Chronic Renal Failure and Shortened Lifespan in COL4A3+/- Mice: An Animal Model for Thin Basement Membrane Nephropathy

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A heterozygous mutation in autosomal Alport genes COL4A3 and COL4A4 can be found in 20 to 50% of individuals with familial benign hematuria and diffuse glomerular basement membrane thinning (thin basement membrane nephropathy [TBMN]). Approximately 1% of humans are heterozygous carriers of mutations in the autosomal Alport genes and at risk for developing renal failure as a result of TBMN. The incidence and pathogenesis of renal failure in heterozygous COL4A3/4 mutation carriers is still unclear and was examined further in this study using COL4A3 knockout mice. In heterozygous COL4A3+/− mice lifespan, hematuria and renal function (serum urea and proteinuria) were monitored during a period of 3 yr, and renal tissue was examined by light and electron microscopy, immunohistochemistry, and Western blot. Lifespan of COL4A3+/− mice was found to be significantly shorter than in healthy controls (21.7 versus 30.3 mo). Persistent glomerular hematuria was detected starting in week 9; proteinuria of >0.1 g/L started after 3 mo of life and increased to >3 g/L after 24 mo. The glomerular basement membrane was significantly thinned (167 versus 200 nm in wild type) in 30-wk-old mice, coinciding with focal glomerulosclerosis, tubulointerstitial fibrosis, and increased levels of TGF-β and connective tissue growth factor. The renal phenotype in COL4A3+/− mice resembled the clinical and histopathologic phenotype of human cases of TBMN with concomitant progression to chronic renal failure. Therefore, the COL4A3+/− mouse model will help in the understanding of the pathogenesis of TBMN in humans and in the evaluation of potential therapies.

The pathogenesis and incidence of progression to renal failure in heterozygous carriers of COL4A3/4 mutations are not clear. It is uncertain whether individuals with TBMN and concurrent renal insufficiency represent conditions that are associated with serendipitously coexisting renal lesions such as IgA glomerulonephritis. Previous studies did not report pathologic alterations in murine heterozygous carriers of the autosomal Alport mutation (COL4A3+/− mice) (2,3). This could be due to the focus on young and thus still asymptomatic COL4A3+/− mice in these investigations.

In this study, we present for the first time a detailed clinical and histomorphologic phenotype characterization of heterozygous Alport mice surveyed during a period of 3 yr. With advancing age, COL4A3+/− mice show hematuria, proteinuria, attenuation of the GBM, and discrete glomerulosclerosis. Therefore COL4A3+/− mice are applicable as an animal model for TBMN and should provide new insights into the pathogenesis and progression of this frequent renal condition that may predispose to more severe disturbances.

Materials and Methods
Breeding and Genotyping of COL4A3 Knockout Mice
Cross-breeding and PCR-based genotyping of heterozygous COL4A3+/−, homozygous COL4A3−/−, and wild-type control mice on Sv1/129 genetic background were described previously (1,29). Serum urea and proteinuria were measured after 7.5, 9.5, 12, and 30 mo of age. COm4A3−/− mice show hematuria, proteinuria, attenuation of the GBM, and discrete glomerulosclerosis. Therefore COL4A3+/− mice are applicable as an animal model for TBMN and should provide new insights into the pathogenesis and progression of this frequent renal condition that may predispose to more severe disturbances.

Clinical Chemistry and Urine Analyses
Proteinuria was measured using a gradient polyacrylamide gel, stained with Coomassie Blue, and analyzed by densitometry, a semi-quantitative method described before (1,29). Serum urea and proteinuria were measured after 7.5, 9.5, 12, and 30 wk and 12, 18, 24, and 30 mo. For immunocytochemistry and Western blotting, mice were killed after 30 wk and for light and electron microscopy after 12 mo. Forty mice were left alive until death to monitor lifespan. No animals were lost as a result of infections during monitoring.

