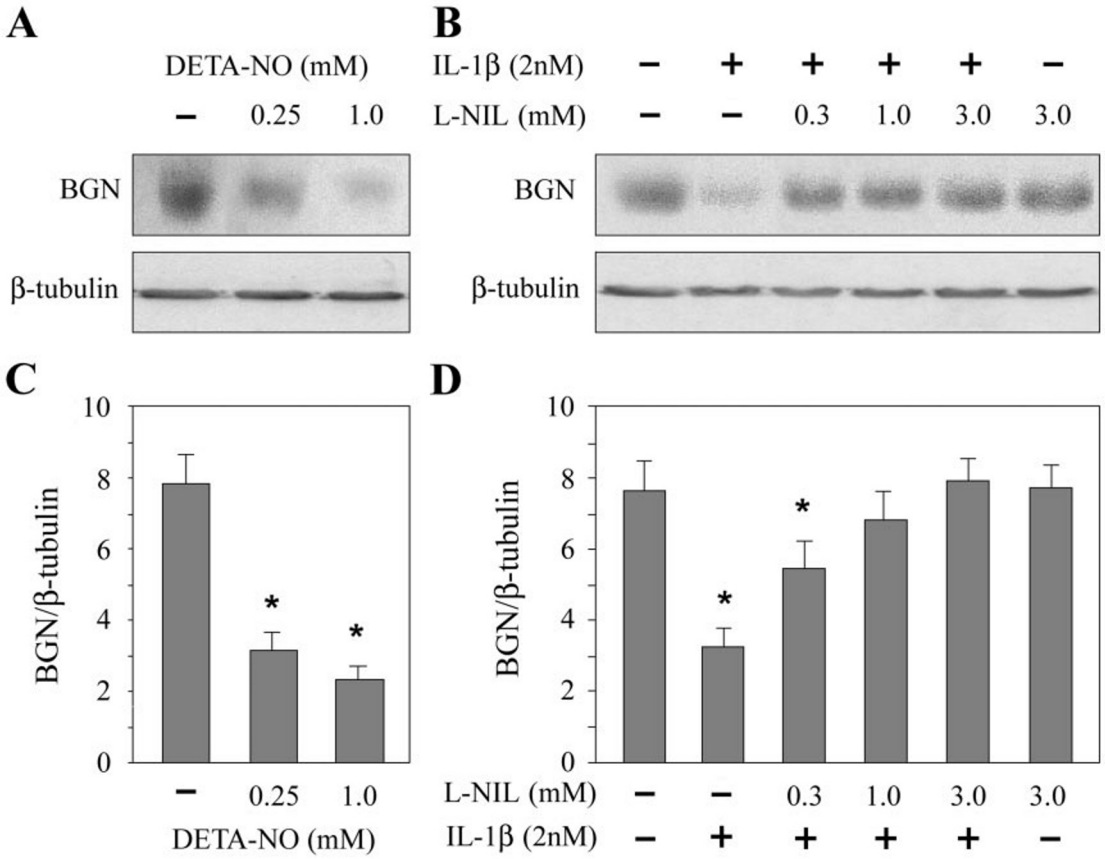

FIG. 2. Western blots for BGN in spent culture media after stimulation of exogenous (*A*) or endogenous (*B*) NO in MCs. Prior to BGN analysis quiescent MCs were treated with DETA-NO for 30 h (*A*) or with IL-1β and l-NIL (*B*). In *C* and *D* BGN was expressed as a ratio of BGN to β-tubulin (mean ± S.D.; $n = 3$; *, $p < 0.05$).

activity of the cytokine, 50 μg of BGN were immobilized on nitrocellulose membranes using a Bio-Dot microfiltration apparatus, and the membrane was blocked with 5% bovine serum albumin for 1 h at 37 °C. The membranes were incubated with 2 nM TNFα in culture medium for 2 h at 37 °C to which, after removal of the membranes, 100 nM of cycloheximide were added. Thereafter, this medium was used for the induction of MC death. Cell death was quantified after 24 h using the cell death detection ELISA mentioned above. To exclude that the effects on adhesion, proliferation, and cell survival of MCs might have been because of other active agents in the BGN preparation, the purity of the BGN preparation was monitored by SDS-PAGE and silver staining (39). Additionally, 50 μg of purified BGN was loaded on a DEAE-Trisacryl M column, and after washing the unbound material was examined for adhesion, proliferation, and cell death.

Apoptotic or necrotic cells, as well as TUNEL-positive nuclei, were evaluated by a blinded observer as percentage of adherent MCs. 400–500 cells/well were evaluated. All trypan blue-positive cells were counted per well. Mean values of three stainings per group were averaged.

Caspase-3 activity was determined in homogenates of MCs by the caspase-3/CPP32 colorimetric assay kit (BioCat, Heidelberg, Germany) as described before (16). The optical density was determined using a microplate reader at 405 nm. Data were calculated as the average of duplicates for each sample per μg of protein. Mean values of four samples per group were averaged.

Other Procedures—Protein concentrations were determined using the BCA protein assay reagent (Pierce). Expression of human BGN in 293 kidney cells and purification of the native proteoglycan were performed as described previously (39).

Statistics—Results are expressed as means ± S.E. Statistical analysis was performed by the unpaired Student's *t* test. Significance was accepted at the 5% level.

RESULTS

Identification of BGN as an NO-regulated Gene by RAP-PCR—Previously we have addressed the effects of the iNOS inhibitor l-NMMA on cytokine-induced gene expression (35, 42). In the present work we investigated whether the NO donor DETA-NO is able to modulate IL-1β-induced mRNA patterns in rat MCs. Therefore, the effects of IL-1β (2.0 nM) and IL-1β + DETA-NO (0.5 mM) were analyzed by RAP-PCR as described (36). The resulting mRNA expression pattern is displayed in Fig. 1*A*. GAPDH primers for low stringency reverse transcriptase-PCR were used to compare differential gene expression directly with the constitutively expressed GAPDH control. A band of 806 bp (Fig. 1*A*), which was considerably down-regulated by IL-1β and IL-1β + DETA-NO, was excised, reamplified, and sequenced. By searching cDNA data bases, the excised band was identified as part of the cDNA for rat or mouse BGN (GenBank™ accession numbers U17834 and X53928; nucleotides 1–793 and 99–904, respectively).

