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Increased Goodpasture Antigen-Binding Protein Expression Induces Type IV Collagen Disorganization and Deposit of Immunoglobulin A in Glomerular Basement Membrane

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Increased expression of Goodpasture antigen-binding protein (GPBP), a protein that binds and phosphorylates basement membrane collagen, has been associated with immune complex-mediated pathogenesis. However, recent reports have questioned this biological function and proposed that GPBP serves as a cytosolic ceramide transporter (CERT_L). Thus, the role of GPBP *in vivo* remains unknown. New Zealand White (NZW) mice are considered healthy animals although they convey a genetic predisposition for immune complex-mediated glomerulonephritis. Here we show that NZW mice developed age-dependent lupus-prone autoimmune response and immune complex-mediated glomerulonephritis characterized by elevated GPBP, glomerular basement membrane (GBM) collagen disorganization and expansion, and deposits of IgA on disrupted GBM. Transgenic overexpression of human GPBP (hGPBP) in non-lupus-prone mice triggered similar glomerular abnormali-

ties including deposits of IgA on a capillary GBM that underwent dissociation, in the absence of an evident autoimmune response. We provide *in vivo* evidence that GPBP regulates GBM collagen organization and its elevated expression causes dissociation and subsequent accumulation of IgA on the GBM. Finally, we describe a previously unrecognized pathogenic mechanism that may be relevant in human primary immune complex-mediated glomerulonephritis. (Am J Pathol 2007, 171:1419–1430; DOI: 10.2353/ajpath.2007.070205)

Basement membrane collagen (type IV collagen) is composed of six distinct α chains ($\alpha 1$ to $\alpha 6$) that apparently form only three types of triple-helical molecules: $\alpha 1. \alpha 1. \alpha 2$ (IV), $\alpha 3. \alpha 4. \alpha 5$ (IV), and $\alpha 5. \alpha 5. \alpha 6$ (IV). The structure of the renal glomerulus is maintained by the glomerular basement membrane (GBM), a peripheral wrapping sheet, and the mesangial matrix, a mesh that cements the core of the capillary tuft. A membrane-organized $\alpha 3. \alpha 4. \alpha 5$ (IV) collagen network supports GBM, and a mesh-organized $\alpha 1. \alpha 1. \alpha 2$ (IV) network scaffolds the mesangial matrix. However, when GBM contacts the capillary wall (capillary GBM), its $\alpha 3. \alpha 4. \alpha 5$ (IV) network (epithelial component) fuses with a membrane-organized version of the $\alpha 1. \alpha 1. \alpha 2$ (IV) network (endothelial component), a phenomenon that is critical to assemble the glomerular filtra-

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tion barrier and does not occur when GBM contacts the mesangial matrix (mesangial GBM).^{1,2}

Goodpasture antigen-binding protein (GPBP) is a non-conventional Ser/Thr kinase that targets the NC1 domain,^{3,4} a key structure in the molecular and supramolecular organization of type IV collagen.² In humans, GPBP is associated with GBM, and increased expression levels of this kinase have been linked with induction of the proautoimmune inflammatory cytokine tumor necrosis factor- α and with Goodpasture and systemic lupus erythematosus diseases,^{4,5} suggesting that GPBP plays a role in GBM collagen organization and in associated immune complex-mediated diseases. However, it has been postulated recently that GPBP Δ 26, a GPBP isoform generated by mRNA alternative splicing, is a cytosolic transporter of ceramide between endoplasmic reticulum and Golgi apparatus, and thus, this isoform has been renamed as CERT.⁶ Based on structural homology and *in vitro* studies using recombinant materials, these authors proposed a similar role for GPBP (CERT_L) and questioned the biological significance of GPBP binding and phosphorylating type IV collagen³ and immunohistochemical evidence revealing GPBP association with human GBM.⁴ Thus, the role of GPBP *in vivo* has remained not only unknown but also controversial.

Predominant IgA deposits at the glomerular mesangium are the histopathological hallmark of IgA nephropathy, the most common primary glomerulonephritis in humans.⁷ Systemic lupus erythematosus is a complex disease displaying IgG autoantibody deposits in multiple organs and tissues including the renal glomerulus (lupus nephritis).⁸ In both instances, glomerulonephritis is thought to be mediated by GBM-associated immune complexes, although the mechanisms responsible for immune complex deposits have not been defined. In Goodpasture disease, IgG autoantibodies bind to GBM in an antigen-antibody manner; however, the mechanisms underlying autoantibody production and binding also remain unknown because the pathogenic epitope(s) residing in the noncollagenous-1 (NC1) domain of the α 3 chain of type IV collagen (the Goodpasture antigen) is cryptic in the quaternary structure.² Nevertheless, deposits of immune complexes associated with GBM cause glomerulonephritis in all three diseases, suggesting the existence of common pathogenic mechanisms.

New Zealand White (NZW) mice are considered healthy animals, although they convey a genetic predisposition for lupus nephritis.⁹ Accordingly, historical reports reveal that aging in NZW mice is associated with autoantibody production and clinically silent immune complex-mediated glomerulonephritis.^{10–12} More recently, a glomerulonephritis with predominant glomerular deposits of IgA and IgM have been reported in NZW mouse-derived models.¹³ These data suggest that genetic background in NZW mice predisposes for all three IgG, IgA, and IgM glomerular deposits. This condition may also extend to human patients because glomerular IgG and IgM deposits are common abnormalities in primary IgA nephropathy, and glomerular IgA and IgM deposits are frequently detected in lupus nephritis.^{7,8}

Here, we report that aging in NZW mice correlates with increased glomerular GPBP expression, GBM disruption and associated IgA deposits, and matrix expansion in the context of a lupus-prone autoimmune response. Moreover, transgenic expression of hGPBP in non-lupus-prone mice induced similar GBM abnormalities, albeit in the absence of an autoimmune response. Thus, our observations provide evidence that *in vivo* GPBP regulates type IV collagen organization and elevated expression of this kinase induces immune complex-mediated glomerulonephritis.