Microscopy
Male COL4A3−/− (n = 5) and wild-type mice (n = 3) were killed by cardiac puncture and intracardially perfused with a solution that contained 10,000 i.e./L heparin (Liquaemin N 25000; Hoffmann-La Roche, Mannheim, Germany) and 1% procainhydrochloride in 0.1 M PBS and half-strength Karnovsky fixative (4% paraformaldehyde and 2% glutardialdehyde in 0.1 M sodium cacodylate [pH 7.3]). Kidney samples were immersion-fixed for 1 to 2 d and processed further for embedding in Araldite as described previously (1,29). Renal cortex from each mouse was cut and at least five glomeruli per mouse were examined using a Zeiss Axiophot light and Zeiss EM 902 electron microscope.

GBM Morphometry
The width of the GBM of COL4A3+/− and age-matched wild-type mice was determined by computerized morphometry on the basis of arithmetic mean measurements (30) using the “arbitrary distance” function of the electron microscopy image-processing software Analy-sis 3.2 (Soft Imaging Systems, Münster, Germany) at a magnification of 20,000. The measurements were performed on the peripheral portions of randomly chosen capillary loops within a glomerulus, where the epithelial and endothelial cell membranes were clearly visible or the endothelial pores circular, thus excluding tangential sectioning. The thickness of GBM was represented by the perpendicular distance between the endothelial cytoplasmic membrane and the outer lining of the lamina rara externa underneath the cytoplasmic membrane of the epithelial foot process. The intervals of repetitive measurements along the GBM were 100 to 500 nm. For each animal, approximately 500 single measurements on five glomeruli were performed and pooled to calculate the arithmetic mean and SD. Results were sorted into subcategories of 50-nm widths to allow the expression of thickness distribution in the form of histograms.

Immunohistochemistry
For paraffin embedding, kidneys were fixed in 4% paraformaldehyde plus 0.1 mol/L PBS and vacuum embedded using a Shandon Citadel 1000 automatic embedding machine (Pittsburgh, PA). Sections of 4 to 5 μm thickness were cut on a Microm HM355S (Walldorf, Germany) microtome. Sections were rehydrated, treated with Trypsin for 7 min, and blocked with 5% BSA at room temperature. The primary antibody was incubated overnight at 4°C (rabbit anti-mouse EHS-laminin and rabbit anti-mouse fibronectin, 1:1000; gift from M. Paulsson, Cologne, Germany). Goat anti-rabbit Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) served as secondary antibody. POX conjugated anti–α-smooth muscle actin (DAKO, Hamburg, Germany) and rat anti-mouse F4/80 with a goat anti-rat IgG horseradish peroxidase secondary antibody (Serotec, Oxford, England) were used to stain activated fibroblasts and macrophages on hemalun-counterstained paraffin sections. Sections were analyzed on a Zeiss Axiophot microscope as described before (31).

Western Blot
Three to four kidneys from either COL4A3+/− or wild-type mice were pooled to minimize interindividual differences. Protein was extracted in a TBS solution, using protease inhibitors PMSF and n-ethylmaleimide. Aliquots that contained 30 μg of protein as shown by BCA protein assay were dissolved in SDS sample buffer, separated by electrophoresis in a 4 to 12% NuPage Novex Bis-Tris Gel (Invitrogen, Carlsbad, CA), transferred to a polyvinylidene fluoride membrane, and blocked with 5% BSA and milk powder. Mouse anti–TGFB1 (1:100; R&D Systems, Minneapolis, MN) and rabbit anti–connective tissue growth factor (CTGF) (1:500; Abcam Limited, Cambridge, UK) were incubated for 60 min. The membrane then was incubated with the secondary antibody conjugated with horseradish peroxidase (Dako, Hamburg, Germany). The blot was developed using chemiluminescence. Protein expression was analyzed densitometrically using gel-pro analyzer software (Media Cybernetics, Silver Spring, MD).

Statistical Analyses
Data were analyzed by log rank statistic (survival analysis) and two-way ANOVA. In case of significant results, pair-wise post hoc t test was performed. P < 0.05 was considered to be significant. For comparison of GBM widths in COL4A3+/− and wild-type kidneys, a paired t test was used.