Effects of NO on BGN mRNA Levels in MCs—To verify the down-regulation of BGN mRNA steady state levels by NO, Northern hybridization experiments were performed with total RNA from MCs that had been treated with different concentrations of DETA-NO for 24 h (Fig. 1, *B* and *D*) or with SNAP (0.5 mM) for various time periods (Fig. 1, *C* and *E*). These experiments confirmed that exogenously administered NO down-regulates BGN mRNA expression in a time- and dose-dependent manner.

Effects of Exogenous and Endogenous NO on BGN Secretion by MCs—To elucidate the effect of exogenous NO on BGN secretion, MCs were stimulated with DETA-NO (0.25 and 1.0 mM) for 30 h. At both doses the amount of BGN protein in the culture supernatants was reduced compared with the untreated control (Fig. 2*A*). Stable levels of β-tubulin in the extracts from MCs in the presence or absence of DETA-NO indicated that the reduced BGN concentration in the culture medium was not because of toxic effects of the NO donor on MCs.

In the next step, endogenous NO production in MCs was stimulated by IL-1β (2.0 nM). After 30 h of incubation, the concentration of BGN in the culture medium was reduced significantly (Fig. 2, *B* and *D*). Furthermore, cytokine-induced endogenous NO production by MCs was blocked using the iNOS inhibitor l-NIL. Interestingly, l-NIL attenuated the IL-1β-induced suppression of BGN expression in a dose-dependent manner (0.3–3.0 mM), suggesting a role for endogenously produced NO in mediating the cytokine-induced reduction of BGN secretion from MCs (Fig. 2*B*). Densitometric quantification of BGN in the culture media from MCs, normalized to β-tubulin, are shown in Fig. 2, *C* and *D*, respectively. Similar results were obtained when secreted BGN was compared with the total protein content of MCs. In none of the experiments BGN was detectable in MCs themselves, neither by Western blotting nor by immunostaining (data not shown), suggesting that the proteoglycan was rapidly secreted into the culture medium and did not associate with cell surface components to a measurable extent.

Effects of Exogenous NO on de Novo Synthesis of BGN—In a further approach, cultured MCs were labeled either by using [4,5-^{3}H]leucine or [^{35}S]sulfate to determine the synthesis of BGN in the presence and absence of DETA-NO. Cells were labeled for 5 h only to exclude the possibility that the reduced amount of BGN in the supernatants from MCs incubated with the NO donor were because of enhanced endocytosis and intracellular degradation. Because the purification protocol for BGN included the immunoprecipitation of decorin, we determined also the influence of NO on the expression of the latter proteoglycan. No NO dependence was observed (data not shown).

After DCN removal, labeled BGN from the conditioned medium was purified on concanavalin A-Sepharose. Upon enzymatic removal of the glycosaminoglycan chains, SDS-PAGE, and fluorography, a 40-kDa band of the BGN core protein could be visualized (Fig. 3*A*). Incubation with 1 mM DETA-NO caused a 3-fold reduction of [^{3}H]leucine-labeled BGN core protein (control, 22 $\pm$ 3 arbitrary units *versus* DETA-NO, 7.4 $\pm$ 2.1 arbitrary units; mean $\pm$ S.D.; $n = 3$, $p < 0.05$; see Fig. 3*A*). Similar effects of the NO donor were observed when the concentration of radiolabeled BGN core protein was normalized by the protein content of cultured MCs (data not shown).

In the standard purification protocol, the decorin-free proteoglycan mixture was chromatographed on a Superose 6 column (Fig. 3*B*). A peak appearing in the V_0 was not investigated in detail, because it contained heparan sulfate and large chondroitin/dermatan sulfate proteoglycans but not BGN. The elution of BGN as the second peak (K_{av} ~0.27) from the media of both MC- and BGN-expressing 293 cells was proven by dot blot analysis with a monospecific anti-BGN antibody. The size of the proteoglycan core protein was independent of the incubation conditions. In case of long term incubation experiments, cell-associated BGN was below the limit of detection after pulse labeling (Fig. 3*A*).

Expression of iNOS and BGN mRNAs in Glomeruli from Rats with anti-Thy 1-Glomerulonephritis—Using Northern blot analysis a marked expression of iNOS could be demonstrated in isolated glomeruli 1 h after injection of the anti-Thy 1 antibody, with maximal expression occurring between 2 and 4 h. The expression of iNOS mRNA remained increased at 8 h but became undetectable 24 h after induction of the anti-Thy 1-glomerulonephritis (Fig. 4*A*). Glomerular expression of BGN mRNA was reduced between 8 and 24 h, with minimal expression 16 h after the initiation of the antibody-mediated inflammation (Fig. 4*A*). At later stages, the expression of BGN increased gradually up to 6-fold at day 7 as has been described previously (37). In contrast, glomerular expression of decorin

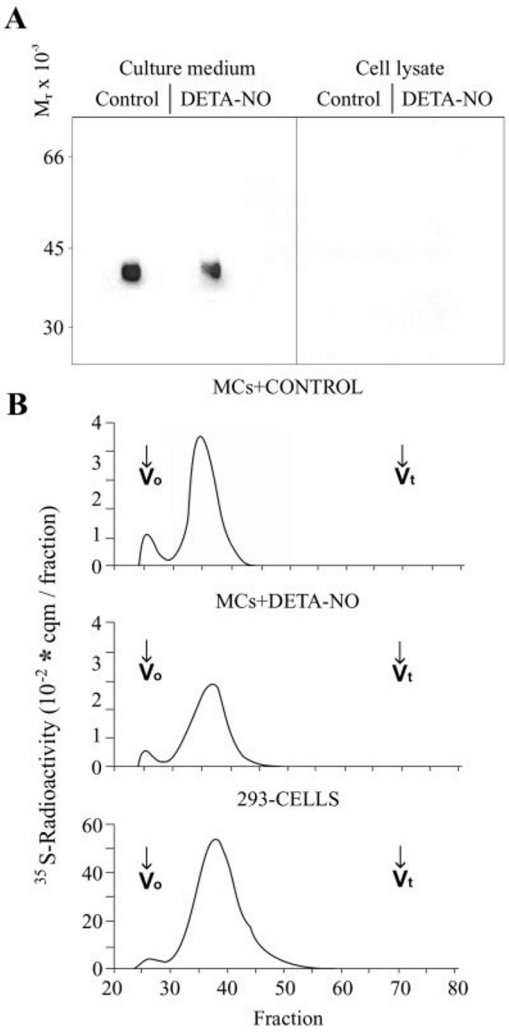

FIG. 3. **Fluorogram of BGN from MC lysates (*A*) and spent culture media (*A* and *B*) after metabolic labeling with [^{3}H]leucine (*A*) or with [^{35}S]sulfate (*B*) and SDS-PAGE.** Quiescent MCs were treated in the presence and absence of DETA-NO (1.0 mM) for 24 h, subsequently labeled with [^{3}H]leucine for 5 h with and without the NO donor, respectively, and further processed as described under "Materials and Methods." Fluorograms of BGN from the respective culture media and cell lysates are shown in *A*. [^{35}S]Sulfate-labeled proteoglycans were size-fractionated on Superose 6 (*B*). As control, [^{35}S]sulfate-labeled proteoglycans from BGN-expressing 293 cells were also subjected to gel filtration. The peak of BGN was found in fractions 38–39.