Materials and Methods

Mice

All of the procedures were performed according to institutional guidelines for the use of animals in experimentation. We used NZW, C57BL/6, and BALB/c inbred mice and transgenic mice expressing hGPBP (Tg-hGPBP) and non-Tg-hGPBP littermate mice. For morphological studies, 60 NZW mice between 2 and 14 months of age were analyzed using standard histochemical and immunofluorescence procedures. Groups of at least three mice representing unaffected (young mice <6 month of age), nodular, or mesangial glomerulonephritis (aged mice >7 month of age) were analyzed by confocal microscopy, and at least two mice from each group were further analyzed by electron microscopy (EM). Control morphological studies were also performed with C57BL/6 mice of 4, 8, and 12 months of age, and, other than IgA mesangial deposits, we found no significant glomerular abnormalities in these mice by light microscopy (LM), confocal, or EM procedures.

To generate Tg-hGPBP mice, we produced pCAGG-FLAG-GPBP by inserting FLAG-GPBP encoding cDNA into EcoRI site of CAGG expression vector provided by Jun-ichi Miyazaki (Osaka University Medical School, Osaka, Japan).¹⁴ A *Sna*BI-HindIII fragment of pCAGG-FLAG-GPBP was subcloned in the pVAX vector (Invitrogen, Carlsbad, CA) to facilitate digestion with *Nru*I and *Pme*I. The resulting DNA fragment (4.2 kb) was isolated and used for pronuclear injection of zygotes, as previously described.¹⁵ We screened the resulting offspring for transgene transmission by polymerase chain reaction (PCR) analysis of genomic DNA extracted from mouse tails using specific GPBP primers (see below). Tg-hGPBP mice (F₁, 50% C57BL/6 and 50% DBA2) were backcrossed with C57BL/6 mice for six generations to acquire C57BL/6 genetic background (F₂ to F₇, 75 to 99.25% C57BL/6 and 25 to 0.75% DBA2). Samples from mice representing each individual generation were analyzed by standard immunofluorescence and confocal microscopy. Mice from F₃ to F₅ generations were further characterized by real-time reverse transcription (RT)-PCR, immunoprecipitation, histochemistry, EM, and enzyme-linked immunosorbent assay and used in the study.

In general, mice were sacrificed monthly commencing at 4 months of age until type IV collagen-based GBM lesions were evident by confocal microscopy,

and the progression of glomerulonephritis was followed for 1 or 2 additional months in littermate mice. Non-Tg-hGPBP littermates raised in parallel were used as controls in the studies. A total of 36 Tg-hGPBP and 22 non-Tg-hGPBP littermates were characterized using confocal microscopy. Moreover, 16 Tg-hGPBP and 6 non-Tg-hGPBP were analyzed by EM. The presence of clinical glomerulonephritis was assessed by estimating proteinuria by determination of urine albumin as previously described.¹³

Antibodies and Conjugates

Anti-FLAG M2 monoclonal antibody coupled to agarose beads or horseradish peroxidase (Sigma Chemical Co., St. Louis, MO) were used for immunoprecipitation or Western blot, respectively. To detect $\alpha 1\text{-}\alpha 2(\text{IV})$ we used a goat anti-type IV collagen polyclonal antibody (AB769; Chemicon, Temecula, CA), unlabeled or labeled with Alexa Fluor 647 (Invitrogen). To detect $\alpha 3(\text{IV})$ we used biotin-labeled mAb3 provided by Jorgen Wieslander (Wieslab AB, Lund, Sweden). For GPBP-specific detection we used chicken antibodies recognizing the 26-residues (GPBPp1) present in GPBP and absent in GPBP Δ 26 (CERT) previously characterized.⁴ Specific antibodies were further purified by adsorption on a GPBPp1-Sepharose column and their specificity determined by Western blot and indirect immunofluorescence using GPBPp1 for competing antibody binding (Supplemental Figure S1, see <http://ajp.amjpathol.org>). The production and characterization of mAb 14, a mouse monoclonal antibody for GPBP have been reported.³ Fluorescein isothiocyanate (FITC)-labeled antibodies for specific immunoglobulins and secondary antibodies were from Sigma. Other conjugates were tetramethyl-rhodamine isothiocyanate (TRITC)-labeled ExtrAvidin (Sigma) and FITC-labeled avidin (Vector Laboratories, Burlingame, CA).

Immunoprecipitation

Kidneys were removed from mice, immediately frozen in liquid nitrogen and ground with pestle and mortar. The resulting kidney powder (50 mg) was homogenized in 10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 1% sodium deoxycholate, 2 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethyl sulfonyl fluoride, and 10 $\mu\text{g/ml}$ leupeptin with a rotor-stator homogenizer (Omni International, Marietta, GA). Homogenates were cleared by centrifugation ($20,000 \times g$, 15 minutes at 4°C), and supernatants were diluted with 2.5 vol of 10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl (Tris-buffered saline) supplemented with 1 mmol/L phenylmethyl sulfonyl fluoride and extracted with 30 μl of agarose-anti FLAG M2 beads for 3 hours at 4°C under continuous end-to-end agitation. Beads were washed three times with ice-cold Tris-buffered saline, and samples were eluted twice with 0.1 mg/ml of FLAG peptide in Tris-buffered saline. Finally, eluted material was analyzed by Western blot using anti-FLAG M2-horseradish peroxidase or mAb 14.