Animal Experiments
All animal experiments were carried out under appropriate German licenses and supervised by veterinarians.
Results
Lifespan of Heterozygous COL4A3+/− Mice Was Significantly Shorter than that of Healthy Controls

Whereas homozygote Alport mice died 70.9 ± 6.0 d after birth as reported before (1), 10 monitored heterozygous COL4A3+/− mice lived for 21.7 ± 2.5 mo (Figure 1). This was significantly shorter (P < 0.05) than 10 wild-type controls that lived for 30.3 ± 2.4 mo. All wild-type and heterozygous mice were autopsied, but a definite basis for death such as severe myocardial infarction, severe stroke, or tumor was not found.

To find explanations for this reduced lifespan, we assessed renal function of COL4A3+/− mice. Phase contrast microscopy revealed that wild-type controls did not show hematuria; however, heterozygous COL4A3+/− mice developed hematuria after 8 wk of life with >10 erythrocytes (and >10% acanthocytes) per visual field (magnification ×400). Mild proteinuria of ≥0.1 g/L started at approximately 3 mo and increased to ≥3 g/L shortly before death (Figure 2A). The general signs of nephrotic syndrome, such as edema and hypercholesterolemia, that were seen in homozygous COL4A3−/− mice could not be detected. Serum urea concentration started to be significantly elevated in heterozygous COL4A3+/− mice at 18 mo of age and increased to levels of >125 mg/dl before death (Figure 2B).

Twelve-Month-Old COL4A3+/− Mice Displayed Glomerular Hypercellularity, Glomerulosclerosis, Tubulointerstitial Cell Alternation, and Significant GBM Thinning

By review of semithin sections in 10-wk-old COL4A3+/− mice, we could not find significant abnormalities in histologic kidney structure either qualitatively or quantitatively (data not shown). However, in 12-mo-old mice, we noted an increase in mesangial cell number, morphologic indications for mild glomerulosclerosis, and thickening of Bowman’s capsule in individual glomeruli (Figure 3), whereas other glomeruli appeared normal. Furthermore, compared with wild-type mice, we observed alternations of tubular epithelial cells with increased cellular size and vast amounts of euchromatin in their nuclei, in keeping with increased intratubular protein load and consistent...
with the above-mentioned proteinuria. Mice that were older than 12 mo were not examined for morphologic changes.

In light of the reported link between TBMN and COL4A3 mutations (4,5,10,26), we measured GBM thickness from five 12-mo-old heterozygous COL4A3+/−/H11001/H11002 mice compared with age-matched wild-type controls. It is interesting that on low-power views, there were no signs of disturbed GBM ultrastructure and no obvious visual evidence for GBM attenuation as described in humans with verified TBMN (21,23,25) (Figure 4). However, arithmetic mean measurements of COL4A3+/−/H11001/H11002 GBM thickness revealed a significant reduction in comparison with wild-type GBM width (Figure 5).

Figure 3. Glomerular hypercellularity, glomerulosclerosis, and tubulointerstitial cell alternation in 12-mo-old COL4A3+/−/H11001/H11002 mice (B) and wild-type controls (A). Whereas glomeruli and tubulointerstitial space appeared regular in wild-types, COL4A3+/− renal cortex showed occasional glomerular hypercellularity and alternation of tubular epithelial cells. Note that tubular epithelial cells generally were increased in size, tubular cell nuclei contained almost exclusively euchromatin, and tubular cylinders were filled with amorphous material. Infrequently, individual glomeruli that were affected by severe glomerulosclerosis could be observed in such areas (inset in B). Magnifications: ×600 in B; ×320 in inset in B.

Figure 4. Electron microscopic overviews of COL4A3+/− peripheral capillary loops. In comparison with wild-type glomerular basement membrane (GBM; A) with the typical trilaminar structure and the adjacent podocyte foot processes or endothelial cytoplasmic layer, GBM ultrastructure in 12-mo-old COL4A3+/−/H11001/H11002 mice appeared normal on gross examination of low-magnification micrographs (B). There is no prominent indication of marked GBM attenuation as in human thin basement membrane nephropathy (TBMN). Magnification, ×3000.