remained unchanged for up to 48 h (Fig. 4*A*), suggesting that the potential effects of iNOS were specific for BGN. Densitometric quantifications of the glomerular expression of iNOS and BGN mRNAs normalized by GAPDH are shown in Fig. 4, *B* and *C*, respectively.

To demonstrate that the reduction of BGN mRNA expression *in vivo* was directly or indirectly caused by iNOS induction, a selective inhibitor of iNOS (l-NIL) was administered intravenously to control and nephritic rats, and the effects were determined 16 h after disease induction. The reduction of glomerular BGN mRNA expression occurring 16 h after administration of the anti-Thy 1 antibody was significantly, albeit not completely, prevented by l-NIL (Fig. 5, *A* and *B*). In contrast, administration of l-NIL had no effect on the expression of BGN in control (non-nephritic) glomeruli. Systemic blood pressure was not different between both groups (data not shown).

In situ hybridization demonstrated weak expression of BGN in some MCs and endothelial cells, as well as in podocytes and

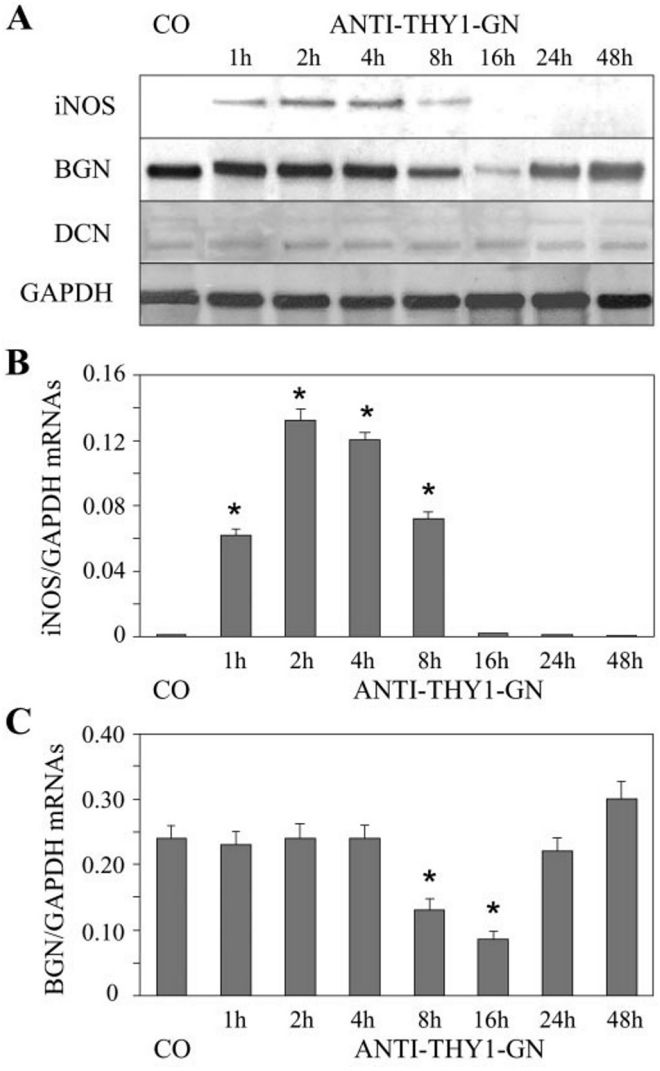

FIG. 4. **Time course of glomerular iNOS, BGN, decorin, and GAPDH mRNA expression in anti-Thy 1-glomerulonephritis.** Glomerular iNOS, BGN, decorin (*DCN*), and GAPDH mRNA levels on Northern blots were visualized (*A*) and evaluated (*B* and *C*) as described in the legend for Fig. 1. Data were expressed as quantitative ratios of iNOS or BGN to GAPDH mRNA (mean ± S.D.; $n = 3$; *, $p < 0.05$). *CO*, control.

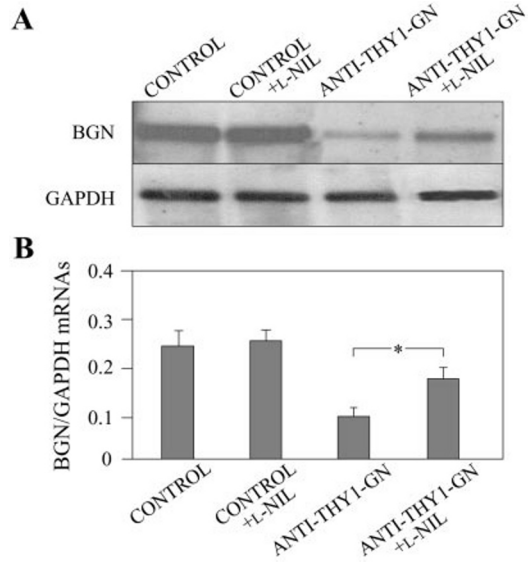

FIG. 5. **Effects of the iNOS inhibitor l-NIL on the expression of glomerular BGN mRNA in anti-Thy 1-glomerulonephritis.** Northern blots of glomerular BGN mRNA from control and anti-Thy 1-GN 16 h after induction of disease were performed (*A*) and evaluated (*B*) as described in the legend for Fig. 1 (mean ± S.D.; $n = 3$; *, $p < 0.05$).

in epithelial cells of Bowman's capsule of normal glomeruli (with and without l-NIL). Sixteen h after induction of the anti-Thy 1-glomerulonephritis, the reduction of BGN mRNA expression was observed in all of the above-mentioned glomerular cells. Administration of l-NIL prevented down-regulation of BGN mRNA only in some of the anti-Thy 1-exposed glomerular cells (Fig. 6).