Serological Studies

Serum levels of total IgG and IgA were determined as previously described,¹³ and results were expressed in mg/ml in reference to a standard curve obtained with a mouse reference serum (MP Biomedicals, Fountain Parkway, OH) (Supplemental Figure S2, see <http://ajp.amjpathol.org>). Circulating IgG and IgA anti-nucleosome or anti-ssDNA autoantibodies were determined following procedures described elsewhere,^{13,16} and results were expressed in titration units based on a standard curve obtained from serial dilutions of a pool of serum from 6- to 8-month-old MRL *lpr/lpr* mice. Circulating IgG and IgA anti-dsDNA autoantibodies were measured by the standard *Crithidia luciliae* immunofluorescence assay (Zentech, Angleur, Belgium), and results were expressed in an scale ranging from 0 to 3 depending on their reactivity at 1:10 (1), 1:50 (2), and 1:250 (3) serum dilutions (Supplemental Figure S2, see <http://ajp.amjpathol.org>). Circulating anti-basement membrane autoantibodies were detected using standard indirect enzyme-linked immunosorbent assay procedures by coating the plates with 2 $\mu\text{g/ml}$ of purified bovine testis NC1 domain, gifted by Billy G. Hudson (Vanderbilt University Medical Center, Nashville, TN), and using Amplex UltraRed reagent (Invitrogen), a fluorogenic horseradish peroxidase substrate, for detection. The coating material contains the hexameric form of the NC1 domain of type IV collagen from $\alpha 1\text{-}\alpha 1\text{-}\alpha 2(\text{IV})$ and $\alpha 3\text{-}\alpha 4\text{-}\alpha 5(\text{IV})$ networks. When indicated the hexamer was denatured for 30 minutes at 100°C in the presence of 6 mol/L guanidine-HCl and similarly used for coating purposes. In these assays, results were expressed as arbitrary units of intensity fluorescence (Supplemental Figure S2, see <http://ajp.amjpathol.org>).

The ability of IgG and IgA from Tg-hGPBP and non-Tg-hGPBP mice to form glomerular deposits was assessed by intravenous injection of serum pools from either of these mouse strains into 2 month-old BALB/c mice. Individual mice received a total volume of 1.5 ml of phosphate-buffered saline or serum pool injected during 3 consecutive days. One day after the last injection, mice were sacrificed, and the presence of IgA and IgG glomerular deposits was examined by immunofluorescence (Supplemental Figure S2, see <http://ajp.amjpathol.org>) following procedures previously described.¹³ Serum pools contained similar IgG or IgA levels.

Histochemistry

Kidneys were fixed in 10% formalin, embedded in paraffin, and sliced on an electronic rotary microtome (Microm, Walldorf, Germany) to generate sections for standard histochemical procedures.

Immunofluorescence

Sections of 6 to 7 μm were obtained by cryostat (Microm) from frozen kidneys embedded in OCT (Sakura, Tokyo, Japan). Cryosections were blocked with an avidin/biotin blocking kit (Vector Laboratories) and also with an irrel-

evant ascites (1:10). Sections were subsequently incubated with primary antibodies and with anti-chicken IgY-TRITC, streptavidin-FITC, fluorescein-avidin, anti-goat TRITC, or combinations thereof and mounted for observation using standard or confocal microscopy. All steps were for 1 hour at room temperature.

Standard fluorescence microscopy was performed with an Axioskop 2 plus microscope (Carl Zeiss, Oberkochen, Germany) combined with a Spot camera and software v2.2 (Diagnostic Instruments, Sterling Heights, MI). All images were acquired using the same settings. Confocal images were acquired using a TCS-SP2 laser-scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) equipped with argon and helium-neon laser beams and attached to a Leica DM1RB inverted microscope. The excitation wavelengths for fluorochromes were 488 nm for FITC, 543 nm for TRITC, and 633 for Alexa Fluor 647. To ensure specificity of fluorochromes, the emission aperture for fluorescence detection were 500 to 543 nm for FITC, 559 to 615 nm for TRITC, and 650 to 750 nm for Alexa Fluor 647. Distribution of fluorescence was analyzed using the Leica Confocal Software version 2.61. Images were acquired using the same settings either for young and aged NZW mice or for Tg-hGPBP and non-Tg-hGPBP mice when comparing intensity of fluorescence.

Electron Microscopy

Slices from mouse kidney were fixed overnight at 4°C in 2% glutaraldehyde and 0.1 mol/L cacodylate, pH 7.5, and rinsed with 0.1 mol/L cacodylate and 0.1 mol/L saccharose, pH 7.5, and kept at 4°C. Postfixation was performed with 1% osmium tetroxide in the same buffer for 1 hour. Dehydration through a graded acetone series was followed by embedding in Epon 812 resin. Selection of the samples was made in semithin sections stained with toluidine blue and evaluated in a light microscope. Ultrathin sections, stained with uranyl acetate and lead citrate, were examined with a 1010 Jeol electron microscope (JEOL Ltd., Tokyo, Japan) at 60 kV.

Laser Microdissection, RNA Purification, Reverse Transcription, and Real-Time PCR

Cryosections of NZW mouse kidney were stained with hematoxylin and eosin following the manufacturer's recommendations for RNA preparation. Approximately 250 to 300 glomerular sections from each mouse were dissected and pooled using a PALM microdissector (P.A.L.M. Instruments, Bernried, Germany). Total RNA was extracted from individual mouse sections using RNeasy Protect mini kit (Qiagen, Valencia, CA). cDNA was synthesized using random hexamer (Applied Biosystems, Foster City, CA) and RTG You Prime RXN beads (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturers' recommendations. Real-time PCRs for GPBP and GAPDH were performed in duplicate with SYBR Green PCR Master Mix and a SDS 5700 ap-

paratus (Applied Biosystems). Primers used were 5'-GG-GAAGCCCATCACCATCT-3' (forward) and 5'-CGA-CATACTCAGCACCGGC-3' (reverse) for GAPDH and 5'-GCTGTTGAAGCTGCTCTTGACA-3' (forward) and 5'-CCTGGGAGCTGAATCTGTGAA-3' (reverse) for GPBP. To determine GPBP expression we used the $\Delta\Delta C_t$ method and GAPDH as a normalizer. Relative expression of GPBP was calculated using 5-month-old mice as reference (GPBP expression = 1). In Tg-hGPBP mice, hGPBP and murine GPBP levels were determined using individual melting curves.

Statistical Analysis

Data are presented as scatter plots with a bar indicating the mean of each series; a dot or circle represents the mean value measured in individual mouse samples. We used unpaired Student's *t*-test or Mann-Whitney test to assess differences between series. A *P* value <0.05 was considered significant. Prism 4.0 software (GraphPad Software, San Diego, CA) was used for all calculations.