Thirty-Week-Old COL4A3+/− Mice Showed Abnormal Glomerular Deposition of Extracellular Matrix Components and Glomerular Invasion of Activated Fibroblasts

Because renal failure in COL4A3 knockout mice morphologically correlates with glomerular deposition of extracellular matrix as a biochemical indicator for glomerulosclerosis (1,2), we further investigated kidney sections from 30-wk-old heterozygous COL4A3+/− mice by immunofluorescence (Figure 7). Whereas wild-type mice showed faint staining for EHS-laminin (staining mainly laminin 1 in the extracellular matrix)
and fibronectin (staining mainly scar tissue) in all glomeruli, the staining intensity of these markers was considerably increased in the intra- and periglomerular matrix in many glomeruli from heterozygous COL4A3+/H11001/H11002 mice (Figure 7, A through F), confirming our results of putative glomerulosclerosis in COL4A3+/H11001/H11002 mice obtained by semithin histology.

To evaluate further the observed glomerular hypercellularity in conjunction with glomerulosclerosis on semithin sections, we performed immunostaining for α-smooth muscle actin (α-SMA), a marker for interstitial myofibroblasts, and for F4/80, a marker for macrophages, which both play a crucial role in the pathogenesis of renal fibrosis (32). We found that in COL4A3+/H11001/H11002 mice, glomeruli that were affected by hypercellularity and sclerosis contained α-SMA– and F4/80-positive cells (Figure 7H). These cells seemed to be arranged concentrically at the outline of the Bowman’s capsule and the tubulointerstitial space. In contrast, α-SMA– and F4/80-positive cells could be seen only rarely in age-matched wild-type mice (Figure 7G).

Profibrotic Cytokines TGF-β1 and CTGF Were Increased in Renal Tissue of COL4A3+/− Mice

TGF-β1 and CTGF are known to be mediators of glomerulosclerosis in chronic kidney disease, and immunoblotting for these markers has been applied successfully in pathogenetic studies using Alport knockout mice to elucidate the pathways that lead to glomerular deposition of extracellular matrix molecules (1,29,31). As presented in Figure 8, TGF-β1 and its downstream regulator, CTGF, were upregulated in 10- and 30-wk-old heterozygous COL4A3+/− mice (lanes 3 and 4) compared with wild-type littermates (lanes 5 and 6). The amounts of TGF-β1 and CTGF in homozygous Alport mice with less fibrosis as a result of Ramipril therapy (lane 2) still are higher than in heterozygous COL4A3+/− mice, indicating less fibrosis and matrix remodeling in these mice. In particular, densitometrically assessed TGF-β1 signal in COL4A3+/− mice was 79% higher than in wild-type controls, and CTGF signal density exceeded the wild-type reference value by 47% in week 30.

Discussion

The basis of these investigations was monitoring life expectancy and the clinical phenotype in heterozygous COL4A3+/− mice during a period of 3 yr. Eight-week-old COL4A3+/− mice developed dysmorphic microhematuria comparable to hematuria in humans with TBMN (18,33). Minimal to mild proteinuria is a common clinical feature of TBMN in adult humans (25), and this condition could be simulated in COL4A3+/− mice that were older than 3 mo. Slight proteinuria increased to 3 g/L after 24 mo, reflecting considerable GBM impairment consistent with reports about occasional progression to nephrotic-range proteinuria in humans (11,15,16,25). However, it has not been clear in the past whether such severe proteinuria is a consequence of TBMN itself, rather than a complication of coincidentally overlapping secondary nephropathology, such as minimal-change nephritis (11,15,16), or as a result of an aggravation after undergoing renal biopsy (25). With our ob-
survival of proteinuria in 24-mo-old COL4A3+/− mice that were not affected by any other renal pathology, it seems likely that elevated urinary protein levels can be caused solely by the TBMN condition. Simultaneously, the expected degree of renal insufficiency in older COL4A3+/− mice could be confirmed by the assessment of serum urea concentrations, where increased levels of >125 mg/dl were detected in 24-mo-old mice that died soon after this time point. Again, some authors believe that impaired renal function parameters are a consequence of a coexisting renal lesion that appeared by chance in patients with TBMN (25). Our observations in COL4A3+/− mice show that older patients with TBMN may require close medical attention. Although TBMN is considered to have a good prognosis owing to a nonprogressive nephropathy, numerous hints in the literature suggest a reduced lifespan in affected individuals.