Previous studies have shown that BGN is not retained in the glomerular extracellular matrix to a significant degree (24). In agreement with these observations the present results of immunological quantification of BGN core protein in glomerular extracts from control and nephritic animals (with and without l-NIL treatment) showed that BGN was not retained in the glomerular matrices from control or nephritic rats (data not shown). It is likely that most of the BGN is eliminated from the glomerulus either via the circulation and/or through glomerular ultrafiltration. However, we were not able to further test this hypothesis as plasma and urinary levels of BGN were below the limits of detection.

Effects of BGN on Adhesion, Proliferation, and Survival of Cultured MCs—The presence of BGN in concentrations of 2.5–50 μg/ml led to a dose-dependent reduction in MC adhesion, when MCs were plated under serum-free conditions on culture wells coated with human non-fibrillar type I collagen or fibronectin. Quantification of the relative attachment of MCs is shown in Fig. 7*A* using the CytoMatrix cell adhesion strips assay. Based on these findings, all further experiments were performed under serum-free conditions using BGN in a concentration of 5 μg/ml. Regarding the underlying mechanism of the antiadhesive properties of BGN, we explored whether BGN binds to type I collagen and/or fibronectin, thereby inhibiting the attachment of MCs to these substrates. In a solid-phase assay binding of [^{35}S]methionine-labeled BGN was detected on both type I collagen and fibronectin substrates. In terms of fibronectin, BGN was capable of binding to the intact molecule, the cell-binding domain, and to both N- and C-terminal heparin-binding domains (Fig. 7*B*, *white bars*). Using glycosaminoglycan-free BGN core protein, produced by incubation with chondroitin ABC lyase immediately before starting the binding studies, we found a significant enhancement of BGN binding to all of the substrates examined (Fig. 7*B*, *gray bars*) as compared with intact BGN (Fig. 7*B*, *white bars*). Coincubation with 25 μg/ml of heparin had no effect on the binding properties of BGN core protein to any of substrates used (Fig. 7*B*, *striped bars*). Similarly, [^{35}S]sulfate-labeled BGN was capable to bind to all tested substrates (Fig. 7*C*, *white bars*), and heparin had no effects on BGN binding (Fig. 7*C*, *black bars*). Radiolabeled BGN-derived glycosaminoglycan chains alone were not capable of binding to any of tested substrates (data not shown).

In the next step we addressed the issue of whether BGN might bind to the cell surface of MCs and thereby exert additional antiadhesive effects. Immunostaining of MCs for BGN grown on fibronectin-coated culture slides with or without exogenous BGN demonstrated the presence of endogenous, as well as exogenous, BGN mainly in the cytoplasm of MCs in a perinuclear localization (Fig. 8*A*). In the presence of exogenous BGN staining was also observed on the fibronectin stratum (Fig. 8*A*, *lower panel*). Occasionally, very weak immunostaining for BGN was detected on the cell surface of MCs (Fig. 8*A*). To quantify the binding of BGN to the cell surface of MCs, FACS analysis was performed. Based on this analysis, endogenous BGN was found on the cell surface of 15% (15.1 ± 6.9%;

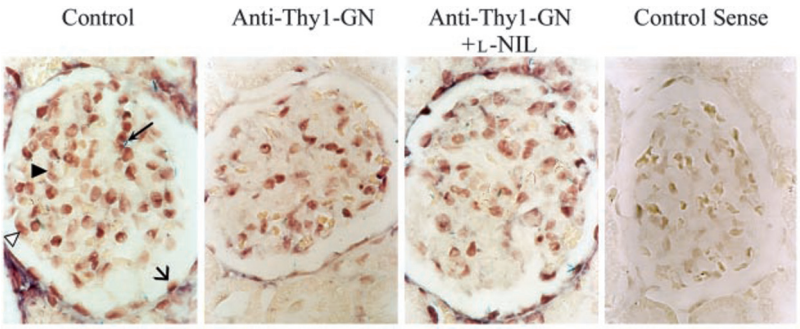

FIG. 6. **Effects of l-NIL on the glomerular expression of BGN in control and anti-Thy 1-glomerulonephritis rats shown by *in situ* hybridization (×1000).** Shown are *In situ* hybridizations with the antisense and sense probes for BGN in renal sections from control and nephritic rats (without or with l-NIL) 16 h after induction of anti-Thy 1-GN. The *long arrow* indicates an MC, the *filled arrowhead* points to an endothelial cell, the *open arrowhead* points to a podocyte, and an epithelial cell of Bowman's capsule is denoted by the *short arrow*.

$n = 3$) of MCs examined (Fig. 8*B*, *upper panel*). Incubation with 10 μg/ml exogenous BGN raised the number of BGN-positive MCs by about 7% (21.1 ± 0.6%; $n = 3$) (Fig. 8*B*, *middle panel*). Cleavage of the glycosaminoglycan chains of BGN enhanced the number of BGN-positive MCs by a further 9% (30.8 ± 2.9%; $n = 3$) (Fig. 8*B*, *lower panel*). These observations strongly implicate that the antiadhesive properties of BGN are mainly because of binding of BGN to matrix components. BGN not only affected initial adhesion of MCs but also reduced their capability to spread properly. This held true either when BGN was added before adhesion had occurred (Fig. 8*A*, *lower panel*) or when MCs were first cultured without BGN for 1 h to allow for proper adhesion and were then incubated with BGN for 18 h (data not shown).

Furthermore, we examined the potential effects of BGN on MC proliferation. *In vivo* under physiological conditions MCs are quiescent, and only during glomerular disease do these cells start to proliferate. Therefore, subconfluent MCs were made quiescent by serum deprivation for 24 h and then treated simultaneously with 10% FCS and 5 μg/ml BGN for 24 h. In pre-studies BGN counteracted the proliferative effect of serum on MCs in a dose-dependent manner at concentrations of 2.5–25 μg/ml. For all further studies concerning antiproliferative effects of BGN a concentration of 5 μg/ml was chosen. Quiescent MCs showed some low level incorporation of [^{3}H]thymidine even in the absence of mitogenic stimuli, which was probably because of some low rate proliferation. In resting MCs proliferation could not be induced by BGN (Table I). As expected, FCS stimulated proliferation of MCs after 24 h as measured by two independent assays based either on direct incorporation of [^{3}H]thymidine or on the activity of mitochondrial dehydrogenases (Table I). Coincubation with FCS and BGN significantly reduced the proliferation rate of MCs (Table I). To evaluate the biological relevance of this finding, we stimulated the proliferation of MCs with PDGF-BB, a major mitogenic growth factor that is frequently up-regulated in glomerular disease and that is considered to be responsible for MC proliferation *in vivo* (43). Incubation with 20 ng/ml of PDGF-BB resulted in marked proliferation of MCs, which was significantly reduced in the presence of BGN (Table I). Because BGN secreted by 293 cells has been found to contain some heparan sulfate incorporated into the glycosaminoglycan side chains (39), which might be responsible for its antiproliferative effects, we also examined BGN treated by heparitinase. Using this particular BGN preparation we observed identical antiproliferative effects on MCs. On the other hand, heparan sulfate in concentrations equivalent to 5–15% of hexouronic acid content of BGN (39) had no antiproliferative effects on MCs (data not shown). These data suggest that it is intact BGN that is capable of inhibiting FCS-induced, and more importantly, PDGF-BB-induced, proliferation of MCs.