Results

NZW Mice Undergo Age-Dependent Glomerulonephritis

LM studies revealed that commencing with 7-months of age, NZW mice developed a glomerulonephritis characterized by hyaline deposits and mesangial matrix expansion (Figure 1A), which progressively affected an increasing number of glomeruli in the absence of significant proteinuria (not shown). Hyaline deposits and matrix components occupied the mesangium or the peripheral region of the glomerulus displaying two distinct morphological patterns, mesangial or nodular, which remained differentiated until glomerular collapse. In general, the nodular pattern was more focal and the mesangial pattern more diffuse, affecting 20 to 40% and 40 to 90% of the glomeruli, respectively. The pathology was more evident with Masson trichrome staining, which revealed that as hyaline fuchsinophilic material (lipstick color) increased, there was also a matricial anilinophilic material (blue color) that constrained and progressively substituted hyaline fuchsinophilic material. The latter virtually disappeared when glomerular architecture was disrupted, as noted by a significant reduction in cells and the collapse of capillary spaces (glomerulosclerosis) (Figure 1A). The pathological pattern varied among mice, and thus, we noted mice with either a prevalent nodular (20 to 30%) or mesangial (70 to 80%) glomerulonephritis, although mice and glomeruli with mixed patterns were also observed. Mesangial cell proliferation (more than three cells per peripheral mesangial area) was found in affected glomeruli of the mice undergoing mesangial glomerulonephritis. In contrast, no mesangial cell proliferation was detected in mice with prevalent nodular glomerulonephritis (not shown). Other histopathological findings included wire loops, hyaline thrombi, subendothelial deposits, silver-stained spikes, and occasional crescents

Figure 1. NZW mice undergo age-dependent glomerulonephritis. **A:** Mason trichromatic staining of glomerular sections representing the indicated mice and glomerulonephritis ($n = 60$). In this and following figures, young is <6 months and aged is >7 months. **B:** Glomerular ultrastructural pathological findings in aged NZW mice ($n = 5$). **a** and **b:** Electron-dense homogenous or vacuolated materials aligned on lamina densa of (para)mesangial GBM are denoted by **arrows** in **b**, which represents the boxed region in **a** analyzed at higher magnification. **c:** Electron-dense homogeneous material filling mesangium and paramesangium. **d** and **e:** Electron-dense vacuolated material occupying paramesangium (**d**) and capillary space (**e**). **f:** Electron-dense material associated with lamina rara of (para)mesangial GBM. In **d-f**, **arrows** and **asterisks** denote electron-dense homogenous and vacuolated material, respectively. Illustrated are selected images from NZW mice of 5, 10, 13, 9, and 9 months (**left to right**) (**A**) and 8 to 9 months (**B**). Scale bars: $1\ \mu\text{m}$ (**a**, **d**, and **f**); $0.5\ \mu\text{m}$ (**b**); $2\ \mu\text{m}$ (**c**); $7\ \mu\text{m}$ (**e**). Original magnifications, $\times 400$ (**A**).

(not shown), all of which are common findings in immune complex-mediated glomerulonephritis. Finally, no inflammatory cell infiltrates (macrophages, lymphocytes, or neutrophils) were detected in these animals. EM analysis revealed that, whereas in young NZW mice the glomerular structure was virtually preserved (not shown), there existed abundant abnormalities in old NZW mice (Figure 1B). The dominant ultrastructural finding included mesangial deposits intimately associated with the lamina densa of a lengthened (para)mesangial GBM (Figure 1Ba). These deposits were either electron dense and homogenous or less electron dense and vacuolated (Figure 1Bb). As the pathology progressed, these deposits could be found filling paramesangium and mesangium and even occluding capillary walls, although with differ-

ing proportions depending on the morphological pattern. In mesangial glomerulonephritis, electron-dense homogeneous deposits were predominant (Figure 1Bc) whereas in nodular glomerulonephritis the predominant deposits were of vacuolated material (Figure 1, Bd and Be). Additional ultrastructural findings included electron-dense deposits associated with lamina rara of (para)mesangial GBM (Figure 1Bf) and with the subepithelial or the sub-endothelial side or within the lamina densa of capillary GBM (not shown). All of these abnormalities are commonly found in human immune complex-mediated glomerulonephritis including IgA nephropathy and systemic lupus erythematosus.^{8,17} Finally, fibrillar collagen was not noted at any disease stage.

Type IV Collagen Disorganization and GBM Disruption in NZW Mouse Glomerulonephritis

LM and EM studies supported that NZW mice underwent age-dependent glomerulonephritis characterized by GBM structural alterations and mesangial matrix expansion. This was further investigated by indirect immunofluorescence procedures in NZW mice of different ages and types of glomerulonephritis (Figure 2). Mesangial matrix expansion in NZW mouse glomerulonephritis was confirmed using standard indirect immunofluorescence and $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ -specific antibodies (Figure 2A). The existence of structural alterations in GBM was, however, explored by confocal microscopy using antibodies specific for $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ or $\alpha 3. \alpha 4. \alpha 5(\text{IV})$ collagen (Figure 2B). Young NZW mice (<6 months of age) displayed normal GBM organization, and thus membrane-organized $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ and $\alpha 3. \alpha 4. \alpha 5(\text{IV})$ collagen networks representing the endothelial and epithelial components of capillary GBM, respectively, showed extensive co-localization. In contrast, in aged NZW mice (>7 months of age) the two networks progressively dissociated, and they were found virtually separated one from the other in terminal glomerulosclerosis. This was further confirmed by ultrastructural analysis that revealed capillary GBM dissociation in endothelial (thin) and epithelial (thick) components and formation of a space within which was often occupied by electron-dense material (Figure 2C).