First, the possibility that TBMN may not be benign in terms of ultimate prognosis has been considered before (13,14,18,30,25), and ESRD similar to that seen in AS was reported in adults with heterozygote COL4A3/A4 mutations (occasionally also referred to as “autosomal dominant Alport syndrome”) (15,34–37). It should be noted that it was suggested that the molecular type of COL4A3/A4 mutations and additional modifier genes are likely to influence the severity of the renal phenotype eventually (35,36,37). Second, hypertension in adults with TBMN was reported previously and is likely to worsen the general prognosis of affected patients (13,25). Third, clinical reports suggest that individuals with TBMN must be viewed to be in a pool of patients who generally display above-average rates of mortality. This means that patients with chronic kidney disease (CKD) such as TBMN are more likely to develop associated cardiovascular disease, and this probably additionally worsens the outcome (31,38,39). Hypertension in patients with TBMN as one contributor to cardiovascular disease might be an essential clue toward this phenomenon.

As a morphologic correlate for persistent hematuria and
chronic renal failure, we found a significant decrease of GBM thickness in 12-mo-old heterozygous COL4A3+/− mice compared with age-matched wild-type controls that was accompanied by glomerular hypercellularity, glomerulosclerosis, and tubulointerstitial cell alteration with advancing age. In adult humans, GBM thickness in peripheral capillary loops of the glomerulus is considered attenuated at <200 to 300 nm, depending on the age, gender, applied measurement method, tissue fixation procedure, and reference values for normal GBM width of individual laboratories (21,25). The diagnosis of TBMN is made when the GBM is thinned in the majority of capillary loops in comparison with control data and at least 50% of the GBM is thinned in individual capillaries. In mice, equal guidelines about when GBM is significantly attenuated do not exist. In contrast to measurements in wild-type mice, in which we obtained an overall mean of 200 nm for GBM thickness in peripheral capillary loops, measurements in COL4A3+/− mice yielded a significant thinning of GBM with an overall mean width of 167 nm. This corresponded to a decrease of approximately 17% in GBM width. Likewise, although the reported mean GBM thickness in adult men and women with TBMN varies widely, comparisons of studies show that >90% of affected individuals have GBM thicknesses between 150 and 300 nm against normal thickness of approximately 370 ± 50 nm in men and 320 ± 50 nm in women (21).

The microscopic findings in COL4A3+/− mice of focal glomerular hypercellularity, sclerosis of individual glomeruli, and diffuse tubulointerstitial cell alternation are in line with existing observations in patients with TBMN, in whom mild mesangial cell proliferation, slight matrix expansion, premature focal global glomerular sclerosis, and tubulointerstitial fibrosis may occur (13,15,25). Moreover, in older adults, nephrosclerosis and tubular atrophy can be observed (21). In this study, in 12-mo-old COL4A3+/− mice, glomerulosclerosis and tubulointerstitial fibrosis that were identified by histology of semithin sections additionally was confirmed by immunohistochemistry. Glomerular hypercellularity was shown to correlate with proliferation of activated fibroblasts and macrophages. Secondary events that led to fibrosis in COL4A3+/− mice were ascribed to upregulation of the profibrotic cytokines TGF-β1 and its downstream mediator, CTGF. Therefore, pathogenesis is much slower but similar to that in AS (1,2,29,31) and might be blocked by potential therapeutic strategies that target profibrotic pathways (see below). As the COL4A3+/− mice transmit a null allele, this might direct to a slightly different phenotype than in-frame mutations in the COL4A3 gene would have had and limits the results of our study. During the entire observation period of 3 yr, no other superimposed renal pathologies were found in the COL4A3+/− mice; neither did this null allele lead to overt tumors or arteriosclerosis or other causes of premature death.