To examine the effects of BGN on the survival of MCs, cells were maintained quiescent for 24 h, and subsequently death was induced by adding 2 nM TNFα and 100 nM cycloheximide in the absence or presence of BGN. Normally, rat MCs are resistant to TNFα-induced cell death but become susceptible to the apoptotic effects of TNFα by treatment with cycloheximide (41). Cell death was monitored after 2–24 h by a cell death detection ELISA and by quantification of trypan blue-positive cells, as well as TUNEL-positive nuclei. Quantification of histone-associated DNA fragments in cytoplasmatic fractions by the cell death detection ELISA showed that BGN strongly protected MCs from dying as long as the proteoglycan was present for at least 4 h (Table II). Maximal effects were observed at a concentration of 10 μg/ml. Higher concentrations of BGN were less effective, probably because of its antiadhesive effects. Without induction of MC death BGN enhanced the survival of MCs after 24 h by 15% (control, 184 ± 17; MCs +10 μg/ml BGN; 212 ± 19 arbitrary units). Quantification of trypan blue-positive MCs and TUNEL-positive nuclei confirmed the protective effects of BGN on MC survival at 6 and 24 h after induction of MC death (Table II). For further differentiation between apoptosis and necrosis, the localization of annexin V in the plasma membrane and the hydrolysis of carboxyfluorescein were studied. Fig. 9*A* shows staining for carboxyfluorescein labeling living MCs and for annexin V stained in apoptotic cells 8 h after the induction of cell death. Coincubation with BGN markedly reduced the number of apoptotic MCs. Apoptotic death of MCs dominated during the first 12 h after the addition of TNFα and cycloheximide. At later time points more necrotic cells were detected (Fig. 9*B*). Quantification of apoptotic, necrotic, and vital cells provided evidence that BGN protected MCs against both forms of death (Fig. 9*B*). The smaller number of apoptotic MCs detected between 16 and 24 h reflects limitations of this method, which assessed only adherent MCs.

To determine how BGN might influence apoptosis, the activity of the effector caspase-3 was measured in MC homogenates at 2, 4, 6, and 12 h following the induction of cell death by TNFα in combination with cycloheximide. A time-dependent increase of caspase-3 activity was observed in TNFα/cycloheximide-treated MCs between 2 and 6 h without a significant effect of BGN at 2 and 4 h (data not shown). However, at 6 h the activity of caspase-3 reached its maximum but was significantly lower in the presence of BGN as compared with MCs incubated without BGN (control MCs, 1.3 ± 0.3 A/μg protein; MCs + TNFα/cycloheximide, 4.3 ± 1.0 A/μg protein; MCs + TNFα/cycloheximide + BGN, 2.6 ± 0.5 A/μg protein ($n = 4, p < 0.05$)). At later time points the caspase-3 activity was decreasing, and the presence of BGN had no effect on its activity. Addition of BGN also had no effect on the activity of caspase-3

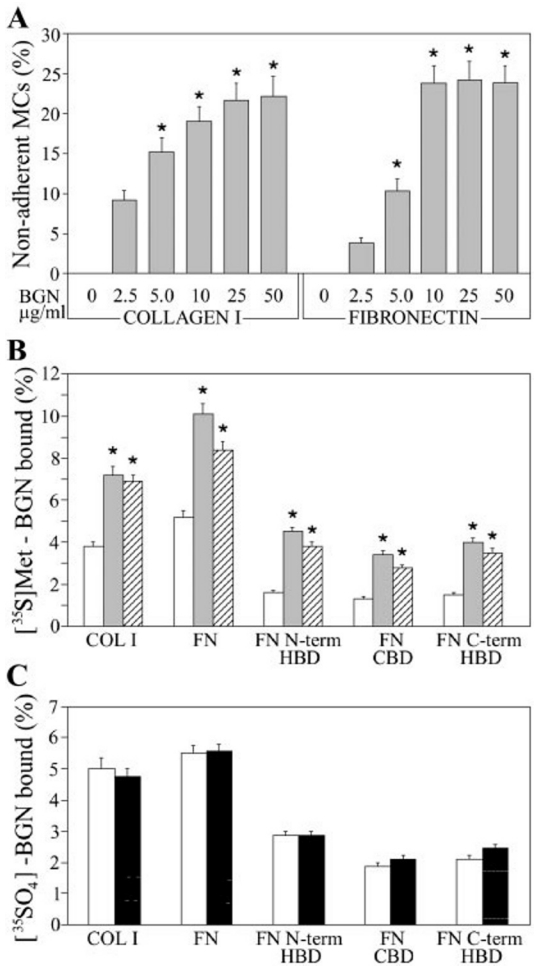

FIG. 7. **Effects of BGN on adhesion of MCs and binding of BGN to different substrates.** *A*, quantitative determinations of adhesion of MCs cultured for 1 h with or without recombinant BGN (2.5–50 μg/ml) on CytoMatrix cell adhesion strips coated with type I collagen or fibronectin. The percentage of non-adherent MCs is presented as the relative difference in adhesion between MCs cultured with or without BGN. The percentage of non-adherent MCs cultured in the absence of BGN was taken as baseline value (mean ± S.D.; $n = 3$; *, $p < 0.05$). *B*, solid-phase assay for binding of [^{35}S]methionine-labeled BGN on type I collagen (*COL I*), fibronectin (*FN*), N- or C-terminal heparin-binding domain (*FN N-term HBD* or *FN C-term HBD*, respectively), and cell-binding domain of fibronectin (*FN CBD*). *White bars* represent the percentage of bound intact BGN (without ABC chondroitin lyase). *Gray bars* show binding of BGN core protein (with ABC chondroitin lyase), and *striped bars* represent binding of BGN core protein in the presence of heparin (25 μg/ml). Data are given as means of duplicates of three independent measurements in each group (*, $p < 0.05$ for *gray* or *striped versus white bars*). *C*, solid-phase assay for binding of [^{35}S]sulfate-labeled BGN to the substrates as in Fig. 7*B*. *White bars* represent the percentage of bound intact BGN, and *black bars* represent binding of intact BGN in the presence of heparin.