Increased GPBP Expression and GBM Disruption Are Associated in NZW Mouse Glomerulonephritis

The expression of GPBP was first explored in NZW mice of different ages by reverse transcription (RT) coupled to real-time PCR using RNA obtained from individual whole kidneys. No significant differences in GPBP mRNA levels were found among mice of different ages (not shown). GPBP expression was subsequently similarly investigated using glomerular RNA extracted from individual mice (Figure 3A, top). Interestingly, aged mice expressed elevated GPBP mRNA levels in the glomeruli when compared with young mice, although with significant differences among individuals. This observation was

also supported by standard indirect immunofluorescence studies (Figure 3A, bottom) performed with GPBP-specific antibodies, which revealed that young mice expressed limited amounts of GPBP in the glomeruli,

whereas aged mice displayed more prominent expression in a number of glomeruli that varied among individuals. Thus, the evidence suggested that elevated GPBP glomerular expression and focal glomerulonephritis were related in NZW mice. This was investigated by confocal microscopy in glomerular sections still maintaining capillary architecture. In young mice, GPBP showed a predominant co-localization with $\alpha 1. \alpha 1. \alpha 2(IV)$ collagen, and thus it was found preferentially in the mesangium (Figure 3Ba) and in more limited amounts in capillary GBM where it also co-localized with $\alpha 3. \alpha 4. \alpha 5(IV)$ collagen network (Figure 3Bb). In contrast, glomeruli expressing elevated GPBP consistently displayed glomerulonephritis in aged NZW mice and GPBP appeared at the interface between an abnormally expanded $\alpha 1. \alpha 1. \alpha 2(IV)$ collagen and the $\alpha 3. \alpha 4. \alpha 5(IV)$ collagen. Thus, GPBP accumulated between the epithelial component of the GBM and either an enlarged mesangium (mesangial glomerulonephritis) or a lengthened and thickened endothelial GBM component (nodular glomerulonephritis) (Figure 3C).

NZW Mice Undergo Age-Dependent Lupus-Prone Autoimmune Response and Immune Complex-Mediated Glomerulonephritis

EM findings also suggested that NZW mice underwent age-dependent immune complex-mediated glomerulonephritis. Consequently, the presence of lupus-prone circulating autoantibodies and glomerular deposits of immunoglobulins were investigated in young and aged NZW mice (Figure 4). The titers of anti-nucleosome and anti-ssDNA autoantibodies were increased in aged NZW mice as compared with young mice of the same strain. The autoimmune response consisted mainly of IgG autoantibodies although significant titers of IgA autoantibodies were also noted (Figure 4A). In addition, aged NZW mice exhibited increased levels of IgG and IgA anti-dsDNA autoantibodies when compared with young NZW mice (Supplemental Figure S2, see <http://ajp.amjpathol.org>). The presence of glomerular immune complex deposits was also investigated (Figure 4B). Direct immunofluorescence studies revealed glomerular deposits of immunoglobulins in NZW mice; however, their nature and distribution varied with age and type of glomerulonephritis. Limited deposits of IgG with mixed capillary and mesangial distribution were observed exclusively in NZW mice displaying mesangial glomerulonephritis. Significant IgM deposits with nodular distribution were present only in

Figure 2. Glomerular type IV collagen disorganization induces capillary GBM dissociation in NZW mouse glomerulonephritis. Glomerular sections representing the indicated mice and glomerulonephritis were analyzed by standard indirect immunofluorescence ($n = 15$) (**A**) or by confocal microscopy ($n = 7$) (**B**). In this and following immunofluorescence studies, labels at left of the composites indicate stained protein(s). Graphs represent fluorescence intensity distributions in the regions indicated by **arrows** in merged images. **C:** Electron microscopy analysis showing capillary GBM dissociation in aged NZW mice ($n = 5$). **Inset** in **left** appears magnified in **right**. **Arrows** denote endothelial (**left**) and epithelial (**right**) components of capillary GBM. Illustrated are selected images from NZW mice of: 5.5, 9, and 8.5 months (**left to right**) (**A** and **B**) and 8.5 months (**C**). Scale bars: 1 μm (**left**); 0.5 μm (**right**). Original magnifications, $\times 400$ (**A**).

Figure 3. GPBP is overexpressed in NZW mouse glomerulonephritis. **A:** Glomerular GPBP mRNA levels in individual NZW mice at the indicated range of age (months) were determined by real-time RT-PCR on laser-dissected glomerular microsections. The relative expression of GPBP in individual animals are represented by **dots** or **circles** and was calculated using 5-month-old mice as reference (GPBP expression = 1). Bars indicate the mean value within a series ($P = 0.0143$). Shown is comparative immunofluorescence analysis of representative glomerular sections of NZW mice of the indicated age (months) ($n = 7$). **B** and **C:** Confocal microscopy analysis of glomerular sections (**left**) or magnified portions thereof (**right**) representing the indicated mice and glomerulonephritis ($n = 9$). Graphs represent fluorescence intensity distributions in the regions indicated by **arrows** in merged images. Illustrated are selected images from NZW mice of 5.5 months (**B**) and 9 and 8.5 months (**left to right**) (**C**). Scale bars = 25 μm . Original magnifications, $\times 400$ (**A**).

NZW mice with nodular glomerulonephritis. Finally, significant deposits of IgA with mixed capillary and either mesangial or nodular distributions were present in NZW mice displaying mesangial or nodular glomerulonephritis, respectively.

The glomerular distribution of IgA was investigated by confocal microscopy on glomerular sections that still maintained capillary structure (Figure 4C). IgA was found aligned on the epithelial component of the GBM at the interface with an enlarged mesangium (mesangial lesion)

or a lengthened and thickened endothelial GBM component (nodular lesion), at positions where $\alpha 3.\alpha 4.\alpha 5(\text{IV})$ and $\alpha 1.\alpha 1.\alpha 2(\text{IV})$ networks appeared separated one from the other. In general, IgA deposits aligned more on (para)mesangial GBM in mesangial glomerulonephritis and on capillary GBM in nodular lesions. In more advanced glomerulonephritis, IgA deposits distributed throughout GBM and formed large aggregates that filled the expanded mesangium (mesangial glomerulonephritis) and the peripheral nodules (nodular glomerulonephritis) (Supplemental Figure S3, see <http://ajp.amjpathol.org>).