The prevalence of TBMN cannot be estimated accurately from population-based studies because many individuals are unaware that they are affected and therefore are not available for statistical registration and medical assessment. It is estimated that TBMN occurs in at least 1% of otherwise normal children and adults (25,27,28). Twenty to 50% of families with biopsy-proven TBMN have hematuria that is thought to segregate with COL4A3/4 mutations (5,7,10,20,25–27). Our study proves the conjectural association between TBMN and the COL4A3 gene on an inductive rather than a deductive level, as done in human clinical studies before, and strengthens the above-mentioned epidemiologic figures for the incidence of TBMN.

Although the synonym “benign familial hematuria” for TBMN connotes a good prognosis of the condition, our results suggest that serious complications in disease progression might occur in older individuals with CKD. At present, it is recommended that patients with TBMN be monitored for the development of hypertension, proteinuria, and renal impairment (20). It must be stressed how important screening and prevention strategies are for detecting those who are at risk for developing CKD (39,40). Such as for other hereditary nephropathies, no specific therapy is available in TBMN if first symptoms appear, but late-onset renal impairment may require preemptive treatment. Because TBMN equals AS in its genetic etiology and in terms of progression to renal insufficiency as a result of a GBM defect and subsequent glomerular sclerosis, although less severe, therapies that are developed for one extreme of the spectrum of collagen type IV gene disease might be applicable to TBMN as the other extreme as well. The COL4A3+/− mouse model is well suited to study this and other clinical questions. Recently, we showed that preemptive therapy with an angiotensin-converting enzyme (ACE) inhibitor or a vasopeptidase inhibitor prolongs lifespan at least by >100% and markedly reduces clinical and morphologic pathology in homozygous COL4A3−/− mice as animal model for AS (1,31). Moreover, substantial lifespan increase and reduction of proteinuria and uremia in Alport mice also have been shown by an angiotensin receptor 1 antagonist and a hydroxyethyl glutaryl co-enzyme reductase inhibitor (29). Preliminary approaches to application of ACE inhibitors in TBMN patients have been undertaken already and proved to be successful by reducing the frequency and the severity of hematuric episodes (41). Applying ACE inhibitors and other drugs in heterozygous COL4A3+/− mice that are more easily amenable to large-scale studies and molecular pathway investigations will lead to new cognitions about putative beneficial effects and mechanisms of action.

Finally, our results also may have implications for renal transplantation. Currently, kidney transplant donation from individuals with TBMN is controversial because the risks for both the allograft recipient and the donor are unknown (42). Our finding of chronic renal failure in COL4A3+/− mice suggests that there might be a likelihood of premature allograft failure that is stronger than previously suspected for recipients of TBMN kidney transplants. However, our findings also indicate a higher risk for progressive renal disease in live donors with TBMN. As a consequence, we support the view that patients who have TBMN and have risk factors for CKD, such as proteinuria, hypertension, or overt renal insufficiency, may not be considered as donors or be informed about an increased risk for premature renal failure in both the kidney donor and the recipient.
Conclusion

We have shown that the clinical and histomorphologic phenotype in heterozygous COL4A3+/− mice in our 3-yr longitudinal study recapitulated essential findings in human cases of TBMN with concomitant progression to chronic renal failure. Therefore, the COL4A3+/− animal model promises to be a useful means for research into this common human entity and closes one more gap where animal models for GBM diseases have not been available yet. We could strengthen the previously stated assumption that the long-term prognosis of carriers of heterozygous mutation in the COL4A3/4 loci might not always be as benign as generally thought. Impaired renal function could be ascribed to increased renal matrix turnover with upregulation of TGF-β and CTGF, partially leading to progressive glomerulosclerosis. This pathogenesis equals disease mechanisms in AS and therefore makes it accessible for potential antifibrotic and nephroprotective therapies.

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