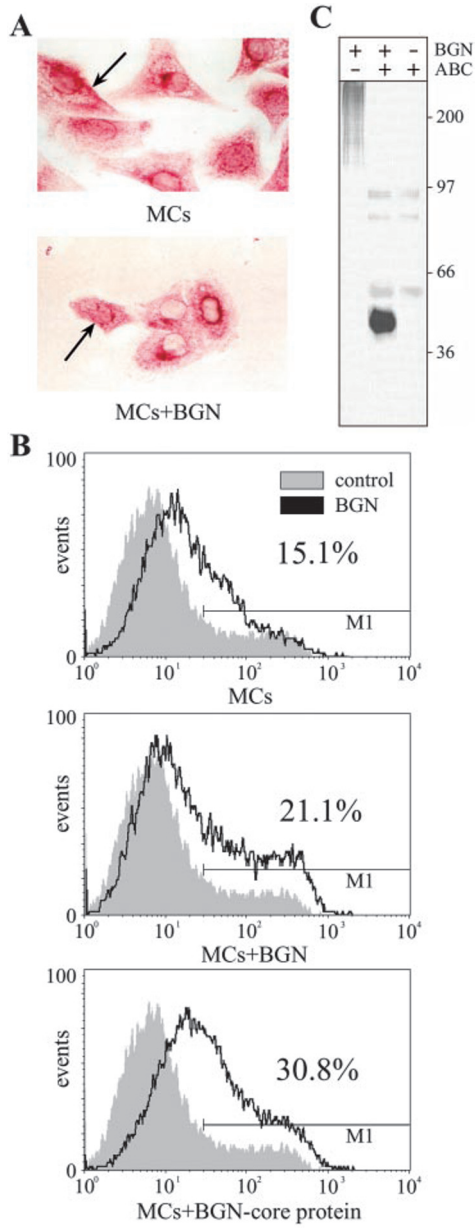

FIG. 8. **Detection of BGN bound to the cell surface of MCs (*A* and *B*) and purity of BGN from 293 cells by silver staining (*C*).** *A*, immunostaining for BGN on MCs cultured for 18 h on fibronectin-coated chamber slides in the presence or absence of 10 μg of BGN (alkaline phosphatase anti-alkaline phosphatase; *red staining*; ×1000). Cell surface-bound BGN is indicated by *arrows*. *B*, FACS analysis of BGN on the surface of MCs. 5×10^5 MCs were either left untreated (*upper histogram*; represents endogenous BGN) or incubated with exogenous human BGN (*middle histogram*) or with BGN core protein derived from exogenous human BGN (*lower histogram*). Cells were stained for BGN on the surface (see "Materials and Methods"). The percent of BGN-positive cells (*open graph*) is indicated. Control (secondary antibody; *filled graph*) is subtracted. One representative experiment of three is shown. *C*, SDS-PAGE and silver staining of BGN from 293 cells.

in control MCs between 2 and 12 h (data not shown). These findings suggest that BGN protects MCs from apoptosis, at least partially, because of inhibition of caspase-3 activity.

We also addressed the possibility that the antiapoptotic effects of BGN might have been caused by complex formation of TNFα with BGN and subsequent inactivation of the cytokine. When TNFα was preincubated with BGN immobilized on nitrocellulose, its ability to induce MC death was not affected, as measured after 24 h by the cell death detection ELISA (preincubation with immobilized BGN, 1018 ± 91 arbitrary units *versus* preincubation with nitrocellulose, 987 ± 98 arbitrary units; $n = 3$, $p > 0.05$). These data demonstrate that the antiapoptotic effect of BGN was not because of binding of TNFα but reflects an intrinsic property of this proteoglycan.

To assess whether other active moieties in our BGN preparation might have been responsible for the effects on adhesion, proliferation, and survival of MCs, we performed SDS-PAGE followed by silver staining to demonstrate the purity of the BGN preparation (Fig. 8*C*). Only the typical staining for BGN could be observed without any additional bands. Moreover, 50 μg of purified BGN were loaded on a DEAE-Trisacryl M column, and after washing the unbound material was examined in adhesion, proliferation, and cell death assays. This unbound

TABLE I
Effects of biglycan on serum- or PDGF-BB-stimulated proliferation of MCs

Subconfluent MCs were deprived of serum for 24 h and then treated simultaneously with 10% FCS or 20 ng/ml PDGF-BB and BGN (5 μg/ml) for 24 h. Proliferation was determined by [^{3}H]thymidine incorporation or by the cell proliferation assay. Data on cell proliferation are given as means ± S.D. from four independent experiments, each of which was performed in duplicate.

FCS	PDGF-BB	BGN	[^{3}H]Thymidine incorporation assay	Cell proliferation assay
10%	*20 ng/ml*	*5 μg/ml*	*cpm × 10^3*	*arbitrary units*
			14.6 ± 2.1	477 ± 41
		+	13.3 ± 2.0	495 ± 48
+			28.2 ± 3.2[a]	771 ± 82[a]
+		+	19.3 ± 3.1[a,b]	545 ± 59[b]
	+		21.2 ± 3.2[a]	621 ± 51[a]
	+	+	15.4 ± 2.5[b]	505 ± 49[b]

[a] $p < 0.05$ for MCs cultured with FCS or PDGF-BB *vs.* MCs without FCS or PDGF-BB, respectivly.
[b] $p < 0.05$ for MCs cultured with FCS or PDGF-BB and BGN *vs.* MCs with FCS or PDGF-BB and without BGN, respectively.

TABLE II
Effects of biglycan on MC death

MC death was induced by TNFα (2 nM) in combination with cycloheximide (100 nM) and monitored by a cell death detection ELISA, as well as by quantification of trypan blue-positive cells and TUNEL-positive nuclei, respectively. Recombinant BGN was added in a concentration of 10 μg/ml. Note that in the cell detection ELISA both adherent and detached cells were evaluated, whereas in trypan blue or TUNEL staining only adherent MCs were assessed. Data are given as means ± S.D. from three independent experiments. Samples in the cell death detection ELISA were measured in duplicate. The values of control MCs measured at the earliest time point by each assay method were taken as 100%.