Transgenic Overexpression of hGPBP Induces Type IV Collagen Disorganization and Deposit of IgA in the GBM of Non-Lupus-Prone Mice

The above data revealed that elevated GPBP expression, GBM disruption, and IgA deposit alignment on disrupted GBM were associated in NZW mouse glomerulonephritis. To investigate further their relationship in pathogenesis, we produced mice expressing hGPBP in a non-lupus-prone genetic background (Tg-hGPBP). Mice expressing hGPBP at ~ 10 -fold the levels of endogenous GPBP as estimated by real-time RT-PCR (not shown) were further used for recombinant protein expression assessment and glomerular phenotype characterization using non-Tg-hGPBP littermates as control mice (Figure 5). Tg-hGPBP mice expressed hGPBP displaying the expected size and antibody reactivity in kidney (Figure 5A) and in liver (not shown). LM analysis revealed association between transgene expression and glomerulonephritis that displayed, as in NZW mice, mesangial and nodular morphological patterns (Figure 5B) and progressively involved 15 to 75% of glomeruli in the absence of proteinuria. A reduced number of Tg-hGPBP mice ($\sim 15\%$) showed mesangial cell proliferation in 15 to 50% of their glomeruli, and no inflammatory cell infiltrates were found in these mice (not shown). EM analysis revealed abundant ultrastructural abnormalities in Tg-hGPBP mice (Figure 5C). Early manifestations of the phenotype mainly involved capillary GBM. Thus, we found endothelial and epithelial components separated one from the other, buttonhole-like disruptions within dissociated epithelial component (Figure 5Ca), horsetail-like unwinding of lamina densa (Figure 5Cb), and electron-dense deposits in between dissociated GBM components (Figure 5Cc). Late manifestations of the phenotype included mesangial deposits associated with (para)mesangial GBM containing electron-dense homogenous or less electron-dense vac-

Figure 4. NZW mice develop age-dependent autoimmune response and glomerular immune complex deposits. **A:** The titration units (TUs) of the indicated circulating autoantibodies in individual mice of indicated age (months) and strain are represented by dots or circles. The bar indicates the mean value in each series (* $P = 0.0031$, ** $P < 0.0001$). **B:** Glomerular sections representing the indicated mice and glomerulonephritis were analyzed by standard direct immunofluorescence ($n = 12$). **C:** Confocal microscopy analysis on glomerular lesions representing aged NZW mice undergoing the indicated glomerulonephritis ($n = 8$). Graphs represent fluorescence intensity distributions in the regions indicated by arrows in merged images. Illustrated are selected images from NZW mice of 5.5, 9, 8, 5.5, 10, 8, 5.5, 13, and 13 months (left to right and top to bottom) (**B**); 13 and 9 months (left to right) (**C**). Original magnifications, $\times 400$ (**B**).

Figure 5. Transgenic hGPBP expression induces glomerulonephritis in non-lupus-prone mice. **A:** Similar amounts of kidney homogenates from the indicated mice were subjected to immunoprecipitation and Western blot analysis of immunoprecipitates using the indicated antibodies. Bars at left of composite denote the positions of 97- and 66-kDa protein standards ($n = 30$). **B:** Mason trichromic staining of glomerular sections representing the indicated mice and glomerulonephritis ($n = 38$). **C:** Ultrastructural pathological findings in Tg-hGPBP mice ($n = 16$). **a:** Arrows indicate endothelial (thin) and epithelial (thick) components in dissociated capillary GBM and box denotes a buttonhole-like disruption within the epithelial component. **b:** A horsetail-like unwinding of the lamina densa in capillary GBM. **c:** Electron-dense material within a disruption of the lamina densa. Arrows denote dissociated GBM components. **d:** Electron-dense homogenous or vacuolated materials aligned on the lamina densa of (para)mesangial GBM. **e:** Electron-dense material filling mesangium and paramesangium. **f:** Vacuolated material protruding in capillary wall. Illustrated are selected images from mice 8-month F_4 (**A**); 8-month F_4 (control and mesangial) and 12-month F_3 (nodular) (**B**); and 8- (**a–c**) and 12-month (**d–f**) F_3 (**C**). Scale bars: 0.2 μm (**a**); 0.5 μm (**b**, **c**, and **f**); 1 μm (**d**); 2 μm (**e**). Original magnifications, $\times 400$ (**B**).

uolated materials (Figure 5C*d*) that, as in NZW mice, progressively occupied paramesangium and mesangium (Figure 5C*e*) or the capillary walls (Figure 5C*f*). In general, glomerulonephritis was less severe in Tg-hGPBP than in aged-matched NZW mice, and mixed mesangial and nodular glomerulonephritis were commonly found. In contrast, we found no significant glomerular pathology in control mice by LM (Figure 5B) and EM (not shown) analysis.

To relate these pathological findings with GPBP overexpression, we performed confocal microscopy analysis (Fig-

ure 6A). These studies confirmed limited GPBP expression and normal GBM collagen organization in control mice. In contrast, we noted a coordinated increased expression of GPBP and $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ collagen in Tg-hGPBP mice. As in aged NZW mice, endothelial and epithelial GBM components progressively separated, and GPBP accumulated between an expanded $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ collagen and the $\alpha 3. \alpha 4. \alpha 5(\text{IV})$ collagen. Thus, differences between control and Tg-hGPBP mice resembled differences between young and aged NZW mice (compare Figure 3, B and C, with Figure 6A), suggesting that increased GPBP expression induced GBM type IV collagen disorganization in mice regardless of genetic background.

We assessed production of lupus-prone circulating autoantibodies and immune complex glomerular deposits in Tg-hGPBP mice. We found no evidence for circulating anti-dsDNA, anti-ssDNA, or anti-nucleosome autoantibodies in these mice. In addition, serum levels of IgG and IgA were virtually the same in Tg-hGPBP mice as in control mice (Supplemental Figure S2, see <http://ajp.amjpathol.org>), suggesting that Tg-hGPBP mice do not undergo lupus-prone autoimmune response. In contrast, Tg-hGPBP mice underwent a generation-dependent progression of glomerular immune complex deposits (Supplemental Figure S4, see <http://ajp.amjpathol.org>) that contained predominantly IgA and IgM whereas deposits of IgG were detected in only a few Tg-hGPBP mice (Figure 6B). Confocal microscopy studies were performed to determine glomerular IgA immune complex distribution (Figure 6, C and D). In control mice, IgA mainly existed in aggregates dispersed in an otherwise normal $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ collagen network. However, in Tg-hGPBP mice these aggregates coexisted with IgA deposits that mapped to histological lesions where they appeared aligned on the epithelial component of GBM at the interface with an enlarged mesangium (mesangial lesion) or a lengthened and thickened endothelial GBM component (nodular lesion), at positions where $\alpha 3. \alpha 4. \alpha 5(\text{IV})$ and $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ networks appeared separated. In more advanced phenotypes, IgA deposits distributed throughout the GBM and formed large aggregates that, as in more advanced glomerulonephritis of NZW mice, filled the expanded mesangium (mesangial glomerulonephritis) and the nodules (nodular glomerulonephritis) (Supplemental Figure S3, see <http://ajp.amjpathol.org>).

Discussion

Here, we show that aging in NZW mice associates with increased glomerular expression of GPBP and glomerular abnormalities including defective fusion of epithelial and endothelial components of capillary GBM and structural collapse by matrix expansion. Transgenic overexpression of hGPBP in non-lupus-prone mice (Tg-hGPBP) induced similar glomerular abnormalities, suggesting that GPBP regulates glomerular type IV collagen organization regardless of genetic background. Accordingly, elevated GPBP glomerular levels induce defective fusion of $\alpha 3. \alpha 4. \alpha 5(\text{IV})$ and $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ networks in capillary GBM and unbalanced expansion of mesh- and membrane-organized $\alpha 1. \alpha 1. \alpha 2(\text{IV})$ collagen in both our mod-

Figure 6. Transgenic hGPBP expression induces immune complex-mediated glomerulonephritis in non-lupus-prone mice. **A:** Comparative confocal microscopy analysis of glomerular sections (left) or magnified portions thereof (right) representing the indicated mice and glomerulonephritis. **B:** Representative glomerular sections from the indicated mice were analyzed by standard direct immunofluorescence. **C:** Confocal microscopy on selected glomerular sections of the indicated mice and glomerulonephritis. **D:** Representative IgA deposits in the indicated lesion of Tg-hGPBP mice. In **C** and **D**, IgA appears in green, $\alpha 1\text{-}\alpha 2(\text{IV})$ in blue, and $\alpha 3(\text{IV})$ in red. **A–D:** $n = 38$. Graphs represent fluorescence intensity distributions in the regions indicated by arrows in merged images. Illustrated are images taken from mice 8-month F_4 (control and nodular) and 12-month F_3 (mesangial) (**A**); 8-month F_5 (**B**); 10-month F_4 , 14-month F_3 , and 7-month F_5 (control, mesangial, and nodular, respectively) (**C**); and 7-month F_5 (**D**). Scale bars = 25 μm . Original magnifications, $\times 400$ (**B**).

els. In nodular lesions, an abnormally long and thick endothelial GBM component substitutes the mesangial matrix. In mesangial lesions, an enlarged mesangium replaces endothelial GBM component. In both types of lesions, there exist a defective glomerular filtration barrier, a lengthening of the mesangial GBM and an overall reduction of the capillary space (Figure 7). The evidence also supports that GBM disruption is an early event in the pathogenic cascade and therefore that glomerular type IV collagen expansion (glomerulosclerosis) might depend on surveillance systems for adequate GBM collagen assembly that signal for more collagen synthesis.

The mechanism by which increased GPBP expression induces GBM disruption is unknown. The tertiary and quaternary structures of the NC1 domain of type IV collagen are stabilized, at least in part, by intra- and intermolecular disulfide bonds, respectively.^{18–20} We have reported that the $\alpha 3(\text{IV})\text{NC1}$ domain undergoes structural diversification and assembles in multiple conformational isoforms (conformers) that diverge in the redox state of specific cysteine residues. These residues can be found engaged in intramolecular disulfide bonds or free and,

therefore, available for intermolecular disulfide bond formation.²¹ Whether GPBP plays a role in the structural diversification of the NC1 domain remains to be determined; however, recent findings using a recombinant model in cultured cells reveal that the levels of GPBP regulate both conformation and disulfide bond connectivity of the $\alpha 3(\text{IV})\text{NC1}$ domain (unpublished results). Conceivably, the $\alpha 3(\text{IV})\text{NC1}$ conformers produced by cells expressing high levels of GPBP could be defective for intermolecular disulfide cross-linking and their assembly to induce $\alpha 1\text{-}\alpha 1\text{-}\alpha 2(\text{IV})$ and $\alpha 3\text{-}\alpha 4\text{-}\alpha 5(\text{IV})$ network disassociation and capillary GBM disruption.

Additional pathological findings associated with aging in NZW mice include production of autoantibodies and glomerular deposits of immune complexes. In contrast, Tg-hGPBP mice display similar glomerular deposits in the absence of an evident autoimmune response. Thus, the evidence suggests that glomerular immune complex deposit formation is more dependent on GPBP overexpression than on autoantibody production in our animal models. This is also supported by independent observations, which include the following: IgA is more prominent than

Figure 7. Model for type IV collagen distribution in normal glomeruli and in GPBP-mediated glomerulonephritis. The clover-like structure represents a section of a renal glomerulus in which type IV collagen is depicted in an unaffected capillary (normal) or in capillary undergoing the indicated GPBP-mediated glomerulonephritis. In black, the $\alpha 3. \alpha 4. \alpha 5(IV)$ collagen (epithelial GBM component). In red, membrane-organized $\alpha 1. \alpha 1. \alpha 2(IV)$ collagen (endothelial GBM component). In blue, mesh-organized $\alpha 1. \alpha 1. \alpha 2(IV)$ collagen (mesangial component). In white, virtual spaces resulting from defective fusion of epithelial and endothelial GBM components that support GPBP accumulation and IgA deposits on orphan epithelial wall. In mesangial glomerulonephritis, the mesangial component progressively replaces the endothelial component whereas in nodular lesions the endothelial component progressively substitutes mesangial component. In advanced glomerulonephritis, a component can entirely substitute the other. In both NZW and Tg-hGPBP mice, there were animals, glomeruli, and even capillaries displaying both types of lesion.