Assay method	Time	Control MCs	MCs + TNFα + cycloheximide	MCs + TNFα + cycloheximide + BGN
	h			
Cell Death Detection ELISA (arbitrary units)	2	155 ± 17 (100%)	299 ± 21 (193%)[a]	231 ± 18 (149%)[a]
	4	159 ± 19 (103%)	481 ± 41 (310%)[a]	328 ± 34 (212%)[a,b]
	6	164 ± 18 (106%)	691 ± 44 (446%)[a]	491 ± 37 (317%)[a,b]
	12	189 ± 20 (122%)	995 ± 69 (642%)[a]	510 ± 57 (329%)[a,b]
	24	235 ± 26 (152%)	1262 ± 79 (814%)[a]	661 ± 93 (426%)[a,b]
Trypan blue-positive MCs (MCs/well)	6	382 ± 29 (100%)	892 ± 73 (234%)[a]	438 ± 28 (115%)[a,b]
	24	412 ± 28 (108%)	1218 ± 107 (319%)[a]	592 ± 71 (155%)[a,b]
TUNEL-positive nuclei (% of MCs)	6	2.1 ± 0.3 (100%)	14.4 ± 2.3 (685%)[a]	5.4 ± 1.3 (257%)[a,b]
	24	2.6 ± 0.3 (124%)	10.8 ± 2.2 (514%)[a]	5.9 ± 1.2 (281%)[a,b]

[a] $p < 0.05$ for MCs with induced death *vs.* controls.
[b] $p < 0.05$ for MCs + TNFα/cycloheximide + BGN *vs.* MCs + TNFα/cycloheximide.

material had no effects in any of the bioassays performed (data not shown) suggesting that the observed alterations in MCs were indeed because of BGN rather than contaminants in the preparation.

DISCUSSION

Our findings provide evidence that NO is capable of down-regulating BGN gene expression in MCs both *in vitro* and *in vivo*. Using two different NO donors we could show that transcriptional regulation occurred in a time- and concentration-dependent manner. However, the underlying mechanism of this regulation is still unknown, because (i) the signaling pathways involved in the regulation of BGN expression are not known, and (ii) the promoter of the rat BGN gene has not yet been characterized. It has been shown, however, that transcriptional down-regulation of BGN resulted also in a reduced *de novo* production of BGN core protein. This was achieved both by the use of exogenous NO donors and by stimulation of iNOS through IL-1β. Down-regulation of BGN was NO-specific and was not caused by a direct effect of IL-1β on BGN gene expression, because l-NIL, a selective inhibitor of iNOS, readily counteracted the NO effect.

Our *in vitro* findings in cultured MCs were confirmed *in vivo* in a rat model of mesangioproliferative glomerulonephritis, characterized by enhanced NO formation during the early phase of mesangiolysis (44, 45). In agreement with previous studies we found an early induction of iNOS mRNA expression in isolated glomeruli from nephritic rats (46). In parallel, down-regulation of BGN mRNA was observed that was at least partially reversible by the systemic administration of l-NIL, again indicating an NO-dependent mechanism. The *in vivo* observation that l-NIL only partially reversed the down-regulation of BGN might be explained by the fact that in glomeruli BGN is expressed not only in MCs but also in endothelial cells and podocytes (22). As BGN was down-regulated in all types of glomerular cells, and l-NIL is a selective inhibitor of iNOS (47), it is conceivable that not all glomerular NO synthases were suppressed. Moreover, there might be other biologically reactive radicals, produced as a consequence of an increased NO production, which may have a strong impact on gene expression (48, 49). In contrast to the immediate down-regulation of BGN upon induction of the anti-Thy 1-induced glomerulonephritis, BGN mRNA became up-regulated 48 h after administration of the antibody. This effect, however, might at least partly be because of an increased TGF-β production, as has been reported previously (37, 50).

Our differential display data indicated that the NO-mediated effects on proteoglycan transcription in MCs seemed to be specific for BGN. The expression of the closely related proteoglycan decorin was not affected by NO neither *in vitro* in cultured MCs nor *in vivo* in isolated glomeruli. However, NO modulates the synthesis of other extracellular matrix components such as collagen, fibronectin, and laminin in MCs (49, 51).

One of the most intensively investigated features of NO is its effect on cell death (33, 34). In glomerular MCs NO is capable of triggering cell death (33, 52). It has been proposed that the balance between NO and superoxide determines either an apoptotic or a necrotic outcome (34). Here we show that BGN displays a protective effect on MCs, preventing both apoptotic and necrotic cell death induced by TNFα and cycloheximide. Normally, rat MCs are resistant to TNFα-induced cell death but become susceptible to the apoptotic effects of TNFα by