IgG in glomerular deposits of aged NZW mice, but circulating IgG autoantibodies are more evident than circulating IgA autoantibodies, and IgA and GPBP deposits occupy the virtual space generated by an abnormally expanded $\alpha 1. \alpha 1. \alpha 2(IV)$ network that does not fuse with the $\alpha 3. \alpha 4. \alpha 5(IV)$ network in both our mouse models. In this regard, an additional relevant factor is the predisposition that specific mouse strains show for glomerular immune complex deposit formation. We have noted that F_1 generation of Tg-hGPBP and non-Tg-hGPBP mice (50% C57BL/6, 50% DBA2) display limited and variable IgA glomerular deposits, a condition that is dependent on C57BL/6 genetic background because mice from F_2 to F_7 generation (75 to 99.25% C57BL/6 and 25 to 0.75% DBA2) displayed progressively more consistent IgA glomerular deposits (Supplemental Figure S4, see <http://ajp.amjpathol.org>). Thus, our data suggest that glomerular immune complex deposit formation depends at least on two main factors: the genetic trait of certain mouse strains (ie, NZW and C57BL/6), which predisposes immunoglobulins to accumulate in the renal glomerulus, and GPBP-dependent type IV collagen-based glomerulonephritis. Consistently, NZW-immunoglobulins form limited glomerular deposits in young normal mice (Figure 4B) and Tg-hGPBP-immunoglobulins do not form significant glomerular deposits when transferred to normal BALB/c mice (Supplemental Figure S2, see <http://ajp.amjpathol.org>). Our evidence also indicates that elevated expression of GPBP does not represent the predisposing trait for IgA deposit formation because non-Tg-hGPBP mice displayed significant IgA deposits (Figure 6B). Finally, this predisposition might be relevant for triggering inflammatory signals that

initiate focal GPBP-mediated glomerulonephritis in NZW mice.

The immune complex deposits in our mouse models are heterogeneous and their composition varies mainly depending on the type of lesion. Specifically, IgA and IgM are the predominant immunoglobulins found in nodular lesions whereas IgA and to a more limited extent IgG are the immunoglobulins deposited in mesangial glomerulonephritis. In any event, IgA is predominant in deposits associated with type IV collagen alterations that underlie capillary GBM and glomerular filtration barrier structural defects. These observations reveal that GPBP-dependent capillary GBM disruption exposes binding sites for IgA immune complexes. Moreover, persistent and elevated GPBP levels further disturb glomerular type IV collagen networks and expose additional IgA immune complex-binding sites throughout GBM and mesangial matrix (Supplemental Figure S3, see <http://ajp.amjpathol.org>). Thus, glomerulonephritis in our mouse models share the triad of elevated GPBP expression, type IV collagen alterations, and IgA deposit formation. Interestingly, IgA glomerular deposits in non-Tg-hGPBP (Figure 6B) and C57BL/6 (not shown) mice are not associated with GPBP-dependent type IV collagen pathology, and we found no type IV collagen-based abnormalities attributable to IgA deposits when comparing Tg-hGPBP mice of different generations (Supplemental Figure S4, see <http://ajp.amjpathol.org>). However, we noted that Tg-hGPBP mice containing more of the C57BL/6 genetic background develop IgA deposits and glomerulonephritis earlier.

Whether binding of antibodies to disrupted GBM occurs through antigen-antibody recognition or alternative mechanisms^{7,22,23} remains to be determined. We have not found evidence for autoimmune responses in Tg-hGPBP mice including presence of anti-GBM autoantibodies (Supplemental Figure S2, see <http://ajp.amjpathol.org>), suggesting that antibody binding to disrupted GBM occurs through an antigen-antibody-independent mechanism. Nevertheless, Tg-hGPBP mice display less severe glomerular lesions than NZW mice, suggesting that autoantibodies are a contributing pathogenic factor in NZW mouse glomerulonephritis. One possibility is that augmented levels of GPBP and GBM disruption are part of a wider, coordinated pathogenic program that includes neoantigen exposure and engagement of the immune system in the production of autoantibodies of different specificities. Alternatively, primary autoantibody production and tissue binding could trigger inflammatory signals (ie, tumor necrosis factor- α) that induce GPBP expression and GBM disruption. In either case, subsequent autoantibody binding to disrupted GBM, mainly through an antigen-antibody-independent mechanism, could maintain GPBP expression at elevated levels, thereby perpetuating and aggravating the glomerulonephritis.

Collectively, our data suggest that elevated GPBP expression induces type IV collagen-based glomerulonephritis and exposure of immune complex binding sites. Subsequent glomerular deposit formation requires a predisposing trait for effective tissue antibody binding. The latter becomes pathogenically more relevant in the presence of circulating autoantibodies.

To our knowledge, increased GPBP expression, capillary GBM dissociation, and deposits of immune complexes on orphan epithelial component represent a previously unrecognized mechanism for immune complex-mediated glomerulonephritis. Whether similar mechanisms operate in human pathogenesis remains to be determined. Nevertheless, EM evidence for GBM disruption and accumulation of electron-dense material between separated GBM components have been reported in patients undergoing IgA nephropathy and lupus nephritis.^{8,17} Moreover, an increased GPBP expression could reduce the reinforcement of the quaternary structure of the NC1 domain of type IV collagen, thereby facilitating epitope exposure, immune system activation, and autoantibody binding in Goodpasture disease.²⁴

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