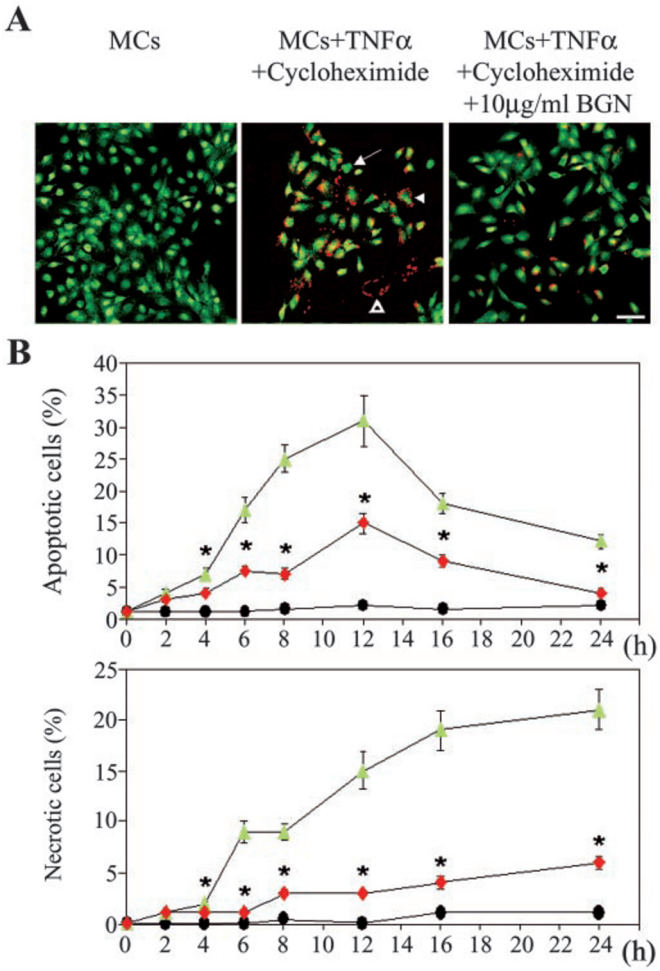

FIG. 9. **Detection of apoptotic, necrotic, and vital MCs.** MCs were incubated with TNFα and cycloheximide in the presence or absence of 10 μg/ml of BGN up to 24 h. Subsequently, MCs were stained with Cy3-labeled annexin V (*red*) and 6-carboxyfluorescein diacetate (*green*) and analyzed by confocal microscopy. In *A* typical stainings after 8 h of incubation are shown. The *arrow* indicates vital MC, the *filled arrowhead* points to an apoptotic MC, and a necrotic MC is indicated by an *open arrowhead* (*bar*, 50 μm). *B*, apoptotic or necrotic MCs were evaluated as the percentage of adherent MCs. Control MCs are indicated in *black*, MCs treated with TNFα and cycloheximide are in *green*, and MCs treated with TNFα, cycloheximide, and BGN are in *red* (mean ± S.D.; $n = 3$; *, $p < 0.05$ for *red versus green staining*).

treatment with cycloheximide because of the sustained activation of c-Jun N-terminal protein kinase (41). In agreement with these studies (41) we observed mainly apoptosis up to 12 h. Moreover, the antiapoptotic effects of BGN on MCs were because of inhibition of the effector caspase-3. The presence of necrotic MCs observed at later stages may reflect the fact that we followed MCs for prolonged periods of time after the stimulation of cell death. Up to now, not much was known about the effects of BGN on cell survival except that the rate of cell death has been found to be enhanced in BGN-deficient bone marrow stromal cells (6). Previous reports (15–17) have primarily dealt with antiapoptotic effects of decorin in selected cell types. During the acute phase of mesangiolysis in the anti-Thy 1-nephritis, when iNOS was overexpressed, and BGN was simultaneously down-regulated, both apoptotic and necrotic cell death have been described previously (53, 54). In light of the present investigation we postulate that NO-induced MC death is at least partially mediated by NO-dependent down-regulation of BGN.

In a number of glomerular diseases apoptosis and/or necrosis of MCs have been observed to be preceeded by lobular disintegration of the mesangium with detachment of MCs (55). The pathogenetic mechanism underlying detachment of MCs is still poorly understood. NO has been reported to inhibit adhesion and spreading of MCs on various extracellular matrices, mediated primarily by cGMP-dependent signaling pathways (32). Conversely, the loss of MC adhesion may contribute to mesangiolysis. In fact, inhibition of NO synthase prevents mesangiolysis in anti-Thy 1-glomerulonephritis (45). Our data provide evidence for an antiadhesive effect of BGN in MCs. Thus, down-regulation of BGN should promote adhesion of MCs. Because NO and BGN both inhibit adhesion of MCs, NO-mediated down-regulation of BGN may be considered as a negative feedback mechanism to attenuate antiadhesive effects of NO on MCs. Antiadhesive properties of decorin and BGN have been shown previously for other cell types, as well (7, 8). In agreement with these reports antiadhesive effects of BGN in MCs required the binding of the BGN core protein to type I collagen, fibronectin, or fibronectin fragments in the substratum. As shown by fluorography, immunostaining, and FACS analysis only limited amounts of BGN bind to the cell surface of MCs, suggesting that cell-bound BGN is probably not responsible for the antiadhesive effects in MCs.

Another important biological effect of NO is related to its antiproliferative properties in MCs (30, 31). BGN has been suggested to act as a growth factor inducing proliferation and differentiation of monocytic cells (21), whereas in pancreatic cancer cells proliferation was suppressed by BGN (19). In resting MCs proliferation could not be induced by BGN. More importantly, BGN counteracted the effects of growth factors being present in fetal calf serum. Serum-treated cells are considered to be exposed to a pro-inflammatory environment because of the presence of growth factors and cytokines released during serum preparation. What is even more important, BGN is capable of inhibiting the proliferation of MCs induced by PDGF-BB, a major mitogenic growth factor frequently involved in glomerular disease (43). Therefore, it is tempting to speculate that BGN might be an important factor for regulating the proliferation in renal diseases. Further studies, however, are needed to elucidate those pathways by which BGN exerts its antiproliferative effects in MCs.

The *in vitro* effects of BGN in MCs described above correspond well with the events occurring *in vivo* in anti-Thy 1-glomerulonephritis, which is characterized by wide-spread MC death, followed by MC migration and proliferation (56). Because NO exerts antiproliferative, antiadhesive, and proapoptotic effects in MCs (30–34), only its influence on MC survival can be explained by the NO-related down-regulation of BGN. Thus, this reflects one of the mechanisms by which NO induces MC death in the early stage of anti-Thy 1-glomerulonephritis. Probably because of enhanced levels of TGF-β1 BGN is overexpressed in Thy 1-nephritic glomeruli at later time points (37, 50). In parallel, there is repopulation of the mesangium involving the migration of MCs from extraglomerular sources in the juxtaglomerular apparatus (56). At this stage, the antiadhesive effects of BGN might promote migration of MCs to start the repair process of the mesangium. Furthermore, PDGF-BB is being overexpressed in the Thy 1-nephritic glomeruli (43), thereby promoting the proliferation of MCs as a further step of regeneration. The glomerular overexpression of BGN observed at this stage might be important to control PDGF-BB-induced proliferation to avoid hyperproliferation of MCs and the development of glomerulosclerosis.

Taken together, our study shows that BGN gene expression is down-regulated by NO in MCs both *in vitro* and *in vivo*. By demonstrating that BGN regulates adhesion, proliferation, and survival of MCs, we are describing a novel mechanism by which

this proteoglycan might influence the course of renal glomerular disease. At an early stage in Thy 1-glomerulonephritis NO-mediated down-regulation of BGN appears to have contributed to MC death, whereas at later stages TGF-β-dependent up-regulation of BGN might have promoted migration of MCs because of its antiadhesive effects and might have counterbalanced PDGF-BB-driven proliferation of MCs.

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